nature portfolio

Peer Review File

Self-priming of Plk1 binding to BubR1 ensures accurate mitotic progression

Corresponding Author: Dr Gang Zhang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript by Song et al. the authors explore the role of additional motifs and neighbouring phosphorylation sites on the efficiency of interaction between the kinetochore protein BUBR1 and the PLK1 kinase. In particular, the authors investigate the role of accessory phosphorylation on BUBR1 T600 and 608 by PLK1 for its kinetochore recruitment and binding to BUBR1. Overall, the authors show that in addition to the canonical STP motif around T620 on BUBR1, T600/608 support the recruitment of PLK1, and the authors propose that this occurs through local structural modifications rather than direct recruitment. Overall, the experiments performed are in line with the hypotheses proposed and the conclusions drawn. However, there are a certain number of concerns that pevent, this reviewer at least ,from accepting the manuscript in its current form.

Major comments
1. Statistical analysis of experiments need to be shown (statistical significance between the conditions that the authors claim are different). The authors also need to indicate in the methods or in the figure legends, the number of replicate experiments that were performed as well as the approximate number of cells analyzed where missing (in particular in the immunofluorescence experiments).
2.Line 112-114: “the mitotic time was significantly elongated compared to cells with wild type BubR1 suggesting the failure of timely checkpoint silencing (Supplementary Fig. 1a).”
This is not shown in 1a, rather in1b. This experiment should be done and included in the main figure 1.
3. Line 148-149: I would argue that all the phosphomutants are as severe as the 620A mutant. Without statistical analysis, the authors’ conclusion might not hold.
4. lines 192-197 and figure 3: There is an important control missing here: WDR47 mutant (without the 2A). Evaluation of the 2A-WDR47 PBD BM chimera should be benchmarked relative to the to the WDR47 PBD BM single mutant and not the WT. Otherwise, the contribution of the 2A in this background cannot be evaluated (the hypothesis being tested according to lines 186-188.
5. Lines 213-214: Unless I am missing something, this is somewhat of an over-inerpretation and it is not clear how their observations rule out other interpretations. In addition, the observation that mitotic timing is reduced to WT levels in the 2A+3E mutant, despite the lack of enhanced PLK1 binding suggests that these sides regulate mitotic timing independently of PLK1 recruitment. The authors need to further clarify this experiment and their conclusion.
Minor comments:
1. Overall, the manuscript could benefit from additional language editing.
2. Line 128: “Largely maintained” is probably a strong interpretation of the results because there appears to be still a significant decrease in signal. The authors may wish to consider ‘partially rescued’ instead.
3. Line 145-146: The proper expression is 2-fold or 100%.
4. Line 288: It is unclear what the authors mean by “very close”.

Reviewer #2

(Remarks to the Author)
In this paper, Song et al show that Plk1 phosphorylates BubR1 at T600/T608 and this double phosphorylation is important for BubR1-Plk1 interaction. Also, a non phosphorylatable mutant T600A/T608A exhibits prolonged metaphase and

chromosome misalignment in metaphase. Moreover, the authors show that BubR1 T600/T608 is required for phosphorylation of BubR1 at T676/T680 and interaction of BubR1 with PP2A/B56 which is required for proper kinetochore-microtubule attachment and SAC silencing.
The authors have constructed several DNA plasmids which really helped them support their idea. Also, they have performed elegant rescue experiments with phosphomimetic constructs. Overall their findings are novel, the manuscript is focused and well organized and I suggest the following few experiments to strengthen their conclusions.

Major points
1. The authors raised a phosphor specific antibody against pT600 of BubR1 but they have not characterized it in unperturbed mitosis. Could the authors show the localization of phosphorylated BubR1 at pT600 in different phases of unperturbed cell cycle (prometaphase, metaphase, anaphase cytokinesis)? Does phosphorylated BubR1 T600 localize to kinetochores only after nocodazole treatment or also in unperturbed prometaphase? A quantification would be nice to compare pT600 in unperturbed versus nocodazole treated prometaphases. Please show both pictures and graphs and an inset of a pair of magnified kinetochores

2. There are no magnified kinetochores (insets) throughout the paper. Please show a pair of magnified kinetochores at least in figures 1c, 2a and 3b.

3. Apart from the elongation of the mitotic duration from NEB to anaphase, what is the phenotype of cells expressing BubR1 2A? For example, do they exhibit lagging chromosomes in anaphase due to mis segregation defects compared to WT and 2E? Do cells expressing BubR1 2A exhibit micronuclei in interphase?

4. Do other spindle checkpoint proteins localize in cells expressing 2A for example Mad2 and ZW10?

Minor points

1. In lines 114, 199 and 209 do the authors mean Supplementary Fig 1b?
2. In line 90, the reference Kruse et al, 2020 is missing from the list of references.
3. In Figure legend of S3a, please state that the cells are treated with nocodazole
4. In line 404 please add nocodazole treated mitotic cells
5. Please show statistical significance in the graphs of figures 2b, 2d, 3c, 4b, 4d, 5c, S3a,b,c,d,e
6. In Figure 2e could you quantify the western blot bands of ΔKARD vs ΔKARD 2A to better show the difference of Plk1 bound protein?

Reviewer #3

(Remarks to the Author)
This manuscript expands the repertoire of phosphorylations on BubR1 that are regulated by Plk1 to ensure accurate mitotic progression. The authors propose that Plk1 phosphorylates T600 and T608 on BubR1 to promote Plk1 interaction with BubR1 and thereby Plk1-mediated phosphorylation of S676 and T680, which are known to be required to recruit PP2A/B56 and counteract phosphorylations driving the destabilization of kinetochore-microtubule attachments and SAC signalling.

To support these conclusions, the authors replaced endogenous BubR1 in HeLA cells by an exogenous unphosphorylatable T600A/T608A double mutant transgene, and observed chromosome congression defects, longer mitotic timing, a stronger mitotic arrest in nocodazole, decreased ability to accumulate Plk1 at unattached kinetochores (in a delta-KARD background) and decreased phosphorylation of pT680. Notably, replacing the native PBD BM of BubR1 by the high-affinity WDR47 PBD BM, elegantly bypassed the requirement of pT600A/pT608 for Plk1 kinetochore recruitment, mitotic progression and SAC-counteracting activity. Likewise, introducing the phospho-mimetic mutations D675E/S676E/R677E in BubR1 to enhance B56 binding, efficiently restored timely mitotic progression and SAC-counteracting activity in a T600A/T608A background.

Intriguingly, the mechanism by which pT600/pT608 enhances Plk1 recruitment and Plk1-mediated phosphorylation of pT680 remains elusive. Peptide binding assay could not detect any interaction between the pT600/pT608 peptide with the recombinant Plk1, indicating that phosphorylation of T600/T608 does not generate an additional binding motif for Plk1 binding.
And here lies my reluctance in presenting these findings as self-priming of Plk1 binding to BubR1, as depicted in the title of the manuscript and written in the text.

I do think this study offers an important contribution to the field of mitosis. The characterization of two new Plk1 phosphorylations on BubR1 that are required for efficient recruitment of Plk1 and PP2A/B56 reveals an additional layer of regulation required for mitotic fidelity. The experiments are well-designed and most of the conclusions are supported by the results. However, some overstatements that are present throughout the manuscript call for some caution and revision. I endorse the publication of this work, but I have some suggestions and concerns, that the authors may wish to consider.

1- Please represent individual points in all the graphs of the manuscript. Violin plots of the data from the immunofluorescence analysis would be much more informative. Statistical significance should be tested and depicted for all datasets in all graphs.

2- Line 106: “In line with live cell imaging results, cells complemented with BubR1 2A mutant showed a clear chromosome alignment defect (Fig. 1c,d).” I do not agree with this statement. From the movie stills presented in Figure 1 a, the congression time seems equivalent for all the transgenes and similar to siControl cells. It would further support the notion of alignment defect in BubR1 2A cells if the time from NEBD to full chromosome alignment was also plotted. Likewise, plotting the metaphase duration could be helpful to show potential SAC silencing defects in BubR1 2A cells.

3- To evaluate SAC strength, the authors measured the time cells spent in mitosis when incubated with low doses of nocodazole. Why did the authors prefer low doses of nocodazole, which due to the partial depolymerisation of tubulin may introduce variability in the cellular response? (Santaguida et al., 2011) Why not using high doses of nocodazole (3.3 uM) to ensure all kinetochores are equally unattached in all cell lines? If the point is to measure SAC strength (and not silencing), I would be more comfortable with a condition where complete depolymerisation of microtubules was certain.

4- Line 111: “In cells complemented with BubR1 2A, the mitotic time was significantly elongated compared to cells with wild type BubR1 suggesting the failure of timely checkpoint silencing (Supplementary Fig. 1a).”
I have some issues in using the term SAC silencing when kinetochores are (potentially) unattached, as in this assay, which actually measures SAC strength. Under these circumstances, what the BubR1 2A mutant is doing is either directly increasing the SAC strength or indirectly due to decreased opposing phosphatases activity. To claim SAC silencing delays, the authors should arrest cells in mitosis with nocodazole, then inhibit MPS1 and monitor the mitotic exit time or the fraction of cells arrested in mitosis along different time points. With the data presented in Supplementary Fig. 1a, one can only conclude that cells expressing BubR1 T2A spend more time in mitosis when treated with low doses of nocodazole.

5- Line 129: “The above results clearly show that BubR1 pT600/pT608 is required for efficient phosphorylation of BubR1 T680 and its interaction with PP2A/B56 as a result.”
I agree with the first part of this conclusion, but the requirement of pT600/pT608 for interaction with PP2A/B56, although expected, was not formally demonstrated. Given the inability of performing IF staining for B56 at kinetochores, could the authors use the phosphorylation status of a well-established substrate of BubR1:B56:PP2A holoenzyme as a readout of PP2A/B56 activity and in this way infer impaired interaction in cells?

6- The observation that BubR1 2A mutation in the delta-KARD background significantly reduces Plk1 kinetochore levels to a similar extent as the T620A mutant (Fig. 2c,d). is very interesting. However, I think this observation should be complemented with Plk1 kinetochore levels in a BubR1 2E mutation as control.

7- To test whether T600 is phosphorylated by Plk1, the authors generated a BubR1 pT600 phospho-antibody and characterized it by immunofluorescence in nocodazole treated cells. It would also be interesting to follow the profile of pT600 throughout mitosis (from prophase to anaphase) and correlate the levels with kinetochore microtubule attachment status.

8- Importantly, the results presented in Supplementary Figure 3 clearly demonstrate that BubR1 pT600 depends on PLK1 activity in cells, however they do not demonstrate direct phosphorylation. If the phospho-antibody works well for immunoblotting, I would use it to probe the phosphorylation in in vitro kinase assays and in this way further support direct phosphorylation by PLK1.

9- The authors propose the following model “Cdk1 phosphorylates BubR1 T620 to generate the docking site for Plk1. In turn, Plk1 transiently bound to pT620 phosphorylates two BubR1 residues T600 and T608 in front which enhances the binding between Plk1 and BubR1. Now, stably bound Plk1 on BubR1 could efficiently phosphorylate BubR1 on T676 and T680 to promote the interaction between BubR1 and PP2A/B56 which is critical for the proper attachment between kinetochores and microtubules as well as the timely silencing of SAC.” My question here is simple, although I admit the answer might be complex. Why would the phosphorylation of T676 and T680 require PLK1 to be more stably bound to BubR1 than the phosphorylations of T600 and T608? Could the authors discuss this?

10- In line 285, the authors hypothesize that the double phosphorylation pT600/p608 may cause conformation changes that expose pT620 more efficiently to Plk1. In that regard, it would be interesting to evaluate pT620 levels in BubR1 2A. Maybe pT600/p608 promote somehow Cyclin:CDK1 binding, leading to higher phosphorylation of T620 and consequently enhanced binding of Plk1.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my comments. The manuscript is significantly imprvied and and I now support it's publication.

Reviewer #2

(Remarks to the Author)
After my recommendations, the authors analyzed localization of phosphorylated BubR1 at pT600 in different phases of unperturbed cell cycle (prometaphase, metaphase, anaphase cytokinesis) and presented a quantification of pT600

phosphorylation in both unperturbed and nocodazole treated cells. They also showed magnified kinetochores (insets) throughout the paper as I suggested. Moreover, the authors showed that cells expressing BubR1 2A exhibit lagging chromosomes in anaphase due to mis segregation defects compared to WT and 2E. They did not examined micronuclei in interphase due to technical difficulties which is fine. They also examined the kinetochore localization and intensity of the checkpoint protein Mad2 and showed statistical significance in all the graphs throughout the paper. The new data strengthened their conclusions, their findings are novel and their experiments are well executed. The quality of confocal microscopy images is also great.
To conclude, the authors responded to all my comments successfully and addressed all the major and minor points. Therefore, I strongly suggest publication of their manuscript in Communications Biology.

Reviewer #3

(Remarks to the Author)
The authors did a good job in addressing most points of criticism. The conclusion of the work was significantly strengthened by the additional data that is now included in the revised version of the manuscript. This is a good study and the findings are relevant to the field of mitosis. However, I remain unease with the author's replies to points "1" and "8" of my previous review report.

Point 1- I do not understand the author's reluctance in presenting Violin plots displaying all individual data points. The argument presented by the authors "We would like to keep the form since this is also consistent to all our previous publications." seems rather unreasonable. I do not agree with the authors on this matter but I will let this topic to be decided by the editorial team.

Point 8- This point concerns the claim that PLK1 is directly phosphorylating BubR1 T600. Showing in cells that pT600 (as measured by the phospho-antibody) is sensitive to Plk1i and reduced in BuBR1 T620A does not prove direct phosphorylation. It merely shows that this phospho-epitope is responsive to PLK1 activity in cells. Of course specificity is always an issue with in vitro kinase assays and proper controls should always be included regardless the detection method (phospho-antibody, radiolabelled ATP, Pro-Q Diamond Gel Stain etc…) My goal with this suggestion was to strengthen the conclusion that PLK1 phosphorylates new sites on BubR1 that are functionally important. I disagree with the authors when they write that "such an experiment adds anything". It is acceptable to disregard the in vitro experiment, but at least I would ease on the direct phosphorylation claim. Again, I will let this topic for editorial decision.

Reviewer #1 (Remarks to the Author):

In this manuscript by Song et al. the authors explore the role of additional motifs and neighbouring phosphorylation sites on the efficiency of interaction between the kinetochore protein BUBR1 and the PLK1 kinase. In particular, the authors investigate the role of accessory phosphorylation on BUBR1 T600 and 608 by PLK1 for its kinetochore recruitment and binding to BUBR1. Overall, the authors show that in addition to the canonical STP motif around T620 on BUBR1, T600/608 support the recruitment of PLK1, and the authors propose that this occurs through local structural modifications rather than direct recruitment. Overall, the experiments performed are in line with the hypotheses proposed and the conclusions drawn. However, there are a certain number of concerns that pevent, this reviewer at least ,from accepting the manuscript in its current form.

We thank the reviewer for the positive comments and constructive comments to improve the manuscript.

Major comments

1. Statistical analysis of experiments need to be shown (statistical significance between the conditions that the authors claim are different). The authors also need to indicate in the methods or in the figure legends, the number of replicate experiments that were performed as well as the approximate number of cells analyzed where missing (in particular in the immunofluorescence experiments).

We thank the reviewer's constructive suggestions. Now we provide the statistics, repeat number and cell number of the related experiment in figures, figure legends and methods.

2.Line 112-114: "the mitotic time was significantly elongated compared to cells with wild type BubR1 suggesting the failure of timely checkpoint silencing (Supplementary Fig. 1a)."
This is not shown in 1a, rather in1b. This experiment should be done and included in the main figure 1.

We performed the SAC examination experiment with only BubR1 WT, 2A, 2E now and presented the result in Fig. 1e. The original Supplementary Fig. 1a is now Supplementary Fig. 4b.

3. Line 148-149: I would argue that all the phosphomutants are as severe as the 620A mutant. Without statistical analysis, the authors' conclusion might not hold.

We thank the reviewer for the comments. The statistical analysis shows T620A and 2A mutants have no significant difference on Plk1 recruitment which is presented in Fig. 2d now.

4. lines 192-197 and figure 3: There is an important control missing here: WDR47 mutant (without the 2A). Evaluation of the 2A-WDR47 PBD BM chimera should be benchmarked relative to the to the WDR47 PBD BM single mutant and not the WT. Otherwise, the contribution of the 2A in this background cannot be evaluated (the hypothesis being tested according to lines 186-188.

We thank the reviewer for the good suggestion. Now we generated new BubR1 mutant with WDR47 PBD BM in Δ KARD background and tested its ability for Plk1 recruitment. The result shows no significant difference to wild type BubR1 or BubR1 2A+WDR47 (all in Δ KARD background) (Fig. 3b,c). Another BubR1 mutant with WDR47 PBD BM in the presence of KARD motif was tested for its ability to support mitotic progression. The result shows this mutant could efficiently support mitosis with the time from NEBD to anaphase around 40 min as the wild type BubR1 (Fig 3d,e).

5. Lines 213-214: Unless I am missing something, this is somewhat of an over-inerpretation and it is not clear how their observations rule out other interpretations. In addition, the observation that mitotic timing is reduced to WT levels in the 2A+3E mutant, despite the lack of enhanced PLK1 binding suggests that these sides regulate mitotic timing independently of PLK1 recruitment. The authors need to further clarify this experiment and their conclusion.

Since BubR1 2A+3E mutant is able to efficiently bind PP2A/B56 but not Plk1, the restoration of normal mitotic timing by this mutant suggests Plk1-BubR1 interaction is dispensible as long as PP2A/B56 efficiently binds BubR1. However, we agree with the reviewer that we cannot rule out other possibilities and we now modified the text to indicate this.

Minor comments:
1. Overall, the manuscript could benefit from additional language editing.

We further edited the manuscript and hope it is better organized now.

2. Line 128: “Largely maintained” is probably a strong interpretation of the results because there appears to be still a significant decrease in signal. The authors may wish to consider ‘partially rescued’ instead.

Thanks for the suggestion. It is changed to partially rescued now.

3. Line 145-146: The proper expression is 2-fold or 100%.

Thanks for the suggestion. It is corrected to 2-fold now.

4. Line 288: It is unclear what the authors mean by “very close”.

We mean the number of the affinity of Bub1-Plk1 and BubR1-Plk1 are very close. We now re-phrased the sentence.

Reviewer #2 (Remarks to the Author):

In this paper, Song et al show that Plk1 phosphorylates BubR1 at T600/T608 and this double phosphorylation is important for BubR1-Plk1 interaction. Also, a non phosphorylatable mutant T600A/T608A exhibits prolonged metaphase and chromosome misalignment in metaphase. Moreover, the authors show that BubR1 T600/T608 is required for phosphorylation of BubR1 at

T676/T680 and interaction of BubR1 with PP2A/B56 which is required for proper kinetochore-microtubule attachment and SAC silencing.
The authors have constructed several DNA plasmids which really helped them support their idea. Also, they have performed elegant rescue experiments with phosphomimetic constructs. Overall their findings are novel, the manuscript is focused and well organized and I suggest the following few experiments to strengthen their conclusions.

We thank the reviewer for the positive comments and constructive points to improve manuscript.

Major points
1. The authors raised a phosphor specific antibody against pT600 of BubR1 but they have not characterized it in unperturbed mitosis. Could the authors show the localization of phosphorylated BubR1 at pT600 in different phases of unperturbed cell cycle (prometaphase, metaphase, anaphase cytokinesis)? Does phosphorylated BubR1 T600 localize to kinetochores only after nocodazole treatment or also in unperturbed prometaphase? A quantification would be nice to compare pT600 in unperturbed versus nocodazole treated prometaphases. Please show both pictures and graphs and an inset of a pair of magnified kinetochores

We thank the good suggestions by the reviewer. It is meaningful to look at the phosphorylation status on BubR1 T600 in unperturbed cell cycle. We now performed the experiment and presented the results in Supplementary Fig. 3f as suggested. We could see the phosphorylation happens as early as in prophase once BubR1 appears on kinetochores. The signals increase at prometaphase and declines continuously till anaphase. The signals at prometaphase and metaphase are higher or close to the signals of nocodazole-treated cells.

2. There are no magnified kinetochores (insets) throughout the paper. Please show a pair of magnified kinetochores at least in figures 1c, 2a and 3b.

Thanks for the suggestions. We now provide insets for all the relevant figures.

3. Apart from the elongation of the mitotic duration from NEB to anaphase, what is the phenotype of cells expressing BubR1 2A? For example, do they exhibit lagging chromosomes in anaphase due to mis segregation defects compared to WT and 2E? Do cells expressing BubR1 2A exhibit micronuclei in interphase?

We thank the reviewer for this suggestion. We have now examined the chromosome segregation in cells expressing BubR1 WT, 2A and 2E. Indeed, 2A mutant has more mis-segregated chromosomes than WT and 2E. The result is now presented in Supplementary Fig. 1a,b. Since BubR1 is largely degraded in interphase, we couldn't precisely distinguish cells with YFP-BubR1 to cells without YFP-BubR1 expressed, thus we did not examine the micronuclei in interphase.

4. Do other spindle checkpoint proteins localize in cells expressing 2A for example Mad2 and ZW10?

Thanks for the suggestion. We now examined the kinetochore localization and intensity of the checkpoint protein Mad2 and presented the results in Supplementary Fig. 1c,d. The kinetochore levels of Mad2 is slightly higher in 2A mutant than wild type or 2E mutant.

Minor points

1. In lines 114, 199 and 209 do the authors mean Supplementary Fig 1b?

Yes, they all referred to the original Supplementary Fig 1b (Supplementary Fig. 4b now). We now presented a separate checkpoint assay with only BubR1 WT, 2A and 2E in Fig. 1c and made changes of the figure reference accordingly.

2. In line 90, the reference Kruse et al, 2020 is missing from the list of references.

Thanks very much for pointing out the mistake. We now added Kruse et al., 2020 in the reference.

3. In Figure legend of S3a, please state that the cells are treated with nocodazole

It is corrected in figure legend.

4. In line 404 please add nocodazole treated mitotic cells

It is added now.

5. Please show statistical significance in the graphs of figures 2b, 2d, 3c, 4b, 4d, 5c, S3a,b,c,d,e

Thanks for the good suggestion. The statiscal analysis is now provided in the relevant figures.

6. In Figure 2e could you quantify the western blot bands of ΔKARD vs ΔKARD 2A to better show the difference of Plk1 bound protein?

The quantification of two replicates is provided now.

Reviewer #3 (Remarks to the Author):

This manuscript expands the repertoire of phosphorylations on BubR1 that are regulated by Plk1 to ensure accurate mitotic progression. The authors propose that Plk1 phosphorylates T600 and T608 on BubR1 to promote Plk1 interaction with BubR1 and thereby Plk1-mediated phosphorylation of S676 and T680, which are known to be required to recruit PP2A/B56 and counteract phosphorylations driving the destabilization of kinetochore-microtubule attachments and SAC signalling.

To support these conclusions, the authors replaced endogenous BubR1 in HeLA cells by an exogenous unphosphorylatable T600A/T608A double mutant transgene, and observed chromosome congression defects, longer mitotic timing, a stronger mitotic arrest in nocodazole, decreased ability to accumulate Plk1 at unattached kinetochores (in a delta-KARD background) and decreased phosphorylation of pT680. Notably, replacing the native PBD BM of BubR1 by the high-affinity WDR47 PBD BM, elegantly bypassed the requirement of pT600A/pT608 for Plk1 kinetochore recruitment, mitotic progression and SAC-counteracting activity. Likewise, introducing the phospho-mimetic mutations D675E/S676E/R677E in BubR1 to enhance B56 binding, efficiently restored timely mitotic progression and SAC-counteracting activity in a T600A/T608A background.

Intriguingly, the mechanism by which pT600/pT608 enhances Plk1 recruitment and Plk1-mediated phosphorylation of pT680 remains elusive. Peptide binding assay could not detect any interaction between the pT600/pT608 peptide with the recombinant Plk1, indicating that phosphorylation of T600/T608 does not generate an additional binding motif for Plk1 binding.
And here lies my reluctance in presenting these findings as self-priming of Plk1 binding to BubR1, as depicted in the title of the manuscript and written in the text.

I do think this study offers an important contribution to the field of mitosis. The characterization of two new Plk1 phosphorylations on BubR1 that are required for efficient recruitment of Plk1 and PP2A/B56 reveals an additional layer of regulation required for mitotic fidelity. The experiments are well-designed and most of the conclusions are supported by the results. However, some overstatements that are present throughout the manuscript call for some caution and revision. I endorse the publication of this work, but I have some suggestions and concerns, that the authors may wish to consider.

We thank the reviewer for the positive comments and constructive suggestions.

1- Please represent individual points in all the graphs of the manuscript. Violin plots of the data from the immunofluorescence analysis would be much more informative. Statistical significance should be tested and depicted for all datasets in all graphs.

We agree with the reviewer that showing all individual points is more informative especially for the assays comparing **median** values. In our case for immunofluorescence quantification, we presented the **mean** values in a more straightforward form as used here. We would like to keep the form since this is also consistent to all our previous publications. We hope the reviewer could support our decision. In case the readers are interested, all the individual points are provided in Supplementary data which could be easily converted.

We thank the reviewer for the suggestion of statistical significance which is now shown for all the relevant figures.

2- Line 106: “In line with live cell imaging results, cells complemented with BubR1 2A mutant showed a clear chromosome alignment defect (Fig. 1c,d).” I do not agree with this statement. From the movie stills presented in Figure 1 a, the congression time seems equivalent for all the transgenes

and similar to siControl cells. It would further support the notion of alignment defect in BubR1 2A cells if the time from NEBD to full chromosome alignment was also plotted. Likewise, plotting the metaphase duration could be helpful to show potential SAC silencing defects in BubR1 2A cells.

BubR1 T600A/T608A mutation causes much milder chromosome alignment defects compared with the defects caused by mutating the core B56 binding motif LxxIxE (KARD motif). This is not surprising as the phosphorylated amino acids close to KARD motif only facilitate but not determine the BubR1-B56 binding. By live cell imaging, it is difficult to precisely judge whether all chromosomes align properly as the mild alignment defect may not be easily detected without scanning the whole cell. By examining all chromosomes in fixed cells, we could detect the alignment defects as shown in Fig. 1c,d. Now we modified the related sentences to make this clearer.

3- To evaluate SAC strength, the authors measured the time cells spent in mitosis when incubated with low doses of nocodazole. Why did the authors prefer low doses of nocodazole, which due to the partial depolymerisation of tubulin may introduce variability in the cellular response? (Santaguida et al., 2011) Why not using high doses of nocodazole (3.3 uM) to ensure all kinetochores are equally unattached in all cell lines? If the point is to measure SAC strength (and not silencing), I would be more comfortable with a condition where complete depolymerisation of microtubules was certain.

High dose of nocodazole normally causes a very long arrest and much more cell death. Thus, low dose of nocodazole (15ng/ml-100ng/ml) is widely used for the mitotic checkpoint study by many labs (10.1038/nature13911; 10.1016/j.cub.2017.08.033; 10.1016/j.devcel.2008.11.004; 10.1038/sj.emboj.7600641) including ours (10.1242/jcs.139725; 10.1038/ncomms6563; 10.1038/ncomms12256; 10.15252/embj.2018100977; 10.1038/ncomms15822; 10.1093/jmcb/mjac062). So far we are not aware of any inconsistency on checkpoint mechanism revealed by low or high dose of nocodazole. We understand the concern of the reviewer and repeated the checkpoint experiment with a relatively higher dose of nocodazole at 100ng/ml (presented now in Fig. 1e) which gave consistent result as we originally showed (Supplementary Fig. 4b now). We hope this addresses the concerns of the reviewer.

4- Line 111: “In cells complemented with BubR1 2A, the mitotic time was significantly elongated compared to cells with wild type BubR1 suggesting the failure of timely checkpoint silencing (Supplementary Fig. 1a).”
I have some issues in using the term SAC silencing when kinetochores are (potentially) unattached, as in this assay, which actually measures SAC strength. Under these circumstances, what the BubR1 2A mutant is doing is either directly increasing the SAC strength or indirectly due to decreased opposing phosphatases activity. To claim SAC silencing delays, the authors should arrest cells in mitosis with nocodazole, then inhibit MPS1 and monitor the mitotic exit time or the fraction of cells arrested in mitosis along different time points. With the data presented in Supplementary Fig. 1a, one can only conclude that cells expressing BubR1 T2A spend more time in mitosis when treated with low doses of nocodazole.

We agree with the reviewer that a strict SAC silencing assay is not conducted here. We claim the SAC silencing defect by BubR1 2A mutant is because this mutant impairs BubR1-PP2A/B56 interaction and PP2A/B56 is well studied of its role in SAC silencing. We now modified the related sentence.

5- Line 129: "The above results clearly show that BubR1 pT600/pT608 is required for efficient phosphorylation of BubR1 T680 and its interaction with PP2A/B56 as a result."
I agree with the first part of this conclusion, but the requirement of pT600/pT608 for interaction with PP2A/B56, although expected, was not formally demonstrated. Given the inability of performing IF staining for B56 at kinetochores, could the authors use the phosphorylation status of a well-established substrate of BubR1:B56:PP2A holoenzyme as a readout of PP2A/B56 activity and in this way infer impaired interaction in cells?

We thank the reviewer for the good advice. We now use the phosphor antibody against Mps1 pT33 as an indicator for PP2A/B56 activity. The new result is presented in Supplemenatary Fig. 1e,f. In 2A mutant, Mps1 pT33 was increased around 30% while in 2E mutant, the signals was reduced around 30% compared to wild type BubR1.

6- The observation that BubR1 2A mutation in the delta-KARD background significantly reduces Plk1 kinetochore levels to a similar extent as the T620A mutant (Fig. 2c,d). is very interesting. However, I think this observation should be complemented with Plk1 kinetochore levels in a BubR1 2E mutation as control.

We thank the reviewer for the good suggestion. Now we generated BubR1 2E mutant in ΔKARD background and examined the kineotochore intensity of Plk1 in cells expressing this mutant. The result is presented in Fig. 2c,d now which shows 2E mutant recruits significantly more Plk1 than 2A mutant.

7- To test whether T600 is phosphorylated by Plk1, the authors generated a BubR1 pT600 phospho-antibody and characterized it by immunofluorescence in nocodazole treated cells. It would also be interesting to follow the profile of pT600 throughout mitosis (from prophase to anaphase) and correlate the levels with kinetochore microtubule attachment status.

We thank the reviewer for the good suggestion. We now examined and quantified the phosphorylation status of BunR1 T600 in one cell cycle and compared to the signals from nocodazole-treated cells. The results are presented in Supplementary Fig. 3f now. We could see the phosphorylation happens as early as in prophase once BubR1 appears on kinetochores. The signals increase at prometaphase and declines continuously till anaphase. The signal levels at prometaphase and metaphase are higher or close to the signals of nocodazole-treated cells.

8- Importantly, the results presented in Supplementary Figure 3 clearly demonstrate that BubR1 pT600 depends on PLK1 activity in cells, however they do not demonstrate direct phosphorylation.

If the phospho-antibody works well for immunoblotting, I would use it to probe the phosphorylation in in vitro kinase assays and in this way further support direct phosphorylation by PLK1.

We disagree with the reviewer on this. In vitro phosphorylation assays are inherently unspecific and given the complexity of Plk1 regulation of BubR1 we do not think such an experiment adds anything. The fact that in cells T600 phosphorylation (as measured by the phospho antibody) is sensitive to Plk1i and reduced in BuBR1 T620 is strongly supporting that Plk1 is phosphorylating T600.

9- The authors propose the following model "Cdk1 phosphorylates BubR1 T620 to generate the docking site for Plk1. In turn, Plk1 transiently bound to pT620 phosphorylates two BubR1 residues T600 and T608 in front which enhances the binding between Plk1 and BubR1. Now, stably bound Plk1 on BubR1 could efficiently phosphorylate BubR1 on T676 and T680 to promote the interaction between BubR1 and PP2A/B56 which is critical for the proper attachment between kinetochores and microtubules as well as the timely silencing of SAC." My question here is simple, although I admit the answer might be complex. Why would the phosphorylation of T676 and T680 require PLK1 to be more stably bound to BubR1 than the phosphorylations of T600 and T608? Could the authors discuss this?

That is a very good question. One explanation could be the distance of those sites to the kinase and phosphatase determines their reliance on the interaction status of BubR1-Plk1. T600/T608 is closer to the kinase (at BubR1 619-621AA), but further away from the phosphatase (at BubR1 669-674AA) while T676/T680 is closer to the phosphatase and further away from the kinase. This creates the distinct dependence on the binding status between Plk1 and BubR1. We now expanded the discussion with this perspective.

10- In line 285, the authors hypothesize that the double phosphorylation pT600/p608 may cause conformation changes that expose pT620 more efficiently to Plk1. In that regard, it would be interesting to evaluate pT620 levels in BubR1 2A. Maybe pT600/p608 promote somehow Cyclin:CDK1 binding, leading to higher phosphorylation of T620 and consequently enhanced binding of Plk1.

We thank the reviewer for this suggestion. We took an unbiased mass spectrometry approach and compared the phosphorylation pattern of BubR1 WT and BubR1 2A. Although we did not directly detect the pT620 peptide we did detect the unphosphorylated form of this peptide. This peptide was highly reduced in BubR1 2A indirectly supporting that pT620 is likely more phosphorylated in BubR1 2A than BubR1 WT. We also detected S670p in this experiment (the SP site in the B56 binding site) which revealed a similar level of this phosphorylation site in BuBR1 2A compared to BubR1 WT (Supplementary Fig. 1 g,h).

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have addressed my comments. The manuscript is significantly imprvied and and I now support it's publication.

We thank this reviewer's constructive suggestions and support for publishing our manuscript.

Reviewer #2 (Remarks to the Author):

After my recommendations, the authors analyzed localization of phosphorylated BubR1 at pT600 in different phases of unperturbed cell cycle (prometaphase, metaphase, anaphase cytokinesis) and presented a quantification of pT600 phosphorylation in both unperturbed and nocodazole treated cells. They also showed magnified kinetochores (insets) throughout the paper as I suggested. Moreover, the authors showed that cells expressing BubR1 2A exhibit lagging chromosomes in anaphase due to mis segregation defects compared to WT and 2E. They did not examined micronuclei in interphase due to technical difficulties which is fine. They also examined the kinetochore localization and intensity of the checkpoint protein Mad2 and showed statistical significance in all the graphs throughout the paper. The new data strengthened their conclusions, their findings are novel and their experiments are well executed. The quality of confocal microscopy images is also great.
To conclude, the authors responded to all my comments successfully and addressed all the major and minor points. Therefore, I strongly suggest publication of their manuscript in Communications Biology.

We thank this reviewer's constructive suggestions and support for publishing our manuscript.

Reviewer #3 (Remarks to the Author):

The authors did a good job in addressing most points of criticism. The conclusion of the work was significantly strengthened by the additional data that is now included in the revised version of the manuscript. This is a good study and the findings are relevant to the field of mitosis. However, I remain unease with the author's replies to points "1" and "8" of my previous review report.

We thank this reviewer's constructive suggestions.

Point 1- I do not understand the author's reluctance in presenting Violin plots displaying all individual data points. The argument presented by the authors "We would like to keep the form since this is also consistent to all our previous publications." seems rather unreasonable. I do not agree with the authors on this matter but I will let this topic to be decided by the editorial team.

We think our format is more straitforward than the violin format. We agree the violin plot could be more informative in some ways and may consider it in the future.

Point 8- This point concerns the claim that PLK1 is directly phosphorylating BubR1 T600. Showing in cells that pT600 (as measured by the phospho-antibody) is sensitive to Plk1i and reduced in BuBR1 T620A does not prove direct phosphorylation. It merely shows that this phospho-epitope is responsive to PLK1 activity in cells. Of course specificity is always an issue with in vitro kinase assays and proper controls should always be included regardless the detection method (phospho-antibody, radiolabelled ATP, Pro-Q Diamond Gel Stain etc…) My goal with this suggestion was to strengthen the conclusion that PLK1 phosphorylates new sites on BubR1 that are functionally important. I disagree with the authors when they write that "such an experiment adds anything". It is acceptable to disregard the in vitro experiment, but at least I would ease on the direct phosphorylation claim. Again, I will let this topic for editorial decision.

Besides the experiments by Plk1i and T620A, both sites fit the Plk1 phosphorylation consensus and we believe the evidences are strong enough to claim Plk1 phosphorylates these two sites. We appreciate the reviewer's intention to strengthen this point by proposing in vitro kinase assay and we now modified the context to avoid claiming a direct phosphorylation of BubR1 T600/T608 by Plk1 kinase (see changes on line 186-187 and 209).

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We now re-formatted all our quantifications into dot plots.

Point 8- This point concerns the claim that PLK1 is directly phosphorylating BubR1 T600. Showing in cells that pT600 (as measured by the phospho-antibody) is sensitive to Plk1i and reduced in BuBR1 T620A does not prove direct phosphorylation. It merely shows that this phospho-epitope is responsive to PLK1 activity in cells. Of course specificity is always an issue with in vitro kinase assays and proper controls should always be included regardless the detection method (phospho-antibody, radiolabelled ATP, Pro-Q Diamond Gel Stain etc…) My goal with this suggestion was to strengthen the conclusion that PLK1 phosphorylates new sites on BubR1 that are functionally important. I disagree with the authors when they write that "such an experiment adds anything". It is acceptable to disregard the in vitro experiment, but at least I would ease on the direct phosphorylation claim. Again, I will let this topic for editorial decision.

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