PP2A-B55γ counteracts Cdk1 and regulates proper spindle orientation through the cortical dynein adaptor NuMA

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AUTHORS: Riya Keshri, Ashwathi Rajeevan, and Sachin Kotak
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

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Reviewer 1

Advance summary and potential significance to field

The authors and others have previously established that cell cycle dependent phosphorylations regulate the recruitment of NuMA and hence also dynein to the cell cortex during mitosis. Cdk1-dependent phosphorylation of a particular site was demonstrated to negatively control cortex recruitment. Here, the authors identify the phosphatase complex that dephosphorylates NuMA at this particular site (T2055) at the transition from metaphase to anaphase thereby promoting cortex recruitment of NuMA. They also show that flanking positively charged sites promote the efficiency of dephosphorylation. This is a nice manuscript with new information on the biochemistry of the control of spindle positioning via dynein recruitement at the cortex. It is focused and useful for the field. It fills a gap in our knowledge of the specificity of dephosphorylations. What is maybe a pity is that we do not learn, why this particular phosphatase complex dephosphorylates this site on NuMA. But a solid description of the role of the responsible phosphatase is a useful first step towards obtaining this answer.

Comments for the author

Overall the data as presented support the conclusions, however the following questions arose upon reading of the manuscript:

1. Categorizations: E.g in Fig. 1C, 1F, S1D and others: What are the criteria determining classification into the categories 'absent', 'weak', 'significant', 'strong'. If someone wanted to reproduce these experiments, how can the categorization be reproduced? Note: the opposite of 'significant' is usually 'non-significant'. Therefore, please consider renaming this category (similar point: line 3, page 8: what does 'significant number of cells' mean?)

2. Fig. 1I/J: It appears that Cdk1 inhibition reduces the degree of NuMA T2055 phosphorylation by a little more than a factor of 2. Where does the remaining phosphorylation come from? Concomitant knockdown of the B55gamma subunit does not seem to change this much (according to the bar graphs), the decrease is now a little less than a factor of 2. Does this experiment really support the conclusion that B55gamma is the major regulatory subunit for NuMA dephosphorylation by PP2A? Please explain why the B55gamma effect in this experiment is less striking than in Fig. 1D/E.

Technical questions:

For fluorescence intensity quantifications at the spindle poles, do the authors use an image taken in one z-position or an image created from a z-stack? In the first case one would have the concern that intensity differences may arise from the pole being more or less positioned in the focal plane.

For fluorescence intensity quantifications at the cortex: the intensity seems to fluctuate locally. Is an average intensity at the cortex for one-half cell calculated (or for an entire cell – if it is round as in Fig.4)? The authors often indicate in their figure only a small area of the cortex by a rectangle. If the intensity was only computed in this part of the cortex, how was the position chosen? Will measurements at other positions produce the same intensities? How exactly is the background intensity treated to arrive at the reported intensities?

Minor points:

Is Fig. 1B useful?

At several instances, the text would profit from correcting grammatical mistakes (e.g. use of plural/singular).

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Reviewer 2

Advance summary and potential significance to field

NuMA plays important roles in spindle orientation and anaphase spindle elongation. These roles require its association with the cell cortex in a highly regulated manner. Cdk1 phosphorylation is known to inhibit cortical localization from phosphorylation at T2055. Here the authors identify the phosphatase complex required for dephosphorylation of this residue and thus regulates NuMA cortical function.

Comments for the author

Major Concerns:

1. Polybasic residues around the T2055 site were suggested to be important for recognition by the phosphatase complex. The authors need to demonstrate that these mutations do not affect cortical localization through direct effects on phospholipid interactions. Ideally this would be through phospholipid interactions which the PI has performed in previous work, or by demonstrating that Thr to Ala mutation of T2055 is able to rescue the cortical localization of the polybasic mutant. Additionally, it would greatly strengthen the manuscript to directly show that the polybasic mutant no longer physically interacts with the phosphatase complex.

2. Throguhout the introduction and results, a clear description of the different requirements for NuMA localization to the cortex in metaphase and anaphase (LGN dependence, etc.) should be given. Metaphase localization is normally thought to be LGN dependent and insensitive to phosphorylation. Thus it is somewhat surprising that effects in metaphase as well as anaphase (where the dephosphorylation is essential for cortical localization) are reported.

3. Controls/Quants. A number of essential controls are missing throughout the mansucript.
- knockdown efficiency of B55 is not demonstrated.
-Experiment in 2C does not include a negative control.
-Figure 2M requires quantitation.
-the dephosphorylation assay in 2I does not have a negative control.

Other concerns

The manuscript needs editing for grammar.

Unless knockdown efficiencies of all phosphatase subunits have been assayed, it is important to state that there are potentially false negatives in the dataset.

The experiment in Figure 2L is hard to interpret. Does the antibody sterically hinder association?

Supplemental Figure S4 - why is there still significant T2055 signal in the NuMA RNAi line?
Also, is the difference between the last two lanes in S4E significant?

Reviewer 3

Advance summary and potential significance to field

This manuscript represents an advance in the field, and builds on earlier work by Kotak from 2013, although the manuscript by Keshri and colleagues is overall poorly proofed, in my opinion. However, the data partly justify the conclusions drawn, which are that the phosphorylation of Thr2055 by CDK1 is counteracted by the B55gamma/PP2Ac complex and the authors deserve credit for trying hard to do a broad range of experiments merging cell biology and analytical in vitro phosphorylation analysis. The study demonstrates that the PP2A catalytic subunit/B55gamma complex (which is depleted in various ways, which will have massive knock-on effects) counteracts the phosphorylation of Thr2055 of NUMA which is a potential CDK1 site of phosphorylation. Cortical NuMA (which is dephosphorylated) is a dynein anchor, and NuMA localisation is negatively regulated

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by this phophsphorylation event, and PP2A depletion of B55gamma, but not other B subunits changes NuMA/dynein localisation, consistent with previous data pertaining to the catalytic subunit. Modulation of cortical NuMA changes spindle orientation and elongation.
Greatwall/ENSA, downstream of CDK1, are not shown to be involved in the NuMA regulation presumably because this is a B55gamma-specific complex.
C-terminal Polybasic residues in the vicinity of Thr2055 are also likely involved.

Major points:
1) In Figure 2, GFP-tagged B55gamma co-IPs NuMA and PP2catalytic subunit. However, no phosphatase-dead control, or beads alone are used in these experiments, which makes the conclusions problematic. Related, Figure 2K is an unconventional approach: why would GFP antibody mask the phosphatase activity? Why not add an inhibitor of the phosphatase or use other controls to demonstrate that the dephosphorylation is direct? Would any CDK1-mediated phosphosubstrate be dephosphorylated? Most notably, testing of the 2K or 4KR mutant in this assay would prove that 1) CDK1-mediated T2055 still occurs and 2) that the phosphatase in the IP can't now dock and dephosphorylate this site. This experiment would add considerably to the manuscript. It would also prove that the pT2055 antibody can still recognise the 2KK or 4KR protein.
2) Given the clear data presented in Figure 1, the authors need to repeat their biochemical analysis with recombinant NuMA with a different B complex (B55alpha/beta), which does not show up in the initial screen. Like 1) above, this would add considerably to the manuscript.

Minor points:
- p.4 ‘Weak and enrich’. What does weak mean in this context?
- P.5 ‘That is consists of’, then the authors fail to mention the catalytic subunit when describing the trimeric complex!
- Figure 1A should label the regulatory (B) subunit
- p.8 B55gamma and PPP2R1B siRNA decrease pT2055. This does not prove it is direct as stated
- Why is it remarkable that the IP’d complex dephosphorylates CDK1-phosphorylated NuMA? What else was tested?
- How do we know that the experiment in Figure 2M lacks PP2CA catalytic subunit?
- In an artificial anaphase, induced with kinesin 5 inhibitor STLC and subsequent RO-3306 addition, siRNA of B55gamma or other members of the complex, change cortical NuMA. Wouldn’t RO-3306 addition do this anyway independent of cells being forced to exit mitosis, since this is a CDK1 site?
- p.12 2KK becomes 4KR in GFP-NuMA construct. This is confusing for readers, but I think it makes sense overall. Why not use these in the pull-down and dephosphorylation scenario discussed in Figure 2 (see major points, above)
- p.16. Why is the B55gamma-PP2A complex ‘unique’?
- Discussion is clear and succinct.
- Tenses change throughout the manuscript.

Comments for the author

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- Discussion is clear and succinct.
- Tenses change throughout the manuscript.

First revision

Author response to reviewers' comments

The point-by-point response to the reviewers

Reviewer #1

We are incredibly grateful to the reviewer for their kind remarks that '*this is a nice manuscript with new information on the biochemistry of the control of spindle positioning via dynein recruitment at the cortex. It is focused and useful for the field*'. The reviewer raised a couple of remaining concerns, which we have addressed as explained below:

Remaining Concerns:

1) Categorizations: E.g in Fig. 1C, 1F, S1D, and others: What are the criteria determining classification into the categories' absent', 'weak', 'significant', 'strong'. If someone wanted to reproduce these experiments, how can the categorization be reproduced? Note: the opposite of 'significant' is usually 'non-significant'. Therefore, please consider renaming this category (similar

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point: line 3, page 8: what does 'significant number of cells'mean?)

Response. We genuinely thank the reviewer for all of their constructive remarks on our paper. To obtain the data that is shown in Figure 1C, 1F, and Supplementary Figure S1D, we conducted a mini-screen based on RNAi, and the slides were analyzed visually by inspecting a large number of cells under the epifluorescence microscope. Therefore, for this purpose, we relied on subjective quantification as done in Kotak et al., 2013; PMID: 23921553 and Sana et al., 2018; PMID: 30456393. However, based on this critical remark, we have now tried to explain this subjective quantification by referring to the images in Figure panels. For instance, please see Figure legend for Figure 1 on p. 37. We have also replaced the word significant with the 'moderate', and we thank the reviewer for alluding this mistake. However, we like to mention that all positive candidates obtained in such initial screens were not only analyzed by such a descriptive method but also by quantifying the signal intensity in the cells. As mentioned by the reviewer, we have also removed 'significant number of cells', and directed the readers to the appropriate Figure and related Figure legend.

2) Fig. 1I/J: It appears that Cdk1 inhibition reduces the degree of NuMA T2055 phosphorylation by a little more than a factor of 2. Where does the remaining phosphorylation come from? Concomitant knockdown of the B55gamma subunit does not seem to change this much (according to the bar graphs), the decrease is now a little less than a factor of 2. Does this experiment really support the conclusion that B55gamma is the major regulatory subunit for NuMA dephosphorylation by PP2A? Please explain why the B55gamma effect in this experiment is less striking than in Fig. 1D/E.

Response. We thank the reviewer for this excellent point. We inactivated Cdk1 acutely for only 5 min in our analysis. Thus the remaining phosphorylation could be because of residual Cdk1 activity. Inactivation of Cdk1 leads to dephosphorylation of pT2055, but due to partial inactivation of Cdk1 for 5 min, some pT2055 signal is still retained. We know from our previous work that longer incubation with Cdk1 inhibitor fully dephosphorylate pT2055 (Kotak et al., 2013; PMID: 23921553). The reviewer is right that the *B55*γ or PPP2CA depletion in cells acutely inactivated with Cdk1 significantly, but not fully rescues the pT2055 mark (Figure 1 and Supplementary Figure S1) at the spindle poles. We believe this is because of partial depletion of PPP2CA or *B55*γ, that is often the case in siRNAs-mediated depletion conditions. The other explanation could be that some other phosphatase, together with *B55*γ could be involved in T2055 dephosphorylation. However, this seems unlikely to us since in our screen where we depleted all the catalytical subunits, and the corresponding regulatory subunits, only PP2A and *B55*γ stabilized the pT2055 signal.

Technical questions

For fluorescence intensity quantifications at the spindle poles, do the authors use an image taken in one z-position or an image created from a z-stack? In the first case, one would have the concern that intensity differences may arise from the pole being more or less positioned in the focal plane.

Response. For analyzing the signal intensity at the spindle poles, we utilized the maximum intensity projected images from z-stacks. We would like to mention that a similar quantification approach was recently utilized in Sana et al., 2018 (PMID: 30456393), and this method is explicitly described on p. 25-26 in the materials and methods section.

For fluorescence intensity quantifications at the cortex: the intensity seems to fluctuate locally. Is an average intensity at the cortex for one-half cell calculated (or for an entire cell - if it is round as in Fig.4)? The authors often indicate in their figure only a small area of the cortex by a rectangle. If the intensity was only computed in this part of the cortex, how was the position chosen? Will measurements at other positions produce the same intensities? How exactly is the background intensity treated to arrive at the reported intensities?

Response. This is again an important point that is made by the reviewer. The reviewer is absolutely right in mentioning that the cortical intensity seems to fluctuate locally. We know from our previous work, and work from other groups that cortical NuMA is significantly enriched at the polar cortical region both in metaphase and anaphase (Woodard et al., 2010; PMID: 20479129; Kotak et al., 2013; PMID: 23921553; Kiyomitsu and Cheeseman, 2013; PMID: 23870127). Therefore, to quantify the

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signal at the cell cortex, we utilized a plane from the optical sectioning of an image where the cortical intensity is brightest. We choose a defined area of the cell cortex (52 μm^2) in an unbiased manner from control and experimental data set, and divided this intensity value with the mean intensity value of the cytoplasm (equal area). We have tried to explain this in the materials and methods on p. 24. A similar approach was used for cortical quantification in Kotak et al., 2014; PMID: 24996901, and Sana et al., 2018; PMID: 30456393.

Minor points

Is Fig. 1B useful?

-We agree with the reviewer that this information is not so informative, and thus as suggested, we have omitted this in the revised figure panel.

At several instances, the text would profit from correcting grammatical mistakes (e.g. use of plural/singular).

Thank you so much, we have now carefully gone through the entire manuscript, and we have corrected the paper for the grammar-related errors.

Reviewer #2

Likewise, this reviewer recognizes that 'the authors identify the phosphatase complex required for dephosphorylation…and this regulates NuMA cortical function'. However, they have raised a few major and minor concerns that we have dealt with in the revised version, as explained below.

Major concerns:

1a) Polybasic residues around the T2055 site were suggested to be important for recognition by the phosphatase complex. The authors need to demonstrate that these mutations do not affect cortical localization through direct effects on phospholipid interactions. Ideally this would be through phospholipid interactions which the PI has performed in previous work, or by demonstrating that Thr to Ala mutation of T2055 is able to rescue the cortical localization of the polybasic mutant.

Response. We thank the reviewer for raising this point and their suggestion. Prompted by this suggestion, we have now introduced Thr to Ala mutation at 2055 in one of the polybasic mutant (RR>AA) (please see below).The data obtained from this analysis clearly demonstrate that the addition of alanine significantly rescues the loss of cortical localization seen upon the expression of the polybasic mutant. This data is shown below, and now added to the new Supplementary Figure S5A-S5D, and discussed on p. 12. In the current circumstances, unfortunately, we could not generate T2055A mutations in all the polybasic mutants because of the unavailability of the primers, and sequencing facility. However, since RR>AA, and 4KR>AA phenocopy each other in terms of cortical NuMA localization, we believe that 4KR>AA would also be rescued by the T>A substitution at 2055 position.

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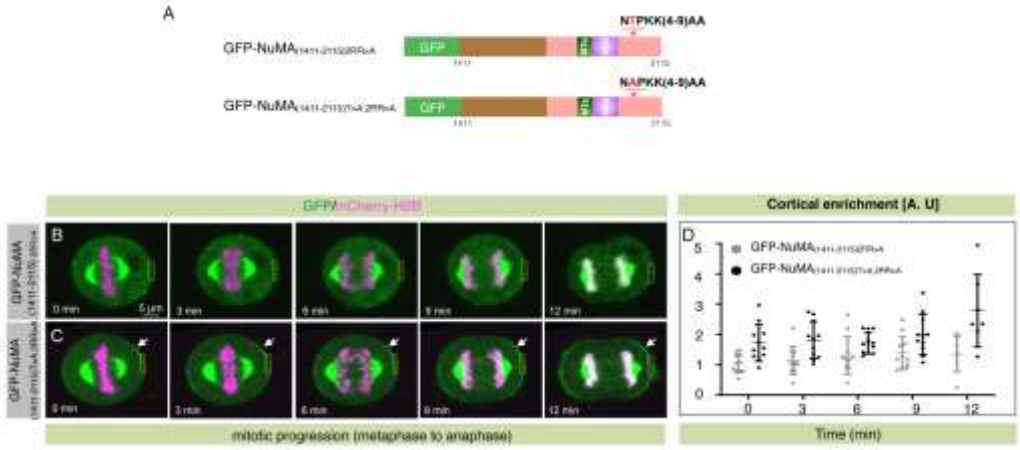


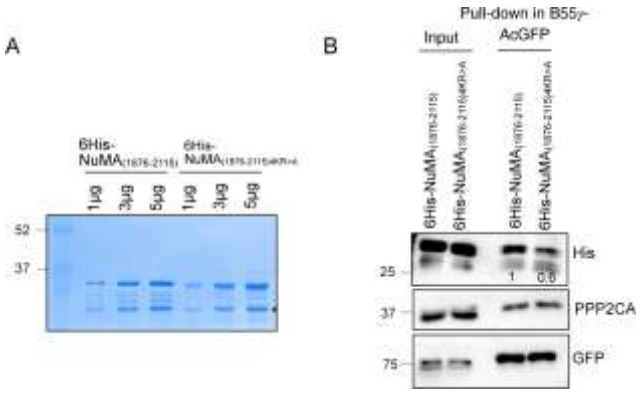
(A) Schematic of AcGFP-NuMA$_{(1411\text{-}2115)2RR>A}$ and AcGFP-NuMA$_{(1411\text{-}2115)T>A;2RR>A}$ construct with mono FLAG (FL) and AcGFP-tag at the N-terminus (A).

(B, C) Images from time-lapse microscopy of HeLa cells stably expressing mCherry-H2B and were transfected either with GFP-NuMA$_{(1411\text{-}2115)2RR>A}$ (B) or GFP-NuMA(1411-2115)T>A;2RR>A (C). The GFP signal is shown in green, and the mCherry signal is in pink. Note the significant loss of cortical NuMA in cells expressing GFP-NuMA$_{(1411\text{-}2115)2RR>A}$. Also, note the considerable rescue of the cortical signal in cells that were expressing GFP-NuMA$_{(1411\text{-}2115)T>A;2RR>A}$ construct wherein addition to the 2RR>A mutations, T2055 was mutated to alanine.

(D) Quantification of cortical enrichment on the right for cells that underwent metaphase to anaphase transition (shown in B, C); see Experimental procedures. $p<0.01$ between GFP-NuMA(1411-2115)2RR>A and GFP-NuMA$_{(1411\text{-}2115)T>A;2RR>A}$ at different time interval as indicated. (error bars: s.d.).

1b) Additionally, it would greatly strengthen the manuscript to directly show that the polybasic mutant no longer physically interacts with the phosphatase complex.

Response. We have followed the suggestion of the reviewer, and we have now tested the interaction between the polybasic mutant and *B55γ*. Results gained from this analysis clearly show that in comparison to the wild-type NuMA fragment, the polybasic mutant is significantly weak in its interaction with the *B55γ*. However, we still found noted some weak interaction between poly-basic mutant protein and *B55γ* in this *in vitro* setting. This data is shown below, and now added to the Supplementary Figure S5E and S5F and discussed on p. 12 of the revised manuscript



(A) Coomassie-stained gel for the wild-type [6HIS-NuMA$_{(1876\text{-}2115)}$] and mutated NuMA fragments [6HIS-NuMA$_{(1876\text{-}2115)4KR>A}$] that were generated and purified from *E.coli* (E). Please note that 6HIS-NuMA$_{(1876\text{-}2115)}$, and 6HIS-NuMA$_{(1876\text{-}2115)4KR>A}$ are unstable, therefore the occurrence of two species in Coomassie-stained gel.

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(B) The above mentioned recombinant proteins were incubated with the mitotically synchronized HeLa cells extracts made from cells that were stably expressing B55γ-AcGFP followed by co-immunoprecipitation (IP) using GFP-Trap. Resulting blots were probed for antibodies raised against 6HIS-tag, PPP2CA, and GFP. NuMA, and GFP as indicated. IN (1% of total), IP: 30% of the total. Please note significantly weak interaction between B55γ and 6HIS-NuMA$_{(1876\text{-}2115)4KR>A}$.

2) Throguhout the introduction and results, a clear description of the different requirements for NuMA localization to the cortex in metaphase and anaphase (LGN dependence, etc.) should be given. Metaphase localization is normally thought to be LGN dependent and insensitive to phosphorylation. Thus it is somewhat surprising that effects in metaphase as well as anaphase (where the dephosphorylation is essential for cortical localization) are reported.

Response. We apologize for not having been sufficiently clear in our earlier manuscript draft that may have caused some misunderstanding with respect to the importance of T2055 phosphorylation for NuMA cortical localization in metaphase and anaphase. We have shown earlier (Kotak et al., 2013; PMID: 23921553) that endogenous pT2055 NuMA species (detected by the phosphospecific NuMA antibody) do not localize at the cell cortex in metaphase. Furthermore, acute inactivation of PP2A using CalA impacted cortical NuMA localization in metaphase. In addition, cells expressing a phosphomimetic mutations (T>D or T>E) in NuMA at T2055 fail to localize NuMA at the cell cortex, in metaphase as well as in anaphase (Kotak et al., 2013; PMID: 23921553 ; Seldin et al., 2013; PMID: 24109598). Overall, these data suggested that NuMA must be in dephosphorylated state at T2055 for its cortical localization both in metaphase (LGN- dependent) and anaphase (phosphoinositides-dependent). Therefore, in our analysis to identify the PP2A-based holoenzyme complex that acts on T2055 residue of NuMA, we relied on two assays: (a) checking the NuMA cortical localization at the metaphase in the absence of the relevant subunits of the PP2A-based phosphatase complex (b) stabilization of pNuMA at T2055 in anaphase like condition upon RNAi-mediated loss of the relevant subunits of the PP2A-based phosphatase complex. In the revised version, we have altered the corresponding section in the introduction to explain this in a clearer manner.

Controls/Quants. A number of essential controls are missing throughout the mansucript. 3a) knockdown efficiency of B55 is not demonstrated.

Response. We tried a couple of commercially available antibodies, but we failed in detecting a specific band at a size corresponding to *B55*γ in western blot analysis. Therefore, we could not determine the *knockdown efficiency of B55*γ using immunoblot or immunostaining. However, to ensure that the loss of *B55*γ lead to the proposed phenotypes (loss of cortical NuMA, and stabilization of pNuMA upon RO-3306 treatment), we made the usage of multiple siRNAs. The information about the siRNAs was provided in the materials and methods section and Supplementary Table 1.

3b) Experiment in 2C does not include a negative control.

Response. Thanks, we have now added a negative control in the 2C experiment.

3c) Figure 2M requires quantitation.

Response. Thanks again, we have provided quantification with 2M, and also in all the western blots where we have conducted dephosphorylation reaction.

3d) the dephosphorylation assay in 2I does not have a negative control.

Response. We thank the reviewer for asking this control; a similar request was also made by the third reviewer (please see major point 1a of the third reviewer). Prompted by this comment, we have now conducted a dephosphorylation reaction with the immunoprecipitated fraction obtained using GFP-trap from the HeLa cells, stably expressing GFP-CAAX protein. As the reviewer will notice that in comparison with the AcGFP-*B55γ* IP fraction (Figure 2J), the IP fraction obtained with GFP-CAAX line is unable to dephosphorylate Cdk1-phosphorylated NuMA at T2055. This data is shown below and now added to the new Supplementary Figure S2B and S2C, and discussed on p. 9.

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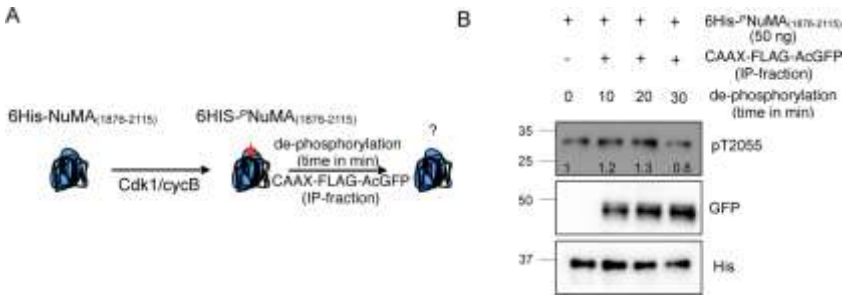

(A, B) Schematic representation of the *in vitro* dephosphorylation assay whereby Hexa-histidine-tagged $NuMA_{(1876-2115)}$ fragment [6HIS-$NuMA_{(1876-2115)}$] which was phosphorylated by Cdk1/cycB was incubated with the IP fraction from HeLa cells stably expressing CIBN-CAAX-FLAG-AcGFP [CAAX-FLAG-AcGFP] (B). Dephosphorylation reaction with Cdk1/cycB phosphorylated 6HIS- $NuMA_{(1876-2115)}$ with CAAX-FLAG-AcGFP IP fraction at a different time as indicated (C). Note that in this and other dephosphorylation experiments, values below the pT2055 western blot represent the band intensity with respect to the initial intensity value, which was kept as 1.

Other concerns

-The manuscript needs editing for grammar.

Thanks, as the reviewer will notice that we have substantially edit our revised manuscript for the errors related to grammar.

-Unless knockdown efficiencies of all phosphatase subunits have been assayed, it is important to state that there are potentially false negatives in the dataset.

As suggested by the reviewer, we have now added this information, thanks.

-The experiment in Figure 2L is hard to interpret. Does the antibody sterically hinder association?

The reviewer is right; we have utilized GFP-antibody to sterically hinder the association of the phosphatase complex that contains GFP-tagged *B55*γ (B55γ-AcGFP) with the phosphorylated NuMA in dephosphorylation reaction.

-Supplemental Figure S4 - why is there still significant T2055 signal in the NuMA RNAi line? Also, is the difference between the last two lanes in S4E significant?

NuMA, in general, is a dramatically hard protein to deplete completely from the cells using siRNAs. Similar to our previously published studies (Kotak et al., 2014; PMID: 23921553; Sana et al., 2018; PMID:30456393), here we have utilized siRNAs targeting 3'UTR of NuMA to knock down the endogenous protein so that we can see the analyze the rescue with the transgenic NuMA expression. We have also calculated the significance between the last two lanes in S4E (now Supplementary Figure S5), and the data reveal that values are statistically significant ($p<0.0001$). This is an expected outcome as the 4KR>A mutation should block efficient dephosphorylation.We have added this information in the Supplementary Figure S5K and discussed this in the corresponding Figure legend.

Reviewer #3

This reviewer recognized that *'this manuscript represents an advance…..the authors deserve credit for trying hard to do broad range of experiments merging cell biology and analytical in vitro phosphorylation analysis'* but requested that some outstanding issues should be addressed, that we have tried to address as mentioned below.

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Major points:

1a) In Figure 2, GFP-tagged B55gamma co-IPs NuMA and PP2catalytic subunit. However, no phosphatase-dead control, or beads alone are used in these experiments, which makes the conclusions problematic.

Response. We thank the reviewer for asking this critical control experiment. To address this issue, we have performed an immunoprecipitation experiment using cells that are expressing AcGFP-CAAX protein or AcGFP. In comparison with the data obtained B55γ- AcGFP IP, none of these immunoprecipitates showed interaction with NuMA, suggesting that the B55γ-AcGFP association with NuMA is not due because of the presence of GFP, or GFP-Trap beads in these experiments. For consistency, we have shown the results only from the cells expressing AcGFP, and these results are now added to the new Supplementary Figure S3A.

1b) Related, Figure 2K is an unconventional approach: why would GFP antibody mask the phosphatase activity? Why not add an inhibitor of the phosphatase or use other controls to demonstrate that the dephosphorylation is direct?

Response. We apologize for not having been sufficiently clear in our writing that may have caused some misunderstanding. We initially utilized immunoprecipitates from a cell line that stably expresses GFP-tagged B55γ (B55γ-AcGFP) for dephosphorylation reaction. We reasoned if the dephosphorylation is dependent on a direction interaction between *B55*γ and pT2055, than masking this recognition using an antibody raised against GFP, should perturb this. Indeed, this is what we found in our experimental set- up (Figure 2L). Moreover, as suggested by the reviewer,we also had an another control previously. We utilized B55γ-AcGFP immunoprecipitates from cells that were depleted of catalytical subunit PPP2CA, and we found that loss of PPP2CA dramatically impairs dephosphorylation reaction (Figure 2M). This experiment is somewhat analogous to as suggested by the reviewer i.e., usage of inhibitor. However, we believe that our experiment is more specific as we were specifically depleting PP2A catalytical subunit, in contrast to treating the immunoprecipitates with phosphatase inhibitors, which may have some non-specific effects.

1c) Would any CDK1-mediated phosphosubstrate be dephosphorylated?

Response. We thank the reviewer for this interesting suggestion. However, we are of the opinion that in the present circumstances doing this experiment is out of the scope of this current paper. However, prompted by the reviewer comment (see our response to point

2), we have now tested the specificity of B55γ and compare this with B55α. This new data clearly shows that B55γ is remarkably efficient in dephosphorylation in contrast to B55γ. We know from several published studies, that B55α-based complex can efficiently dephosphorylate several Cdk1 phosphorylated substrates for instance PRC1 (Cundell et al., 2013; PMID:24120663). However, we found that B55γ, but not B55α is capable of efficient dephosphorylation of T2055. This observation suggests that different Cdk1- substrates may not get recognized by the same B55 subunits, and thus divergent B55 may have remarkable specificity.

1d) Most notably, testing of the 2K or 4KR mutant in this assay would prove that 1) CDK1- mediated T2055 still occurs and 2) that the phosphatase in the IP can't now dock and dephosphorylate this site. This experiment would add considerably to the manuscript. It would also prove that the pT2055 antibody can still recognise the 2KK or 4KR protein.

Response. We are grateful to the reviewer for bringing up this important point. However, we like to mention that we have shown in the earlier version of the manuscripts that 4KR mutant can get phosphorylated at T2055 *in vivo* (now new Supplementary Figure S5G- S5K). To further strengthen this, we have now tested T2055 phosphorylation at cells over- expressing 4KR mutant. As shown below, this data clearly shows that 4KR (and likely 2KK, or 2RR) are not deficient in phosphorylation at T2055. We are of the opinion that these data are redundant with Figure S5G-S5K and therefore we have decided not to add this in the revised manuscript, if the reviewer thinks otherwise, we will be happy to add this data.

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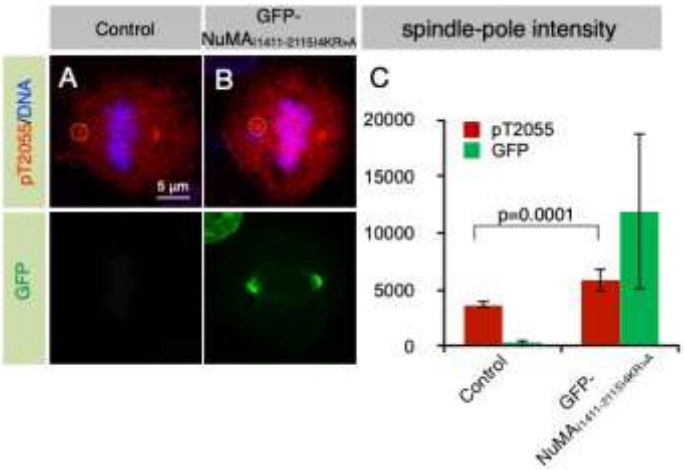
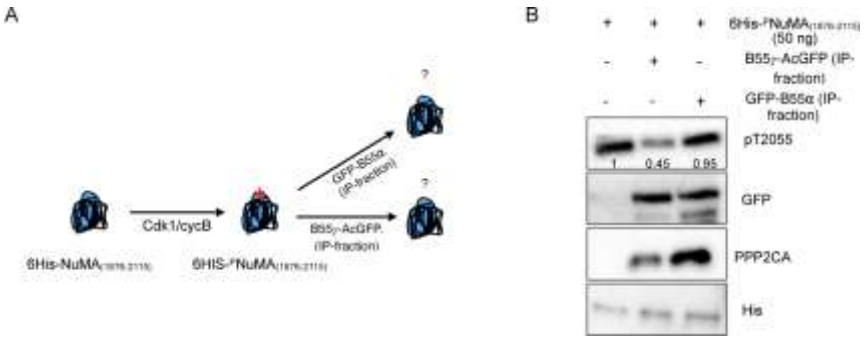


(A-C) HeLa' Kyoto' cells in metaphase which were either non-transfected (Control) (A), or transfected with GFP-NuMA(1411-2115)4KR>A plasmid (B). Cells were fixed after 36 hr of transfection and thereafter stained for pT2055 (red) and GFP (green). Quantification on the right reveals the spindle pole intensity of pT2055 under these conditions (C). Note that cells expressing GFP- NuMA(1411-2115)4KR>A are sufficiently phosphorylated by Cdk1 at; error bars: s.d.

Moreover, prompted by the second reviewer comment (point 1b), we have now tested the interaction of the wild-type NuMA fragment, and 4KR mutant, and we found that 4KR mutant of NuMA weakly associate with B55γ. These data are now added to the Supplementary Figure S5E and S5F and discussed on p. 12 of the revised manuscript.

2) Given the clear data presented in Figure 1, the authors need to repeat their biochemical analysis with recombinant NuMA with a different B complex (B55alpha/beta), which does not show up in the initial screen. Like 1) above, this would add considerably to the manuscript.

Response. We thank the reviewer for this great suggestion. As suggested, we have conducted dephosphorylation reaction with the immunoprecipitates obtained from the mitotically synchronized cells that were expressing B55α. Interestingly, we uncovered that B55α cannot efficiently dephosphorylate pT2055 *in vitro* (please see below). This data suggest that Cdk1-substrates requires divergent B55 subunits for efficient dephosphorylation. We thank the reviewer for asking this essential experiment, and these results are now added to Supplementary Figure S3D and S3E, and discussed on p. 9 of the revised manuscript.



(A, B) Schematic representation of the *in vitro* dephosphorylation assay as mentioned for Figure 2. However, here the dephosphorylation reactions were performed by either incubating with B55γ-AcGFP IP fraction or GFP-B55α IP fraction for 10 min (A). Dephosphorylation reaction with Cdk1/cycB phosphorylated 6HIS-NuMA$_{(1876-2115)}$ either with the B55γ-AcGFP IP or with GFP-B55α IP (B). Please note almost negligible dephosphorylation potential of the B55α in contrast to B55γ.

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Please also check the similar suggestion made by the second reviewer (point 3d) where the reviewer asked us to include a negative control in our dephosporylation reaction. For this we have included dephosphorylation reaction with the IP fraction obtained from the cells expressing CAAX-FLAG-AcGFP. There we uncovered that a non-specific protein such as CAAX-FLAG-AcGFP is incapable of dephosphorylating pT2055.

Minor point:

-p.4 'Weak and enrich'. What does weak mean in this context?

Thanks, weak in our sentence meant that NuMA levels are low in metaphase, and its levels are increased in anaphase. We have now changed this sentence a little bit to express this in a clearer manner.

-p.5 'That is consists of', then the authors fail to mention the catalytic subunit when describing the trimeric complex!

Thank you so much for mentioning this; this is now corrected in the revised manuscript.

-Figure 1A should label the regulatory (B) subunit

Thanks, corrected

-p.8 B55gamma and PPP2R1B siRNA decrease pT2055. This does not prove it is direct, as stated

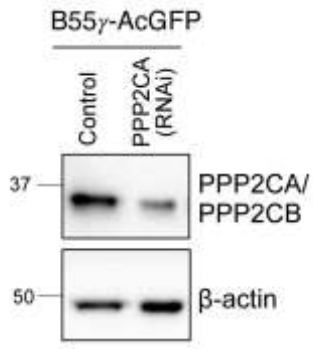
Thanks again, corrected.

-Why is it remarkable that the IP'd complex dephosphorylates CDK1-phosphorylated NuMA? What else was tested?

Thanks, we have now modified this sentence a bit, and we are hoping that the current text sounds logical. As mentioned earlier, we have now tested the IP fraction made from cells either expressing AcGFP-FLAG-CAAX or GFP-B55α and used this fraction for the dephosphorylation assays.

-How do we know that the experiment in Figure 2M lacks PP2CA catalytic subunit?

Thanks for this remark. In our laboratory as a routine work if the antibodies are available we always check the depletion efficiency in our siRNAs-based experimental set-up. It is usually done by western blot analysis or western blotting. In the previous version of the manuscript, we did not include the data related with PPP2CA depletion in AcGFP-B55γ transgenic line. However, as requested by the reviewer we have included this data as a Supplementary Figure S3F. Please note that PPP2CA antibody that we have in the lab (Cell Signalling; 2038S) also recognizes PPP2CB, and thus, the residual protein band may reflect PPP2CB as both subunits have a similar molecular weight.



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-In an artificial anaphase, induced with kinesin 5 inhibitor STLC and subsequent RO- 3306 addition, siRNA of B55gamma or other members of the complex, change cortical NuMA. Wouldn't RO-3306 addition do this anyway independent of cells being forced to exit mitosis, since this is a CDK1 site?

This is again an excellent point made by the reviewer. In a good fraction of cells that were depleted for B55γ by two different siRNAs, we observed chromosomes instability, and thus these cells which are presumably strongly depleted for B55γ were not progressing to anaphase. Similar observations were made in cells depleted for the catalytical subunit PPP2CA. Therefore, to analyze an impact of B55γ depletion on anaphase like condition, we decided to look at cortical NuMA in (control and B55γ depleted) cells which are blocked in prometaphase like stage (using overnight treatment with STLC), and are forced to exit mitosis so that we get Cdk1 inactivation in a synchronized mitotic population. Of course, as the reviewer expected we know that that Cdk1 inactivation at any stage (prometaphase or metaphase) of mitosis, gives similar outcome as shown earlier in Kotak et al., 2013; PMID: 23921553.

-p.12 2KK becomes 4KR in GFP-NuMA construct. This is confusing for readers, but I think it makes sense overall. Why not use these in the pull-down and dephosphorylation scenario discussed in Figure 2 (see major points, above)

A similar remark was made earlier, (please see point 1b of the second reviewer). In the revised manuscript, we have now tested the interaction of the wild-type NuMA fragment, and 4KR mutant, and we found as expected 4KR weakly associate with B55γ (please see new Supplementary Figure S5E and S5F).

- p.16. Why is the B55gamma-PP2A complex 'unique'?

We described this complex unique because, to the best of our knowledge, this will be a first report where B55γ is linked to a mitotic substate (NuMA), also, it appears that it functions independent of the Greatwall kinase pathway.

10. Discussion is clear and succinct.

Thank you so much!

11. Tenses change throughout the manuscript.

Corrected, thanks!

Second decision letter

MS ID#: JOCES/2020/243857

MS TITLE: PP2A-B55γ counteracts Cdk1 and regulates proper spindle orientation through cortical dynein adaptor NuMA

AUTHORS: Riya Keshri, Ashwathi Rajeevan, and Sachin Kotak
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

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As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Cdk1-dependent phosphorylation of a particular site in NuMA (T2055) was previously demonstrated to negatively control cortex recruitment of NuMA and hence also dynein. Here, the authors identify the phosphatase complex that dephosphorylates NuMA at this particular site at the transition from metaphase to anaphase thereby promoting cortex recruitment of NuMA. They also show that flanking positively charged sites promote the efficiency of dephosphorylation. This manuscript provides new information on the biochemistry of the control of spindle positioning via dynein recruitment to the cortex. It is focused and useful for the field, filling a gap in our knowledge of the specificity of dephosphorylations.

Comments for the author

The authors have addressed most of my concerns. They can still be clearer about their categorizations and quantifications, which should be easy to address.

Regarding the categorizations in Fig. 1, the authors should please add a statement to the Legend or Method how exactly this was done. It appears that some criterion that can be described in words must have been used. The term "subjective quantification" in the response letter seems to be an oxymoron and does not promote clarity. If no criterion is provided, it seems that this part of the study remains ambiguous and cannot be reproduced by others. Claiming that the same procedure was used in previously published work does not appear to be a valid argument given that quality standards increase over time as our technical abilities (and expectations) advance. The authors should please also state what are the error bars in panels B and E. Which data sets were used to calculate them?

The authors did not respond to the question how the background correction was performed when quantifying the cortical intensity. They should please be more explicit in their Method on p24. Has background been subtracted from raw values or have raw values been divided by background? Has the background correction been done after or before calculating the ratio of cortex vs cytosol intensity.

Reviewer 2

Advance summary and potential significance to field

The manuscript is much improved and all my major criticisms have been dealt with.

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Comments for the author

The manuscript is much improved and all my major criticisms have been dealt with.

Reviewer 3

Advance summary and potential significance to field

This improved study shows that the phosphorylation of Thr2055 by CDK1 is counteracted by the B55gamma/PP2Ac complex. All my major and minor comments have been addressed appropriately, including changes to the text (highlighted in the new manuscript), both minor and major, and close attention to detail in terms of the Scientific English.

Comments for the author

This improved study, which subject to minor proofing is acceptable for publication, shows that the phosphorylation of Thr2055 by CDK1 is counteracted by the B55gamma/PP2Ac complex.
All my major and minor comments have been addressed appropriately, including changes to the text (highlighted in the new manuscript), both minor and major, and close attention to detail in terms of the Scientific English.

Second revision

Author response to reviewers' comments

Reviewer #1

We express our sincere gratitude to the reviewer for their kind remarks that '*This manuscript provides new information on the biochemistry of the control of spindle positioning via dynein recruitment to the cortex. It is focused and useful for the field, filling a gap in our knowledge of the specificity of dephosphorylations*' However, the reviewer raised a couple of minor concerns which we have addressed, as explained below.

Minor Concerns:

Regarding the categorizations in Fig. 1, the authors should please add a statement to the Legend or Method how exactly this was done. It appears that some criterion that can be described in words must have been used. The term "subjective quantification" in the response letter seems to be an oxymoron and does not promote clarity. If no criterion is provided, it seems that this part of the study remains ambiguous and cannot be reproduced by others. Claiming that the same procedure was used in previously published work does not appear to be a valid argument given that quality standards increase over time as our technical abilities (and expectations) advance.

Response: We thank the reviewer for raising these points. The reviewer will notice that we have now explained more clearly in the respective Figure legends (1B, 1E, and S1D), how these quantifications were done. For instance, we have now mentioned that cells that were depleted for the given phosphatase in RNAi-screen were visually quantified by inspecting a large number of cells under the epifluorescence microscope. These categories either for cortical NuMA quantification (weak or absent) or for spindle pole pT2055 quantification (weak, moderate, or strong) are now better explained by referring to the images in the respective Figure panels. This will help readers to associate these categories with the data. We have also mentioned this quantification method on p. 24 in the revised materials and methods section.

The authors should please also state what are the error bars in panels B and E. Which data sets

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were used to calculate them?

Response: We further thank the reviewer for indicating this point. For panels 1B and IE, an RNAi-based mini-screen was performed thrice, and at every time, we visually analyzed more than 50 cells using the epifluorescence microscope. The error bars in Figure panels 1B and 1E are calculated from the [%] of cells for each category (weak and absent as in Figure 1B or strong, moderate, and weak as in Figure 1E) from three independent experiments. This information is now added to the associated Figure legend on p. 37, 38.

The authors did not respond to the question how the background correction was performed when quantifying the cortical intensity. They should please be more explicit in their Method on p24. Has background been subtracted from raw values or have raw values been divided by background? Has the background correction been done after or before calculating the ratio of cortex vs cytosol intensity.

Response: We apologize to the reviewer for not explaining this in our previously revised manuscript. For the background correction, the raw intensity values from the region of interest (ROI) that were used to measure cortical intensity (I_{cortex}) and cytoplasmic intensity ($I_{cytosol}$) were subtracted from the background intensity values (I_{bg}). The background intensity values were obtained using a similar area outside the cell. Thus, we used equation $(I_{Cortex} - I_{bg})/(I_{cytosol} - I_{bg})$ for calculating the cortical enrichment. We have explained this more clearly now in the revised material and method on p. 25, and we thank the reviewer for raising this concern.

Reviewer #2, and #3

We sincerely thank the reviewer #2 and #3 for their kind remarks on our revised manuscript.

Third decision letter

MS ID#: JOCES/2020/243857

MS TITLE: PP2A-B55γ counteracts Cdk1 and regulates proper spindle orientation through cortical dynein adaptor NuMA

AUTHORS: Riya Keshri, Ashwathi Rajeevan, and Sachin Kotak
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

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