

Activator mimicry by PP6 regulatory subunits directs Aurora A dephosphorylation in mitosis

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Prof. Francis A. Barr
University of Oxford
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South Parks Road
Oxford OX1 3QU
United Kingdom

5th Mar 2026

Re: EMBOJ-2026-124008

Activator mimicry by PP6 regulatory subunits directs selective dephosphorylation of Aurora A

Dear Dr. Barr,

Thank you for submitting your manuscript to our editorial office. We have now considered the study within our editorial team, and unfortunately come to the conclusion that we cannot offer to publish it. We realize that you have further extended your work on Aurora A dephosphorylation by PP6 in the control of mitotic spindle size, by carefully dissecting Aurora A recognition determinants in specific PP6 regulatory subunits. However, we were not convinced that the results from these analyses have provided sufficiently striking new insights to warrant publication in The EMBO Journal at this point. As it stands, we note that functionally, the consequence of PP6R3 depletion on Aurora A activation and nuclear morphologies had already been reported by Heo et al 2020, while Kettenbach et al 2015 already established that PP6-PP6R2 can dephosphorylate Aurora A unless kept in check by Plk1. While we appreciate that your detailed mapping and mutational characterization, guided by AlphaFold models and reported structures and validated by motifs swaps between PP6R2 and PP6R3, has allowed you to define differential interaction determinants and propose the potentially interesting concept of activating-subunit mimicry, we feel that this -although certainly interesting to the field- does not offer a compelling advance over your and others' various earlier works regarding the functional/regulatory connections or physiological outcomes of Aurora A/Plk1/PP6 interplay.

Given the relevance of the topic and the potential interest of the molecular analyses, we feel that your study might nevertheless be suitable for our editorially independent sister journal, EMBO Reports. If you would be interested in the EMBO Reports editors taking a look at your study, you may simply use the hyperlink below to transfer your submission over to them; no reformatting would be required. Please understand, though, that I am not in a position to predict the outcome of any assessment by EMBO Reports.

I am sorry that I cannot be more positive for The EMBO Journal on this occasion.

Yours sincerely,

Hartmut Vodermaier

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
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Link Not Available

Dear Hartmut

Given other recent work published in the EMBO Journal by the Pintard and Musacchio groups I am really surprised and disappointed by this decision.

Pintard and Musacchio described the mechanistic basis for Aurora A regulation of Plk1 and the requirement for the protein Bora using very similar approaches to our work. The roles of all those proteins in cells as key players in mitosis was known and their studies build on many previous studies of Bora, Aurora A and Plk1. Their conceptual advance was the demonstration that Bora positions the Plk1 T-loop so that it can be phosphorylated by Aurora A. That was important and to my mind justified publication in the EMBO Journal.

We have a similar situation in that the key point is that the mechanism of Aurora A regulation by PP6 is not known. To draw a parallel to the Musacchio and Pintard papers, we show how binding of Aurora A to PP6 positions it to promote dephosphorylation. This is achieved via mimicry of the activation proteins for Aurora A which is a conceptual advance with broad implications for future work.

In your decision letter, the two papers from Heo and Kettenbach are mentioned out of context since neither investigate the mechanism of PP6 recognition of Aurora A. Kettenbach propose that Plk1 blocks PP6 activity by phosphorylation not by binding to the Aurora A binding site which our data clearly demonstrates.

I hope that you strive for consistency in editorial decision making and will reconsider the decision. If reviewers in the field agree with your assessment then we will have to accept their decision. If not, then the EMBO Journal will be home to an important study on cell cycle biology.

Best wishes Francis

Francis Barr

EP Abraham Professor of Mechanistic Cell Biology

Department of Biochemistry & Trinity College, University of Oxford,
South Parks Road, Oxford, OX1 3QU

Dear Francis,

Thank you again for submitting your manuscript on PP6 and Aurora A dephosphorylation to The EMBO Journal. I have now received feedback from three expert referees, copied below for your information. The referees all acknowledge the importance and potential interest of the work, but also raise a number of substantive concerns. In particular, the two reviewers with structural/biochemical expertise (refs 1 and 3) bring up important issues related to the structural modeling and its validation. Likewise, all three referees are missing follow-up investigation of PPP6R3 competition with Aurora interactors such as TPX2.

With the focus of this study, as discussed, being the mechanism of PP6 recognition of Aurora A and the molecular positioning promoting dephosphorylation, rather than their general interplay, I feel that strengthening these aspects would be crucial for further consideration at our journal. I would therefore invite you to get back to me with a tentative point-by-point response proposing how you might answer each of the referees' criticisms, so that I could get a better understanding of whether a revision for The EMBO Journal would seem realistic, or whether a less substantively revised, toned-down version would be better suited for rapid publication elsewhere, e.g. in our sister journal EMBO Reports. I would appreciate if you could send me such a revision plan by mid-next week.

Looking forward to hearing from you,

Best regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

Referee #1 (Report for Author)

Precisely orchestrated mitotic spindle assembly and stabilisation are crucial for accurate chromosome segregation. Aurora A kinase is a conserved major regulator of the mitotic spindle. Aurora A localisation and activation are modulated by various regulators such as

TPX2, CENP192, TACC3 and phosphatase PP6. While mechanisms influencing Aurora A localisation and activation, the latter via T-loop phosphorylation, are well studied, mechanisms that counteract Aurora A activation by specifically dephosphorylating the T-loop are not well understood. This is an important knowledge gap, as a precise balance of kinase-phosphatase activity is critical for the stabilisation of a correct spindle structure suitable for facilitating accurate segregation of chromosomes to the daughter cells. Here, Yang et al. show, by combining biochemistry, structural modelling and functional rescue assays, that PP6 holoenzymes containing regulatory subunits R2 and R3 are capable of directly interacting and dephosphorylating the Aurora-A T-loop phosphorylation in vitro. However, in cells, the interaction of PP6-R3 with Aurora-A is the sole contributor to Aurora-A T-loop dephosphorylation. Their data also suggest that PP6-R3 facilitates this by directly interacting with Aurora-A via its C-terminal disordered region, namely A- and P-boxes. This mode of binding appears to mimic that of Aurora-A activators such as TPX2. Their data suggest that R3 A-box interaction is also required to selectively dephosphorylate Aurora A T-loop. As far as R2 is concerned, they show that, though R2 can interact with Aurora A in vitro (likely via the R2 P box), in the cellular context this interaction is outcompeted by PLK1, which interferes with R2 binding to Aurora A (via a possible molecular mimicry involving the R2 P box).

Strength: The findings that PP6-R3 is the sole contributor for dephosphorylating Aurora A T-loop and PP6-R2 - PLK1 interaction inhibit PP6-R2-mediated dephosphorylation of Aurora T loop.

Weakness: In the current form, conclusions around the structural basis for R3 binding to Aurora A and molecular mimicry as a rationale for PLK1-mediated PP6-R2 are not convincing and would require further biochemical and structural characterisation. Also, in the absence of strong evidence that the putative PLK1 PBD motif of R2 is phosphorylated in cells (or even in vitro biochemical analysis of the molecular interplay among phosphorylated R2, PLK1 PBD, and Aurora A), the proposed mechanism remains speculative or correlative.

Concerns:

The predicted structures from AlphaFold 3 modelling need to be accompanied by their PAE plots. It is also important to include the pTM and iPTM scores. Without explicit statements on the confidence scores, it is difficult to assess the conclusions or the structural rationale provided.

Close-up views of the predicted binding interfaces, with amino acids making direct contacts shown in stick representation, will help appreciate the nature of the binding interface.

R3-Aurora A interaction: According to the models, both A and P boxes contribute to binding; however, the cellular biochemistry suggests that the interaction is mainly mediated via the A box. It would be helpful to describe the nature of the interaction interfaces - contribution of A and P boxes to the total buried surface area of the complex will be informative.

Biochemical evaluation of the direct interaction between R3 A box and Aurora A - It is not clear why the Coomassie gel is not shown. As this experiment was carried out using recombinantly purified proteins, the Coomassie-stained SDS-PAGE of the pull-down will strengthen the conclusion.

Figure 5. It is not clear if the Aurora A binding modes of R3 514-616 (shown in Fig 5A) and R3 557-616 (shown in Fig. 5D) are identical or different, and why two different AF3 models were generated?

Mutating the Cysteine residue (C519) to a Histidine in the 'CHIH' motif in the context of A box abolishes R3 interaction with Aurora-A. It is unclear whether the predicted structural model can explain this observation. The C159 is not highlighted in any of the structural models, and the text also does not describe the intermolecular contacts mediated by this Cys residue.

The models used in the manuscript appear to suggest that there will be partial overlap between the TPX2 binding interface and R3 binding interface on Aurora A. If this is correct, will R3 binding to Aurora-A interfere with TPX2 binding? Have the authors attempted to model R3-Aurora A binding in the presence of TPX2?

What is the amino acid sequence similarity/identity between the A and P sites of R2 and R3? Can the authors provide any reasoning for the variations in the Aurora A binding mode of R2 and R3?

The speculation that R2-PLK1 binding interferes with R2-Aurora A interaction (as the P box region is involved in both PLK1 and Aurora A binding) needs to be supported by further competitive binding analysis using recombinantly purified proteins in either Size Exclusion Chromatography (SEC) or affinity pull-down experiments.

Figure EV8A shows that R2 447-966 (a fragment containing both A and P boxes) can interact with both Aurora A and PLK1. Won't this contradict the idea that Aurora A and PLK1 binding regions R2 overlap, and PLK1 binding will likely interfere with Aurora A binding to R2? Or PLK1 binding remodels R2-Aurora A interaction? Was there any attempt to model the Aurora A - PLK1 - R2 complex using AlphaFold?

Typos:

Line 231: '.... box A, as the minimal Aurora B-binding domain in PPP6R3' - it has to be Aurora A.

Line 381: 'whereas 724AA mutant had not effect.' - 'no effect'

Referee #2 (Report for Author)

The authors describe the regulation of Aurora A in mitosis, looking into the specific contributions of PP6 trimeric holoenzymes. Using a microscopy image-based readout of mitotic progression and Aurora localization/phosphorylation, they authors find that PP6-R3 is the sole contributor to Aurora A dephosphorylation in vivo, whereas in vitro PP6-R2 can function as a phosphatase as well. Through elegant truncation and mutation analyses based on AF3 modeling, the authors pinpoint a region in the PP6-R3 (the A-box) that mediates the interaction between Aurora A and PP6. The authors further demonstrate that PP6-R2 does not interact with Aurora A in vivo due to the presence of a Plk1 docking site, which is suggested to outcompete Aurora A binding. Altogether, the authors have identified a motif in a proposed intrinsically disordered region of PP6-R3 that mediates binding with Aurora A and provides mechanistic insight in a biologically relevant regulatory mechanism. This is a strong and interesting piece of work and would be a great fit with EMBO J. Below are some suggestions for improving the manuscript and making it more accessible to the readers.

Major:

An interesting unexplored aspect is whether R3 competes with all known AurA interactors or only a subset. Would it be possible to test the ability of the AurA TPX2, CEP192 and TACC3 binding fragments their ability to compete with the R3-AurA interaction?

An important aspect missing is the affinity of the R3 and R2 binding elements for AurA and how this compares to known interactors such as TPX2. Since they have recombinant

proteins, I suggest measuring affinity of the interactions.

The proximity of the AurA binding site and the ANKRD binding site in R3 is intriguing and there seems to be an indication in their experiments that ANKRD binding to R3 might facilitate AurA binding. Have the authors tried to model the ANKRD interaction with the R3 binding region?

I could not find a useful excel file for the MS data provided.

Specific comments:

Line 24: Can the authors really claim that the A-box positions the T-loop for efficient dephosphorylation? There is no evidence provided on any conformational changes of the T-loop, so in my opinion this claim is not supported by experimental data.

Starting line 43: the description of Aurora-Plk1-PP6 interplay could use a schematic to help the reader. It is quite complex for the non-specialist.

Line 204-220: The AF3 models are nice and are well supported by actual experimental data in many cases. However, it would be important to report model confidence including iPTM and PTM scores, providing PAE plots as supplementary data. In addition, it would be great to see whether all 5 models return the same findings, or rather only a few do so.

Line 106: typo, dependant > dependent

Line 120-121: Figure 1B-D is an unusual way of showing statistical tests. Unclear what the comparisons are.

Line 158-172: Can you make a statement on Aurora A binding here?

Line 161-165: I do not understand this reasoning. If the equivalent relationship was observed for ANKRD's and PPR1-3, why would this explain one being a scaffold and the other not?

Line 221 and further: For the interaction experiments (3E, but mainly 3F), I had a hard time getting my head around the different domains. Would it be possible to use a graphical representation to label the different lanes, rather than their residue numbers? This can potentially be obtained by incorporating (a reworked version of) the graphical representation in fig 3B?

Line 256-278: Regarding the blots in Figure 4, does one of the antibodies (R3?) cross react with TPX2? Or why is it showing in all blots with R3? Is the epitope of the R3 antibody known? Could it be they have a shared binding Aurora A binding motif that the antibody recognizes?

Line 347: typo, phenylamine > phenylalanine

Referee #3 (Report for Author)

This interesting study follows on from previous work by the same team that identified PP6 as the key mitotic phosphatase for Aurora A. Here, the team has delved more deeply into the mechanism and made some important new discoveries. First, it is not only clear that PPP6R3 is the only regulatory subunit that acts on Aurora A in cells, but that (and how) PPP6R2 is blocked from this function by PLK1. This is an especially elegant part of the study. Second, Using AlphaFold3 to model the complex, the team made mutations in PPP6R3 that disrupt the interaction with Aurora A, which are then unable to sustain Aurora A dephosphorylation in cells. One of the mutations disrupts the interaction but enhances dephosphorylation, which is interesting but the mechanism is not nailed down. Another major strength of the study is the robust and beautiful cell work, especially with the knockout/rescue studies in Figure 8.

The study has some limitations that need to be addressed:

Line 199. Figure 3A does not show comparable amounts of PPP6C recovered from co-IPs, the R1 has a much more intense band. Is this because there is more R1 in the cell lysate, or does it suggest there is an excess of R2/R3 subunits that are not part of a holoenzyme complex with the catalytic subunit?

There is insufficient information about the AlphaFold3 models for the work to be reproduced or to have confidence in the result. Was the modelling done using the server or a local installation and what was the input (sequences, ligands, ions, PTMs)? Was the modelling done once or repeated. If repeated, how were models analysed and selected? What is the level of confidence in the modelling (pair-wise iPTM, ipSAE), and can the authors discuss how readers should regard the model (is it a possibility, is the main chain probably correct, are the side chains probably correct, which are the more reliable parts of the model). Will the models be included in the "source data"?

Another missing aspect of the paper is validation of the AlphaFold model from the Aurora A side. Can the interaction with PPP6R3 be disrupted with point mutations in Aurora A? Alternatively, the model predicts competition between PPP6R3 and other Aurora A interactors, notably TPX2. This would be easy to test in a competition binding/IP experiment.

The more detailed model figure EV5A shows Aurora A was doubly phosphorylated (T287, T288). Why was the modelling done this way, was it needed to make the modelling work?

T288 is the established, critical site for activation of Aurora A , and the experimental data in the study, quite rightly, focussed entirely on that modification. Could phospho-T288 fit into the PPP6C active site, perhaps in an alternative model? There is currently a disconnect between the structural modelling and the experimental data.

The experiment shown in Figure 4 is consistent with the C-terminal region of PPP6R3 as being critical for Aurora A dephosphorylation. An alternative explanation is that this truncated protein is less active overall than wild-type - can the authors rule out this possibility?

Figure 6 has interesting but seemingly contradictory results: the FA1 mutant has no Aurora A binding, and reduced Aurora A dephosphorylation activity; but the CA CH CCAA mutant also shows no Aurora A binding, but has increased Aurora A dephosphorylation activity. The suggested mechanistic explanation of product dissociation being limiting is interesting but further biochemical work is needed to support this conclusion. Is there any evidence that the CA CH CCAA mutant retains some binding to Aurora A? Is unphosphorylated Aurora A a potent inhibitor of Aurora A dephosphorylation

The study leaves one big question unanswered - how is the PP6 holoenzyme able to dephosphorylate TPX2/Aurora-A which is resistant to general phosphatases. Is it simply that it is a highly effective phosphatase for Aurora-A, or does the PPP6R3 interaction with the kinase somehow loosen TPX2/Aurora-A complexes. This could be addressed with further biochemical studies, and perhaps modelling, but certainly needs to be discussed in the text.

Minor errors:

Line 231, correct Aurora B to Aurora A.

Line 270, correct binging to binding.

Line 312, correct Aurora to Aurora A

Dear Hartmut

Thank you for your helpful decision letter and for sending the referees comments. I have taken the time to contact all the co-authors of the manuscript and obtain their views before replying. They felt the comments were fair and made good suggestions that we can follow to address the concerns that you highlighted.

To address the specific concerns raised we propose the add data showing:

- a. Competition binding assays showing that the binding site region of PPP6R3 can displace the main activator TPX2 from Aurora A.
- b. Aurora A binding affinity measurements using ITC for PPP6R3 and key mutants, compared to TPX2.
- c. Phosphorylation-dependent interaction of PLK1 with PPP6R2, including the identity of the kinase responsible.
- d. Confidence scores for AlphaFold3 models, and some additional modelling of the interactions between ANKRD and PPP6R3, and PPP6R2 with PLK1 and Aurora A.

We will need to rework some main and supplemental figures to include this information, so I have not at this stage provided the precise detail of where this data would be placed.

A more detailed explanation of the proposed major changes and initial response to the comments is provided in the accompanying file. Other corrections are fairly minor and will be addressed in full with revisions to the manuscript text and some figures to include additional schematics. We hope these combined changes which address the major and minor points raised would sufficiently satisfy the referees and give you the confidence to make a final decision on the suitability of our work for EMBOJ. This is the option my co-authors have asked me to support.

In terms of changes for EMBO Reports do you see these as mainly to the text plus changes to presentation of the figures? Based on the comments, I think that any revision needs to include the AF3 confidence scores for the models shown (d), and probably include some extra data on the PLK1-PPP6R2 interaction which we already have (c). If you strongly feel this is the more appropriate option, then let me know and I will speak with my co-authors again.

Best wishes Francis

EMBOJ-2026-124008R-Q. Correspondence with editor – initial author responses.

Referee #1 (Report for Author)

Precisely orchestrated mitotic spindle assembly and stabilisation are crucial for accurate chromosome segregation. Aurora A kinase is a conserved major regulator of the mitotic spindle. Aurora A localisation and activation are modulated by various regulators such as TPX2, CENP192, TACC3 and phosphatase PP6. While mechanisms influencing Aurora A localisation and activation, the latter via T-loop phosphorylation, are well studied, mechanisms that counteract Aurora A activation by specifically dephosphorylating the T-loop are not well understood. This is an important knowledge gap, as a precise balance of kinase-phosphatase activity is critical for the stabilisation of a correct spindle structure suitable for facilitating accurate segregation of chromosomes to the daughter cells. Here, Yang et al. show, by combining biochemistry, structural modelling and functional rescue assays, that PP6 holoenzymes containing regulatory subunits R2 and R3 are capable of directly interacting and dephosphorylating the Aurora-A T-loop phosphorylation in vitro. However, in cells, the interaction of PP6-R3 with Aurora-A is the sole contributor to Aurora-A T-loop dephosphorylation. Their data also suggest that PP6-R3 facilitates this by directly interacting with Aurora-A via its C-terminal disordered region, namely A- and P-boxes. This mode of binding appears to mimic that of Aurora-A activators such as TPX2. Their data suggest that R3 A-box interaction is also required to selectively dephosphorylate Aurora A T-loop. As far as R2 is concerned, they show that, though R2 can interact with Aurora A in vitro (likely via the R2 P box), in the cellular context this interaction is outcompeted by PLK1, which interferes with R2 binding to Aurora A (via a possible molecular mimicry involving the R2 P box).

Strength: The findings that PP6-R3 is the sole contributor for dephosphorylating Aurora A T-loop and PP6-R2 - PLK1 interaction inhibit PP6-R2-mediated dephosphorylation of Aurora T loop.

Weakness: In the current form, conclusions around the structural basis for R3 binding to Aurora A and molecular mimicry as a rationale for PLK1-mediated PP6-R2 are not convincing and would require further biochemical and structural characterisation. Also, in the absence of strong evidence that the putative PLK1 PBD motif of R2 is phosphorylated in cells (or even in vitro biochemical analysis of the molecular interplay among phosphorylated R2, PLK1 PBD, and Aurora A), the proposed mechanism remains speculative or correlative.

>> Multiple lines of evidence support our conclusions, so we do not agree with the view that the proposed mechanisms are overly correlative or speculative. We a perform combination of binding and phosphatase assays to determine how PP6 recognises Aurora A, and cell-based functional tests to confirm the cellular importance of PP6-R3 holoenzymes. Furthermore, we do not claim that PLK1 regulation is via molecular mimicry, it occludes a

binding site which overlaps with that for Aurora A. We generate specific point mutants that uncouple PLK1-binding and maintain Aurora A binding and phosphatase activity. Figure 7 shows that the well-characterised HK mutant of PLK1 which abolishes phospho-selectivity results in loss of interaction with PPP6R2 suggesting the docking motif is phosphorylated. We can add data that shows PLK1 phosphorylates PPP6R2 at this site, and that this is lost in the mutant or when a PLK1 inhibitor is added.

To address the specific concerns raised by reviewer 1 we propose the add data showing:

- A. Competition binding assays testing if the binding site region of PPP6R3 can displace TPX2 from Aurora A.
- B. Phosphorylation-dependent interaction of PLK1 with PPP6R2.
- C. Confidence scores for Alphafold3 models with some additional analysis as requested.

Concerns:

The predicted structures from AlphaFold 3 modelling need to be accompanied by their PAE plots. It is also important to include the pTM and iTM scores. Without explicit statements on the confidence scores, it is difficult to assess the conclusions or the structural rationale provided.

>> We can add this information as requested.

Close-up views of the predicted binding interfaces, with amino acids making direct contacts shown in stick representation, will help appreciate the nature of the binding interface.

>> We show the interaction surface on Aurora A and specific contact aromatic residues on PPP6R3 in Figure 5D. The interfaces are extensive and a more detailed figure showing the specific pockets on Aurora A for each of the aromatic residues would occupy a lot of space without adding much information. This could be extended in a supplemental figure to show the detail the referee asks for. We do provide sufficient information in the manuscript that readers can always run the AF3 models to see this for themselves.

R3-Aurora A interaction: According to the models, both A and P boxes contribute to binding; however, the cellular biochemistry suggests that the interaction is mainly mediated via the A box. It would be helpful to describe the nature of the interaction interfaces - contribution of A and P boxes to the total buried surface area of the complex will be informative.

>> We use the AF3 structural models to help guide the reader and understand our mapping and point mutant binding assay data. The models show that the A and P boxes could in principle contribute to Aurora A binding. However, the biochemical data clearly show that for PPP6R3 only the A-box is crucial, whereas for PPP6R2 the P-box plays an important and

additional regulatory role. We can calculate the contributions of the A and P-boxes to the “total buried surface area of the complex” for the AF3 predictions.

Biochemical evaluation of the direct interaction between R3 A box and Aurora A - It is not clear why the Coomassie gel is not shown.

As this experiment was carried out using recombinantly purified proteins, the Coomassie-stained SDS-PAGE of the pull-down will strengthen the conclusion.

>> Purified proteins with positive and negative controls were used for the binding assays supporting the conclusion that the interaction is direct. Western blot was used for detection since some of the binding fragments (e.g. TPX2) run at the same size on SDS-PAGE gels as the Aurora A construct used. To avoid this issue we decided to detect binding with Western blot. CBB gels can be added if required.

Figure 5. It is not clear if the Aurora A binding modes of R3 514-616 (shown in Fig 5A) and R3 557-616 (shown in Fig. 5D) are identical or different, and why two different AF3 models were generated?

>> Figure 5A and Figure 5D address different points using the same model. Figure 5A is a comparison of PPP6R3 with known structures from the PDB and gives an overview of the interaction surfaces. Figure 5D highlights specific PPP6R3 residues in the model which are identified in the biochemical assays (Figure 5C) as being important for the interaction.

Mutating the Cysteine residue (C519) to a Histidine in the 'CHIH' motif in the context of A box abolishes R3 interaction with Aurora-A. It is unclear whether the predicted structural model can explain this observation. The C519 is not highlighted in any of the structural models, and the text also does not describe the intermolecular contacts mediated by this Cys residue.

>> We can highlight C519 on the model in Figure 5A. One can infer the position from the current model which shows the R3 514-616 fragment.

The models used in the manuscript appear to suggest that there will be partial overlap between the TPX2 binding interface and R3 binding interface on Aurora A. If this is correct, will R3 binding to Aurora-A interfere with TPX2 binding? Have the authors attempted to model R3-Aurora A binding in the presence of TPX2?

>> We can overlay the two models to show this directly. AF3 was trained on data including the Aurora A/TPX2 structure so this is going to be biased towards that outcome. This seems to go beyond the capabilities of AF3 modelling.

What is the amino acid sequence similarity/identity between the A and P sites of R2 and R3?
Can the authors provide any reasoning for the variations in the Aurora A binding mode of R2 and R3?

>> We can show sequence alignments of the A-box and P-box and add this to the supplemental information.

The speculation that R2-PLK1 binding interferes with R2-Aurora A interaction (as the P box region is involved in both PLK1 and Aurora A binding) needs to be supported by further competitive binding analysis using recombinantly purified proteins in either Size Exclusion Chromatography (SEC) or affinity pull-down experiments.

>> We don't agree with the claim that our conclusions are speculative given the evidence in the manuscript. First, we show that PLK1-bound PP6 cannot act to dephosphorylate Aurora A. Second, we show that this inhibition is lost when a PLK1 docking site mutant of PPP6R2 is used. Third, we show in pull down assays that this mutant can bind to Aurora A but not to PLK1. Additional data on the nature of the requirements for the PPP6R2-PLK1 interaction can be provided, including the regulatory kinase.

Figure EV8A shows that R2 447-966 (a fragment containing both A and P boxes) can interact with both Aurora A and PLK1. Won't this contradict the idea that Aurora A and PLK1 binding regions R2 overlap, and PLK1 binding will likely interfere with Aurora A binding to R2? Or PLK1 binding remodels R2-Aurora A interaction? Was there any attempt to model the Aurora A - PLK1 - R2 complex using AlphaFold?

>> PPP6R2 is used as the bait in Figure EV8A and can bind to either PLK1 or Aurora A in this approach. Therefore, the experiment does not show that the same molecule of R2 is binding to both PLK1 and Aurora A at the same time.

We have used AlphaFold3 to model PLK1 binding and Aurora A binding to PPP6R2. These models reveal overlapping binding sites that match the biochemical data provided in the figures. However, we do not believe AlphaFold 3 is the appropriate tool to model binding competition, that would require additional sophisticated approaches.

Typos:

Line 231: '.... box A, as the minimal Aurora B-binding domain in PPP6R3' - it has to be Aurora A.

Line 381: 'whereas 724AA mutant had not effect.' - 'no effect'

>> All corrected.

Referee #2 (Report for Author)

The authors describe the regulation of Aurora A in mitosis, looking into the specific contributions of PP6 trimeric holoenzymes. Using a microscopy image-based readout of mitotic progression and Aurora localization/phosphorylation, they authors find that PP6-R3 is the sole contributor to Aurora A dephosphorylation in vivo, whereas in vitro PP6-R2 can function as a phosphatase as well. Through elegant truncation and mutation analyses based on AF3 modeling, the authors pinpoint a region in the PP6-R3 (the A-box) that mediates the interaction between Aurora A and PP6. The authors further demonstrate that PP6-R2 does not interact with Aurora A in vivo due to the presence of a Plk1 docking site, which is suggested to outcompete Aurora A binding. Altogether, the authors have identified a motif in a proposed intrinsically disordered region of PP6-R3 that mediates binding with Aurora A and provides mechanistic insight in a biologically relevant regulatory mechanism. This is a strong and interesting piece of work and would be a great fit with EMBO J. Below are some suggestions for improving the manuscript and making it more accessible to the readers.

Major:

An interesting unexplored aspect is whether R3 competes with all known AurA interactors or only a subset. Would it be possible to test the ability of the AurA TPX2, CEP192 and TACC3 binding fragments their ability to compete with the R3-AurA interaction?

>> To address this question we will add titration binding assays testing whether a PPP6R3 fragment can compete TPX2 from Aurora A. This is the reverse to the referees suggestion but we believe is more informative since the phosphatase approaches the substrate, i.e. in cells we imagine PPP6R3 must be able to displace TPX2 from Aurora A.

An important aspect missing is the affinity of the R3 and R2 binding elements for AurA and how this compares to known interactors such as TPX2. Since they have recombinant proteins, I suggest measuring affinity of the interactions.

>> We agree affinity measurements would be informative and will add these as request.

The proximity of the AurA binding site and the ANKRD binding site in R3 is intriguing and there seems to be an indication in their experiments that ANKRD binding to R3 might facilitate AurA binding. Have the authors tried to model the ANKRD interaction with the R3 binding region?

>> Yes, we have modelled the interaction of ANKRD subunits with PPP6R3. This could be added to a supplemental figure. We agree with the referee that it is possible that ANKRD subunits facilitate substrate binding. Alternatively, they may be important for stabilising the PPP6R3 substrate binding region in the absence of substrate.

I could not find a useful excel file for the MS data provided.

>> We will provide the MS data file as source data.

Specific comments:

Line 24: Can the authors really claim that the A-box positions the T-loop for efficient dephosphorylation? There is no evidence provided on any conformational changes of the T-loop, so in my opinion this claim is not supported by experimental data.

>> We can revise the text to remove the specific point about the T-loop and implication that there is a conformational change: “the A-box positions Aurora A for efficient dephosphorylation”.

Starting line 43: the description of Aurora-Plk1-PP6 interplay could use a schematic to help the reader. It is quite complex for the non-specialist.

>> We can add a schematic of the interplay between Aurora-Plk1-PP6 as suggested.

Line 204-220: The AF3 models are nice and are well supported by actual experimental data in many cases. However, it would be important to report model confidence including iPTM and PTM scores, providing PAE plots as supplementary data. In addition, it would be great to see whether all 5 models return the same findings, or rather only a few do so.

>> Alphafold 3 generate model confidence scores and we can add that information as requested.

Line 106: typo, dependant > dependent

Line 120-121: Figure 1B-D is an unusual way of showing statistical tests. Unclear what the comparisons are.

>> As described in the legend the statistical tests are all against control, i.e. multiple pairwise comparison with the appropriate correction. The format was used to reduce the space taken by the comparison bars.

Line 158-172: Can you make a statement on Aurora A binding here?

>> This section is focussed on the composition of different PP6 holoenzymes rather than Aurora A binding. That is addressed in the next section. We could add some text here to make it clear that the purpose of the analysis is to understand what might be relevant for Aurora A recognition by the PP6.

Line 161-165: I do not understand this reasoning. If the equivalent relationship was observed for ANKRD's and PPR1-3, why would this explain one being a scaffold and the other not?

>> As for other phosphatases, the regulatory subunits of PP6 are defined as those which impart specific substrate recognition properties. Our data show that ANKRD scaffold subunits are common across PP6 holoenzymes containing the different regulatory subunits, despite the fact the holoenzymes have different substrate recognition properties.

Line 221 and further: For the interaction experiments (3E, but mainly 3F), I had a hard time getting my head around the different domains. Would it be possible to use a graphical representation to label the different lanes, rather than their residue numbers? This can potentially be obtained by incorporating (a reworked version of) the graphical representation in fig 3B?

>> We can do this easily for both Figure 3E and 3F. It was originally decided against since it increased the size of the figure.

Line 256-278: Regarding the blots in Figure 4, does one of the antibodies (R3?) cross react with TPX2? Or why is it showing in all blots with R3? Is the epitope of the R3 antibody known? Could it be they have a shared binding Aurora A binding motif that the antibody recognizes?

>> We don't believe this is the case. A FLAG antibody was used to detect FLAG-TPX2 from the substrate complexes, and FLAG-PPP6R3 from the enzyme complexes. We will improve the labelling to mark non-specific bands i.e. IgG HC to make the figure panel easier to understand.

Line 347: typo, phenylamine > phenylalanine

>> Corrected.

Referee #3 (Report for Author)

This interesting study follows on from previous work by the same team that identified PP6 as the key mitotic phosphatase for Aurora A. Here, the team has delved more deeply into the mechanism and made some important new discoveries. First, it is not only clear that PPP6R3 is the only regulatory subunit that acts on Aurora A in cells, but that (and how) PPP6R2 is blocked from this function by PLK1. This is an especially elegant part of the study. Second, Using AlphaFold3 to model the complex, the team made mutations in PPP6R3 that disrupt the interaction with Aurora A, which are then unable to sustain Aurora A dephosphorylation in cells. One of the mutations disrupts the interaction but enhances dephosphorylation, which is interesting but the mechanism is not nailed down. Another major strength of the study is the robust and beautiful cell work, especially with the knockout/rescue studies in Figure 8.

The study has some limitations that need to be addressed:

Line 199. Figure 3A does not show comparable amounts of PPP6C recovered from co-IPs, the R1 has a much more intense band. Is this because there is more R1 in the cell lysate, or does it suggest there is an excess of R2/R3 subunits that are not part of a holoenzyme complex with the catalytic subunit?

>> The different PP6 regulatory subunits are present at different levels in cells and we believe have different stability and regulation. For this reason, we used FLAG-tagged exogenous proteins for the experiment in Figure 3A. Despite this, we find there is more PPP6R1 in the cell extract and correspondingly more PPP6C is brought down, albeit it brings down much less Aurora A.

There is insufficient information about the AlphaFold3 models for the work to be reproduced or to have confidence in the result. Was the modelling done using the server or a local installation and what was the input (sequences, ligands, ions, PTMs)? Was the modelling done once or repeated. If repeated, how were models analysed and selected? What is the level of confidence in the modelling (pair-wise iPTM, ipSAE), and can the authors discuss how readers should regard the model (is it a possibility, is the main chain probably correct, are the side chains probably correct, which are the more reliable parts of the model). Will the models be included in the "source data"?

>> We used the public version of AF3 with default configuration provided by Google DeepMind for this reason. Others can simply repeat the modelling without having to run a local version of AF3. The specific sequences used in the modelling are described in the figures with the specific ions noted. We can bring this information together in the methods section more clearly to describe all relevant details for each model shown.

>> We can add the models to the source data. The general point raised by the referee relates to the interpretation of the AF3 models. We have used them as possibilities to help understand our mutational and functional analysis. As such we would agree with the view that the main chains are probably correct, and that some caution about specific side chain positioning is required.

Another missing aspect of the paper is validation of the AlphaFold model from the Aurora A side. Can the interaction with PPP6R3 be disrupted with point mutations in Aurora A? Alternatively, the model predicts competition between PPP6R3 and other Aurora A interactors, notably TPX2. This would be easy to test in a competition binding/IP experiment.

>> We have not looked at Aurora A pockets further and feel it goes beyond the scope of the current work due to the number of mutations required in Aurora A to remove all the binding sites, and technical challenges. We have previously made multiple mutations to Aurora A surface residues involved in binding and observed a strong impact on protein expression and stability. Study of these unstable proteins would not deliver any useful information.

>> We can carry out competition pulldown assays using purified proteins to show for example that TPX2 and PPP6R3 compete with one another for Aurora A binding.

The more detailed model figure EV5A shows Aurora A was doubly phosphorylated (T287, T288). Why was the modelling done this way, was it needed to make the modelling work? T288 is the established, critical site for activation of Aurora A, and the experimental data in the study, quite rightly, focussed entirely on that modification. Could phospho-T288 fit into the PPP6C active site, perhaps in an alternative model? There is a currently a disconnect between the structural modelling and the experimental data.

>> Both T287 and T288 are reported to be phosphorylated in vivo, with T288 being viewed as the major modification required for Aurora A activation. That model shows both T287 and T288 are close to the PPP6C catalytic site and contact residues previously shown to be important for catalysis. We can also show a model for T288 alone if that is required. It is important to stress that these are structural models that have helped guide our study, and in this case the key point is the proximity of the key residues in Aurora A to the PPP6C catalytic site. Whether the precise positions are accurately captured by the model is less clear.

The experiment shown in Figure 4 is consistent with the C-terminal region of PPP6R3 as being critical for Aurora A dephosphorylation. An alternative explanation is that this truncated protein is less active overall than wild-type - can the authors rule out this possibility?

>> For these experiments we carefully control the amount of PP6 complex and precise level of the catalytic subunit in the assays. Thus, all assays are done under conditions where the level of activity should be the same. These experiments

therefore test the importance of substrate binding. In line with that suggestion, WT PP6 R3 and the R3 1-616 complexes have similar activity, whereas an R3 1-513 complex has the same amount of catalytic subunit but no measurable activity towards Aurora A. Biological replicates were performed with different substrate and enzymes preparations to create the graphed data showing the activity measurements are both robust and reproducible.

Figure 6 has interesting but seemingly contradictory results: the FA1 mutant has no Aurora A binding, and reduced Aurora A dephosphorylation activity; but the CA CH CCAA mutant also shows no Aurora A binding, but has increased Aurora A dephosphorylation activity. The suggested mechanistic explanation of product dissociation being limiting is interesting but further biochemical work is needed to support this conclusion. Is there any evidence that the CA CH CCAA mutant retains some binding to Aurora A? Is unphosphorylated Aurora A a potent inhibitor of Aurora A dephosphorylation

>> The CA CH CCAA in Figure 6 retains the conserved aromatic residues and still has residual Aurora A binding (see Figure 5 R3 CA = same mutations). The FA1 mutant lacks both detectable Aurora A binding and activity towards Aurora A. We don't know if Aurora A inhibits Aurora A pT288 dephosphorylation. That is an interesting proposal which we have not tested. It would require high Aurora A concentrations that would probably not occur within cells under normal conditions.

The study leaves one big question unanswered - how is the PP6 holoenzyme able to dephosphorylate TPX2/Aurora-A which is resistant to general phosphatases. Is it simply that it is a highly effective phosphatase for Aurora-A, or does the PPP6R3 interaction with the kinase somehow loosen TPX2/Aurora-A complexes. This could be addressed with further biochemical studies, and perhaps modelling, but certainly needs to be discussed in the text.

>> Our proposal is that PP6 can achieve what a general phosphatase cannot because of its ability to occupy sites overlapping with Aurora A activators, i.e. as the referee suggests it "loosens" the interaction with TPX2. Pulldown data shows that TPX2 is no longer bound to the Aurora A that interacts with PP6. A further piece of evidence in support of this is the minimal PP6 R3 1-513 phosphatase which is effectively reduced to a "general phosphatase" core. This is unable to act on Aurora A/TPx2 complexes. We also believe this is where the role of the ANKRD subunit may be important in chaperoning the exchange of Aurora A activators with the PP6 regulatory subunits. These ideas were raised in the discussion section of the manuscript to highlight the need for further work on this important question, and can be extended further as requested by the referee.

>> Competition binding assays and affinity measurements would also help address this point.

Minor errors:

Line 231, correct Aurora B to Aurora A.

Line 270, correct binging to binding.

Line 312, correct Aurora to Aurora A

>> All corrected.

Prof. Francis A. Barr
University of Oxford
Department of Biochemistry
South Parks Road
Oxford OX1 3QU
United Kingdom

19th Apr 2026

Re: EMBOJ-2026-124008R-Q
Activator mimicry by PP6 regulatory subunits directs selective dephosphorylation of Aurora A

Dear Francis,

Thank you for your letter outlining how you might address the referee comments on your recent EMBO Journal submission. I have now had a chance to go through your revision plan, and I appreciate that you appear to be able to respond well to the majority of points raised by the reviewers. In particular, the addition of the main items highlighted in your letter (competition assays, affinity measurements, phospho-dependence of PLK1-PPP6R2 interaction, and AlphaFold-related aspects) should go along way towards satisfying key issues raised in the reports. In this light, we would be interested in pursuing a new version revised along the proposed lines further for EMBO Journal publication.

I should remind you that It is our policy to allow only a single round of (major) revision, so please keep me updated should there be any unexpected problems with the revisions, or should you require an extension beyond the default 3-months deadline. As always, competing manuscript published elsewhere during the course of this revision will not affect our eventual decision on your study. Finally, please note the detailed information and guidelines on how to prepare a revision below (and in our online Guide to Authors) - closely adhering to them shall greatly facilitate the editorial process at the time of resubmission. In particular, please look at our options for 'layering' data according to importance (main figures, Expanded View figures & tables, Appendix data, referee-response data) when considering how to best organize a revised manuscript.

Thank you again for the opportunity to consider this work, and I look forward to receiving your revision in due time.

With kind regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

*** PLEASE NOTE: All revised manuscript are subject to initial checks for completeness and adherence to our formatting guidelines. Revisions may be returned to the authors and delayed in their editorial re-evaluation if they fail to comply to the following requirements. As a first step please read our guidelines for revised submissions:
<https://link.springer.com/journal/44318/submission-guidelines#cms-Revised-submissions>

1) Every manuscript requires a Data Availability section (even if only stating that no deposited datasets are included). Primary datasets or computer code produced in the current study have to be deposited in appropriate public repositories prior to resubmission, and reviewer access details provided in case that public access is not yet allowed.

2) Each figure legend must specify

- size of the scale bars that are mandatory for all micrograph panels
- the statistical test used to generate error bars and P-values
- the type error bars (e.g., S.E.M., S.D.)
- the number (n) and nature (biological or technical replicate) of independent experiments underlying each data point
- Figures may not include error bars for experiments with $n < 3$; scatter plots showing individual data points should be used instead.

3) Revised manuscript text (including main tables, and figure legends for main and EV figures) has to be submitted as editable text file (e.g., .docx format). We encourage highlighting of changes (e.g., via text color) for the referees' reference.

4) Each main and each Expanded View (EV) figure should be uploaded as individual production-quality files (preferably in .eps,

.tif, .jpg formats). For suggestions on figure preparation/layout, please refer to our Figure Preparation Guidelines: <https://media.springernature.com/original/springer-cms/rest/v1/content/27825798/data/v1>

- 5) Point-by-point response letters should include the original referee comments in full together with your detailed responses to them (and to specific editor requests if applicable), and also be uploaded as editable (e.g., .docx) text files.
- 6) Please complete our Author Checklist, and make sure that information entered into the checklist is also reflected in the manuscript; the checklist will be available to readers as part of the Review Process File.
- 7) All authors listed as (co-)corresponding need to deposit, in their respective author profiles in our submission system, a unique ORCID identifier linked to their name. Please see our Guide to Authors for detailed instructions.
- 8) Please note that supplementary information at EMBO Press has been superseded by the 'Expanded View' for inclusion of additional figures, tables, movies or datasets; with up to five EV Figures being typeset and directly accessible in the HTML version of the article.
- 9) To facilitate reproducibility and cross-laboratory adoption of methodologies, please structure the Materials & Methods section as outlined in our guide to authors, including a completed Reagents and Tools Table.
- 10) Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be clearly noted in the figure legend and/or the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure. Finally, we generally encourage uploading of numerical as well as gel/blot image source data.

At EMBO Press we ask authors to provide source data for the main manuscript figures. You will receive a separate email with instructions for providing source data with your revised manuscript, including how to upload and organize the files.

In the interest of ensuring the conceptual advance provided by the work, we recommend submitting a revision within 3 months (18th Jul 2026). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

EMBOJ-2026-124008R-Q. Correspondence with editor – initial author responses.

Referee #1 (Report for Author)

Precisely orchestrated mitotic spindle assembly and stabilisation are crucial for accurate chromosome segregation. Aurora A kinase is a conserved major regulator of the mitotic spindle. Aurora A localisation and activation are modulated by various regulators such as TPX2, CENP192, TACC3 and phosphatase PP6. While mechanisms influencing Aurora A localisation and activation, the latter via T-loop phosphorylation, are well studied, mechanisms that counteract Aurora A activation by specifically dephosphorylating the T-loop are not well understood. This is an important knowledge gap, as a precise balance of kinase-phosphatase activity is critical for the stabilisation of a correct spindle structure suitable for facilitating accurate segregation of chromosomes to the daughter cells. Here, Yang et al. show, by combining biochemistry, structural modelling and functional rescue assays, that PP6 holoenzymes containing regulatory subunits R2 and R3 are capable of directly interacting and dephosphorylating the Aurora-A T-loop phosphorylation in vitro. However, in cells, the interaction of PP6-R3 with Aurora-A is the sole contributor to Aurora-A T-loop dephosphorylation. Their data also suggest that PP6-R3 facilitates this by directly interacting with Aurora-A via its C-terminal disordered region, namely A- and P-boxes. This mode of binding appears to mimic that of Aurora-A activators such as TPX2. Their data suggest that R3 A-box interaction is also required to selectively dephosphorylate Aurora A T-loop. As far as R2 is concerned, they show that, though R2 can interact with Aurora A in vitro (likely via the R2 P box), in the cellular context this interaction is outcompeted by PLK1, which interferes with R2 binding to Aurora A (via a possible molecular mimicry involving the R2 P box).

Strength: The findings that PP6-R3 is the sole contributor for dephosphorylating Aurora A T-loop and PP6-R2 - PLK1 interaction inhibit PP6-R2-mediated dephosphorylation of Aurora T loop.

Weakness: In the current form, conclusions around the structural basis for R3 binding to Aurora A and molecular mimicry as a rationale for PLK1-mediated PP6-R2 are not convincing and would require further biochemical and structural characterisation. Also, in the absence of strong evidence that the putative PLK1 PBD motif of R2 is phosphorylated in cells (or even in vitro biochemical analysis of the molecular interplay among phosphorylated R2, PLK1 PBD, and Aurora A), the proposed mechanism remains speculative or correlative.

>> To address the specific concerns raised by reviewer 1 we have fully revised the manuscript Figures 3-7 to add new data showing:

- A. Competition binding assays with purified proteins showing PPP6R3 and activators TPX2, CEP192 and TACC3 compete for binding to Aurora A. This is accompanied by

additional AlphaFold 3 modelling to better describe the binding interfaces including calculations for buried surface areas and interface overlap (Figure 6 and EV3). Tables S1-S4 provide a more detailed description.

- B. Competition binding assays with purified proteins showing that PLK1 and Aurora A compete for overlapping binding sites on PPP6R2. Additional analysis of this interaction shows the PLK1 polo-box domain recognition of the phosphorylated pS771 residue in the C-terminus of PPP6R2 is necessary for competition. This data is accompanied by additional AlphaFold 3 modelling to better describe the binding interfaces to PLK1 and Aurora A (Figure 7 and EV7). Tables S5-S6 provide a more detailed description.
- C. We have fully revised the AlphaFold3 modelling to use 5 random seeds and the top 5 models per seed for the analysis. This allows us to more clearly separate confident seed independent interactions from “noise”. PAE plots are shown with plots of confidence scores for binding interfaces (Figures EV3, EV4, EV7). Tables S1-S6 provide per residue confidence scores for the interfaces. Input files for creating AlphaFold 3 server batch jobs, the search output files, Python processing scripts, and data tables are all provided in source data or with the manuscript.

Concerns:

The predicted structures from AlphaFold 3 modelling need to be accompanied by their PAE plots. It is also important to include the pTM and iPTM scores. Without explicit statements on the confidence scores, it is difficult to assess the conclusions or the structural rationale provided.

>> PAE plots for the best models per seed have been added to the relevant figures (Figures EV3, EV4, EV7) and confidence scores are given in those figure legends and main results text (lines 245, 252-254). These plots are derived from fully revised the AlphaFold3 modelling using 5 random seeds and the top 5 models per seed for the analysis. As explained, this allows us to more clearly separate confident seed independent interactions from “noise”. Tables S1-S6 provide per residue confidence scores, contact probabilities and distances for the interfaces. Input files for creating AlphaFold 3 server batch jobs, the search output files, Python processing scripts, and data tables are all provided in source data or with the manuscript.

Close-up views of the predicted binding interfaces, with amino acids making direct contacts shown in stick representation, will help appreciate the nature of the binding interface.

>> We show the conserved interaction surface on Aurora A and specific contact aromatic residues on PPP6R3 and PPP6R2 (Figure 5A, 6A and EV3H). We have added a more detailed comparison of these surfaces with crystal structures for TPX2, CEP192 and TACC3 including

comparison of the buried surface areas (Figure 6B-6C). We have provided sufficient information with the manuscript so that readers can run the AF3 models or view the PyMOL sessions to see this for themselves.

R3-Aurora A interaction: According to the models, both A and P boxes contribute to binding; however, the cellular biochemistry suggests that the interaction is mainly mediated via the A box. It would be helpful to describe the nature of the interaction interfaces - contribution of A and P boxes to the total buried surface area of the complex will be informative.

>> Our biochemical data show that the A-box is the major essential binding site for both PPP6R3 and PPP6R2 (Figures 3-6, EV3), whereas the P-box imparts PLK1 regulation to PPP6R2 (Figure 7, EV3 and EV7). The revised AlphaFold 3 structural modelling did not support a high confidence interaction for the PPP6R3 P-box region to Aurora A, there was some variability between seeds and it fell just below the 60% occupancy threshold and had a lower mean pLDDT than for PPP6R2 (Figure EV3E-3G).

We have calculated the contributions of the A and P-boxes to the total buried surface areas for the different PPP6R3 and PPP6R2 complexes and compared these to crystal structures for TPX2, CEP192 and TACC3 (Figure 6A-6D), see results text (lines 306-333).

Biochemical evaluation of the direct interaction between R3 A box and Aurora A - It is not clear why the Coomassie gel is not shown.

As this experiment was carried out using recombinantly purified proteins, the Coomassie-stained SDS-PAGE of the pull-down will strengthen the conclusion.

>> A CBB stained gel has been added as requested (Figure 3I). Purified proteins with positive and negative controls were used for the binding assays supporting the conclusion that the interaction is direct. Western blot was used for detection since the TPX2 fragments runs at nearly the same size on SDS-PAGE as the Aurora A construct used.

Figure 5. It is not clear if the Aurora A binding modes of R3 514-616 (shown in Fig 5A) and R3 557-616 (shown in Fig. 5D) are identical or different, and why two different AF3 models were generated?

>> We have fully revised the AlphaFold 3 modelling as explained above. The original models were identical with different views shown to highlight different aspects of the interaction.

Mutating the Cysteine residue (C519) to a Histidine in the 'CHIH' motif in the context of A box abolishes R3 interaction with Aurora-A. It is unclear whether the predicted structural model can explain this observation. The C519 is not highlighted in any of the structural

models, and the text also does not describe the intermolecular contacts mediated by this Cys residue.

>> This section of the manuscript has been fully revised to add additional in vitro binding data using cysteine point mutants to better address these questions (Figure EV5). Analysis of the PPP6R3 CA CH CCAA ('CA all') in the original Figures 5 & 6 had residual Aurora A binding and elevated phosphatase activity. We find that the PPP6R3 'CA all' mutant holoenzymes show less interaction with Aurora A (Figure EV5A). However, a purified recombinant R3 binding fragment, in the absence of other components, shows the same binding to Aurora A as wild-type PPP6R3 (Figure EV5B). Therefore, as the referee suggested the mutant does retain Aurora A binding activity. This mutant is interesting because it shows enhanced phosphatase activity in vitro and in cells (Figure EV5B-C and Figure 8). We have also extended the analysis of the ANKRD containing holoenzymes which is potentially relevant for understanding the role of the cysteine motifs (Figure EV4). The text has been updated to reflect these results (lines 335-373).

The models used in the manuscript appear to suggest that there will be partial overlap between the TPX2 binding interface and R3 binding interface on Aurora A. If this is correct, will R3 binding to Aurora-A interfere with TPX2 binding? Have the authors attempted to model R3-Aurora A binding in the presence of TPX2?

>> Yes, there is overlap between the PPP6R and Aurora A activator binding surfaces, including TPX2. As mentioned above in reference to another questions, we show the conserved interaction surface on Aurora A and specific contact aromatic residues on PPP6R3 and PPP6R2 (Figure 5A, 6A and EV3H). We have added a more detailed comparison of these surfaces with crystal structures for TPX2, CEP192 and TACC3 including comparison of the buried surface areas (Figure 6B-6C). Modelling Aurora A in the presence of TPX2 and PPP6R3 gives outputs like the TPX2-Aurora A crystal structure which we assume is in the AlphaFold 3 training data. More sophisticated molecular dynamics simulations would be needed to give further insights into binding site competition.

What is the amino acid sequence similarity/identity between the A and P sites of R2 and R3? Can the authors provide any reasoning for the variations in the Aurora A binding mode of R2 and R3?

>> Sequence alignments of the A-box and P-box are in the supplemental information (Appendix Figure S1A-S1B).

The speculation that R2-PLK1 binding interferes with R2-Aurora A interaction (as the P box region is involved in both PLK1 and Aurora A binding) needs to be supported by further

competitive binding analysis using recombinantly purified proteins in either Size Exclusion Chromatography (SEC) or affinity pull-down experiments.

>> We have added competition binding assays with purified proteins showing that PLK1 and Aurora A compete for overlapping binding sites on PPP6R2. Additional analysis of this interaction shows the PLK1 polo-box domain recognition of the phosphorylated pS771 residue in the C-terminus of PPP6R2 is necessary for competition. This data is accompanied by additional AlphaFold 3 modelling to better describe the binding interfaces to PLK1 and Aurora A (Figure 7 and EV7). Tables S5-S6 provide a more detailed description.

Figure EV8A shows that R2 447-966 (a fragment containing both A and P boxes) can interact with both Aurora A and PLK1. Won't this contradict the idea that Aurora A and PLK1 binding regions R2 overlap, and PLK1 binding will likely interfere with Aurora A binding to R2? Or PLK1 binding remodels R2-Aurora A interaction? Was there any attempt to model the Aurora A - PLK1 - R2 complex using AlphaFold?

>> PPP6R2 used as the bait in the original Figure EV8A was in excess and can bind to either PLK1 or Aurora A in this approach. **Therefore, the experiment does not show that the same molecule of R2 is binding to both PLK1 and Aurora A at the same time. New data shows that the PLK1 polo-box domain can directly compete for Aurora A binding to PPP6R3 via the P-box (Figure 7G).** We have used AlphaFold3 to model PLK1 binding and Aurora A binding to PPP6R2. These models reveal overlapping binding sites that match the biochemical data provided in the figures. However, AlphaFold 3 is not the appropriate tool to model binding competition, that would require additional sophisticated molecular dynamics approaches.

Typos:

>> All corrected.

Line 231: '.... box A, as the minimal Aurora B-binding domain in PPP6R3' - it has to be Aurora A.

Line 381: 'whereas 724AA mutant had not effect.' - 'no effect'

Referee #2 (Report for Author)

The authors describe the regulation of Aurora A in mitosis, looking into the specific contributions of PP6 trimeric holoenzymes. Using a microscopy image-based readout of mitotic progression and Aurora localization/phosphorylation, they authors find that PP6-R3 is the sole contributor to Aurora A dephosphorylation in vivo, whereas in vitro PP6-R2 can function as a phosphatase as well. Through elegant truncation and mutation analyses based on AF3 modeling, the authors pinpoint a region in the PP6-R3 (the A-box) that mediates the interaction between Aurora A and PP6. The authors further demonstrate that PP6-R2 does not interact with Aurora A in vivo due to the presence of a Plk1 docking site, which is suggested to outcompete Aurora A binding. Altogether, the authors have identified a motif in a proposed intrinsically disordered region of PP6-R3 that mediates binding with Aurora A and provides mechanistic insight in a biologically relevant regulatory mechanism. This is a strong and interesting piece of work and would be a great fit with EMBO J. Below are some suggestions for improving the manuscript and making it more accessible to the readers.

Major:

An interesting unexplored aspect is whether R3 competes with all known AurA interactors or only a subset. Would it be possible to test the ability of the AurA TPX2, CEP192 and TACC3 binding fragments their ability to compete with the R3-AurA interaction?

>> Competition binding assays with purified proteins showing PPP6R3 and activators TPX2, CEP192 and TACC3 compete for binding to Aurora A. This is accompanied by additional AlphaFold 3 modelling to better describe the binding interfaces including calculations for buried surface areas and interface overlap (Figure 6 and EV3). Tables S1-S4 provide a more detailed description.

An important aspect missing is the affinity of the R3 and R2 binding elements for AurA and how this compares to known interactors such as TPX2. Since they have recombinant proteins, I suggest measuring affinity of the interactions.

>> We have carried out SPR and added affinity measurements for PPP6R3-Aurora A (Figure 5D-5E).

The proximity of the AurA binding site and the ANKRD binding site in R3 is intriguing and there seems to be an indication in their experiments that ANKRD binding to R3 might facilitate AurA binding. Have the authors tried to model the ANKRD interaction with the R3 binding region?

>> We have modelled the interaction of ANKRD subunits with PPP6R3 holoenzymes (Figures EV4 and EV5). As suggested by the referee it is possible that ANKRD subunits facilitate substrate binding or activity in some way, and this may be related to the role of the cysteine CHIH motif. Alternatively, they may be important for stabilising the PPP6R3 substrate binding

region in the absence of substrate. These possibilities are described in the text (lines 335-373).

I could not find a useful excel file for the MS data provided.

>> The MS data tables are provided as source data.

Specific comments:

Line 24: Can the authors really claim that the A-box positions the T-loop for efficient dephosphorylation? There is no evidence provided on any conformational changes of the T-loop, so in my opinion this claim is not supported by experimental data.

>> This is an important point and we thank the referee for raising it. The extended AlphaFold 3 analysis using multiple seeds makes it clear that there are three robust interfaces PPP6C to the SAPS domain of PPP6R subunits, between the C-terminal A-box region of PPP6R2/3 and Aurora A or ANKRD subunits (we use ANKRD52 as representative. However, we find that between model seeds the position of the PPP6C catalytic subunit and Aurora A or ANKRD-bound A-box is variable (Figure EV3G). Therefore, although the modelling shows that PPP6C can approach the T-loop it does not support a defined position and we have therefore removed this figure panel and revised the text (lines 239-267).

Starting line 43: the description of Aurora-Plk1-PP6 interplay could use a schematic to help the reader. It is quite complex for the non-specialist.

>> We have fully revised the presentation of this data and hope the breakdown into modelling and data elements comparing Aurora A-PPP6R2 then PLK1-PPP6R2 less confusing than our original presentation (Figure 7 and EV7).

Line 204-220: The AF3 models are nice and are well supported by actual experimental data in many cases. However, it would be important to report model confidence including iPTM and PTM scores, providing PAE plots as supplementary data. In addition, it would be great to see whether all 5 models return the same findings, or rather only a few do so.

>> We have fully revised the AlphaFold3 modelling to use 5 random seeds and the top 5 models per seed for the analysis. This allows us to more clearly separate confident seed independent interactions from “noise”. PAE plots are shown with plots of confidence scores for binding interfaces (Figures EV3, EV4, EV7). Tables S1-S6 provide per residue confidence scores for the interfaces. Input files for creating AlphaFold 3 server batch jobs, the search output files, Python processing scripts, and data tables are all provided in source data or with the manuscript.

Line 106: typo, dependant > dependent

Line 120-121: Figure 1B-D is an unusual way of showing statistical tests. Unclear what the comparisons are.

>> As described in the legend the statistical tests are all against control, i.e. multiple pairwise comparison with the appropriate correction. The format was used to reduce the space taken by the comparison bars.

Line 158-172: Can you make a statement on Aurora A binding here?

>> This section of text was removed during revisions. Aurora A binding is mentioned immediately after this (lines 166-190).

Line 161-165: I do not understand this reasoning. If the equivalent relationship was observed for ANKRD's and PPR1-3, why would this explain one being a scaffold and the other not?

>> As for other phosphatases, the regulatory subunits of PP6 are defined as those which impart specific substrate recognition properties. Our data show that ANKRD scaffold subunits are common across PP6 holoenzymes containing the different regulatory subunits, despite the fact the holoenzymes have different substrate recognition properties. We also show that ANKRD binding although important in cells is not essential for Aurora A binding.

Line 221 and further: For the interaction experiments (3E, but mainly 3F), I had a hard time getting my head around the different domains. Would it be possible to use a graphical representation to label the different lanes, rather than their residue numbers? This can potentially be obtained by incorporating (a reworked version of) the graphical representation in fig 3B?

>> We have added some additional graphical elements to mark constructs that do/don't contain the A- or P-boxes. This helps gain an overview of the results.

Line 256-278: Regarding the blots in Figure 4, does one of the antibodies (R3?) cross react with TPX2? Or why is it showing in all blots with R3? Is the epitope of the R3 antibody known? Could it be they have a shared binding Aurora A binding motif that the antibody recognizes?

>> We don't believe this is the case. A FLAG antibody was used to detect FLAG-TPX2 from the substrate complexes and FLAG-PPP6R3 from the enzyme complexes. We have improved the labelling to mark the specific bands.

Line 347: typo, phenylamine > phenylalanine

>> Corrected.

Referee #3 (Report for Author)

This interesting study follows on from previous work by the same team that identified PP6 as the key mitotic phosphatase for Aurora A. Here, the team has delved more deeply into the mechanism and made some important new discoveries. First, it is not only clear that PPP6R3 is the only regulatory subunit that acts on Aurora A in cells, but that (and how) PPP6R2 is blocked from this function by PLK1. This is an especially elegant part of the study. Second, Using AlphaFold3 to model the complex, the team made mutations in PPP6R3 that disrupt the interaction with Aurora A, which are then unable to sustain Aurora A dephosphorylation in cells. One of the mutations disrupts the interaction but enhances dephosphorylation, which is interesting but the mechanism is not nailed down. Another major strength of the study is the robust and beautiful cell work, especially with the knockout/rescue studies in Figure 8.

The study has some limitations that need to be addressed:

Line 199. Figure 3A does not show comparable amounts of PPP6C recovered from co-IPs, the R1 has a much more intense band. Is this because there is more R1 in the cell lysate, or does it suggest there is an excess of R2/R3 subunits that are not part of a holoenzyme complex with the catalytic subunit?

>> The different PP6 regulatory subunits are present at different levels in cells and we believe have different stability and regulation. For this reason, we used FLAG-tagged exogenous proteins for the experiment in Figure 3A. Despite this, we find there is more PPP6R1 in the cell extract and correspondingly more PPP6C is brought down, albeit it brings down much less Aurora A.

There is insufficient information about the AlphaFold3 models for the work to be reproduced or to have confidence in the result. Was the modelling done using the server or a local installation and what was the input (sequences, ligands, ions, PTMs)? Was the modelling done once or repeated. If repeated, how were models analysed and selected? What is the level of confidence in the modelling (pair-wise iPTM, ipSAE), and can the authors discuss how readers should regard the model (is it a possibility, is the main chain probably correct, are the side chains probably correct, which are the more reliable parts of the model). Will the models be included in the "source data"?

>> We have fully revised the AlphaFold3 modelling to use 5 random seeds and the top 5 models per seed for the analysis. This allows us to more clearly separate confident seed independent interactions from "noise". PAE plots are shown with plots of confidence scores for binding interfaces (Figures EV3, EV4, EV7). Tables S1-S6 provide per residue confidence scores for the interfaces. Input files for creating AlphaFold 3 server batch jobs, the search output files, Python processing scripts, and data tables are all provided in source data or

with the manuscript. This information is described in the methods section more clearly to describe all relevant details for each model shown.

>> The general point raised by the referee relates to the interpretation of the AlphaFold 3 models. We have used them as possibilities to help understand our mutational and functional analysis. As such we would agree with the view that the main chains are probably correct, and that some caution about specific side chain positioning is required, even when multiple model seeds are used.

Another missing aspect of the paper is validation of the AlphaFold model from the Aurora A side. Can the interaction with PPP6R3 be disrupted with point mutations in Aurora A? Alternatively, the model predicts competition between PPP6R3 and other Aurora A interactors, notably TPX2. This would be easy to test in a competition binding/IP experiment.

>> We added biochemical analysis of the Aurora A surface, focussing on the S155R mutation which sits at a surface important for TPX2 interaction. In brief, yes mutation also abolishes PPP6R3 binding as well TPX2 ([Figure 6F-6H](#)).

>> We have added competition binding assays with purified proteins showing PPP6R3 and activators TPX2, CEP192 and TAC3 compete for binding to Aurora A. This is accompanied by additional AlphaFold 3 modelling to better describe the binding interfaces including calculations for buried surface areas and interface overlap ([Figure 6 and EV3](#)). Tables S1-S4 provide a more detailed description.

>> We have also added competition binding assays with purified proteins showing that PLK1 and Aurora A compete for overlapping binding sites on PPP6R2. Additional analysis of this interaction shows the PLK1 polo-box domain recognition of the phosphorylated pS771 residue in the C-terminus of PPP6R2 is necessary for competition. This data is accompanied by additional AlphaFold 3 modelling to better describe the binding interfaces to PLK1 and Aurora A ([Figure 7 and EV7](#)). Tables S5-S6 provide a more detailed description.

The more detailed model figure EV5A shows Aurora A was doubly phosphorylated (T287, T288). Why was the modelling done this way, was it needed to make the modelling work? T288 is the established, critical site for activation of Aurora A , and the experimental data in the study, quite rightly, focussed entirely on that modification. Could phospho-T288 fit into the PPP6C active site, perhaps in an alternative model? There is a currently a disconnect between the structural modelling and the experimental data.

>> This is an important point related to one raised by referee 2, and we thank referee 3 for raising it. We have now performed all modelling with only pT288. The extended AlphaFold 3 analysis using multiple seeds makes it clear that there are three robust interfaces PPP6C to the SAPS domain of PPP6R subunits, between the C-terminal A-box region of PPP6R2/3 and Aurora A or ANKRD subunits (we use ANKRD52 as representative. However, we find that

between model seeds the position of the PPP6C catalytic subunit and Aurora A or ANKRD-bound A-box is variable (Figure EV3G). Therefore, although the modelling shows that PPP6C can approach the T-loop it does not support a defined position and we have therefore removed this figure panel and revised the text (lines 239-267).

The experiment shown in Figure 4 is consistent with the C-terminal region of PPP6R3 as being critical for Aurora A dephosphorylation. An alternative explanation is that this truncated protein is less active overall than wild-type - can the authors rule out this possibility?

>> For these experiments we carefully control the amount of PP6 complex and precise level of the PPP6C catalytic subunit in the assays. Thus, all assays are done under conditions where the level of activity should be the same. These experiments therefore test the importance of substrate binding. In line with that suggestion, WT PP6 R3 and the R3 1-616 complexes have similar activity. **whereas an R3 1-513 complex lacking the substrate binding region has the same amount of catalytic subunit but no measurable activity towards Aurora A** (Figures 4A-4C). In addition, the Aurora A binding mutant FA form of holoenzyme contains full length PPP6R3 and has no activity above baseline (Figure 5F and 5G). Additionally, the truncated PPP6R3 1-616 holoenzymes bind the same amount of substrate as the full-length (Figure 3H, whereas the 1-513 and FA mutants do not bind Aurora A (Figure 3H and 5B-5C). Biological replicates were performed with different substrate and enzymes preparations to create the graphed data showing the activity measurements are both robust and reproducible (Figure 4C and Figure 5G). We conclude that simplest explanation for these findings is that the C-terminal region is the substrate binding site.

Figure 6 has interesting but seemingly contradictory results: the FA1 mutant has no Aurora A binding, and reduced Aurora A dephosphorylation activity; but the CA CH CCAA mutant also shows no Aurora A binding, but has increased Aurora A dephosphorylation activity. The suggested mechanistic explanation of product dissociation being limiting is interesting but further biochemical work is needed to support this conclusion. Is there any evidence that the CA CH CCAA mutant retains some binding to Aurora A? Is unphosphorylated Aurora A a potent inhibitor of Aurora A dephosphorylation

>> This section of the manuscript has been fully revised to add additional in vitro binding data using cysteine point mutants to better address these questions (Figure EV5). Analysis of the PPP6R3 CA CH CCAA ('CA all') in the original Figures 5 & 6 had residual Aurora A binding and elevated phosphatase activity. We find that the PPP6R3 'CA all' mutant holoenzymes show less interaction with Aurora A (Figure EV5A). However, a purified recombinant R3 binding fragment, in the absence of other components, shows the same binding to Aurora A as wild-type PPP6R3 (Figure EV5B). Therefore, as the referee suggested the mutant does retain Aurora A binding activity. This mutant is interesting because it shows enhanced phosphatase activity in vitro and in cells (Figure EV5B-C and Figure 8). We have also

extended the analysis of the ANKRD containing holoenzymes which is potentially relevant for understanding the role of the cysteine motifs (Figure EV4). The text has been updated to reflect these results (lines 335-373).

The study leaves one big question unanswered - how is the PP6 holoenzyme able to dephosphorylate TPX2/Aurora-A which is resistant to general phosphatases. Is it simply that it is a highly effective phosphatase for Aurora-A, or does the PPP6R3 interaction with the kinase somehow loosen TPX2/Aurora-A complexes. This could be addressed with further biochemical studies, and perhaps modelling, but certainly needs to be discussed in the text.

>> We show that PPP6R3 and Aurora A activators compete for the same sites. Our proposal is that PP6 can achieve what a general phosphatase cannot because of this ability to mimic and occupy sites overlapping with Aurora A activators, i.e. as the referee suggests it “loosens” the interaction with TPX2. Pulldown data shows that TPX2 is no longer bound to the Aurora A that interacts with PP6. A further piece of evidence in support of this is the minimal PP6 R3 1-513 phosphatase which is effectively reduced to a “general phosphatase” core. This is unable to act on Aurora A-TPX2 complexes. We also think this is where the role of the ANKRD subunit may be important in chaperoning the exchange of Aurora A activators with the PP6 regulatory subunits. These ideas and previous work on allosteric control of Aurora A are raised in the discussion section of the manuscript to highlight the need for further work on this important question.

>> Competition binding assays and affinity measurements using TPX2, CEP192 and TACC3 and additional AlphaFold 3 modelling of the overlapping binding interfaces have been added to directly address these points (Figure 5, Figure 6 and Figure EV3).

Minor errors:

Line 231, correct Aurora B to Aurora A.

Line 270, correct binging to binding.

Line 312, correct Aurora to Aurora A

>> All corrected.

Prof. Francis A. Barr
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South Parks Road
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14th Sep 2026

Re: EMBOJ-2026-124008R1
Activator mimicry by PP6 regulatory subunits directs Aurora A dephosphorylation in mitosis

Dear Francis,

Thank you for submitting your revised manuscript on PP6 in Aurora A regulation to The EMBO Journal. It has now been seen once more by the three original referees (see comments below), and I am happy to say that they were fully satisfied with the revisions. We shall therefore be ready to accept the manuscript for publication, following incorporation of the following remaining editorial issues:

- Please carefully go through the reference list and make sure that all references have their complete citation information - including year, journal name & volume, and page/locator numbers - especially the latter information is currently missing for a number of references.
- For preprint citations: Please change in-text reference format according to "preprint: Meeusen et al, 2025"; and in the reference list, name of the platform plus DOI + preprint label - e.g. "bioRxiv doi: 1234/002.dj123 [PREPRINT]" - but first double-check if there is in the meantime already a formal peer-reviewed publication of any cited preprints.
- The supplementary tables S1-6 should be converted into Expanded View Datasets - requiring renaming them (in the files names, legends, and in-text references) into 'Dataset EV1-EV6'. Each of the XLSX files should include a separate 'Legend' tab containing the dataset title/number and any legend text as appropriate.
- Finally, please provide suggestions for a short 'blurb' text prefacing and summing up the conceptual aspect of the study in two sentences (max. 250 characters), followed by 3-5 one-sentence 'bullet points' with brief factual statements of key results of the paper; they will form the basis of an editor-written 'Synopsis' accompanying the already provided graphical abstract/synopsis image in the online version of the article.

I am returning the manuscript to you for a final round of minor revision, solely to allow you to upload the required modified final files; once we will have received them, we should be ready to swiftly proceed with formal acceptance and production of the manuscript.

With kind regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

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1) Every manuscript requires a Data Availability section (even if only stating that no deposited datasets are included). Primary datasets or computer code produced in the current study have to be deposited in appropriate public repositories prior to resubmission, and reviewer access details provided in case that public access is not yet allowed.

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- size of the scale bars that are mandatory for all micrograph panels

- the statistical test used to generate error bars and P-values
- the type error bars (e.g., S.E.M., S.D.)
- the number (n) and nature (biological or technical replicate) of independent experiments underlying each data point
- Figures may not include error bars for experiments with $n < 3$; scatter plots showing individual data points should be used instead.

3) Revised manuscript text (including main tables, and figure legends for main and EV figures) has to be submitted as editable text file (e.g., .docx format). We encourage highlighting of changes (e.g., via text color) for the referees' reference.

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5) Point-by-point response letters should include the original referee comments in full together with your detailed responses to them (and to specific editor requests if applicable), and also be uploaded as editable (e.g., .docx) text files.

6) Please complete our Author Checklist, and make sure that information entered into the checklist is also reflected in the manuscript; the checklist will be available to readers as part of the Review Process File.

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Referee #1:

I find the revision satisfactory and hence support the publication of the work.

Referee #2:

The authors have done a great job in addressing my concerns and that of the other reviewers. This is a nice manuscript with deep mechanistic insights on how AurA is dephosphorylated by PP6 and I think it should be published in EMBO J.

Referee #3:

The authors have addressed all of my concerns in full. The additional experiments strengthen the manuscript, and I am fully convinced of the whole story from structural modelling to cell-based assays. The study reveals an intricate and elegant mechanism that adds a major new dimension to our understanding of Aurora A kinase.

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Journal Submitted to: The EMBO Journal

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- if $n < 5$, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
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Include a statement about blinding even if no blinding was done.	Yes	Materials and methods
Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not Applicable	
If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.		
For every figure, are statistical tests justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and methods and Figure legends

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In the figure legends: state number of times the experiment was replicated in laboratory.	Yes	Figure legends and associated Figures
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Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have primary datasets been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	All source data provided in this study have been deposited to the BioStudies.
Were human clinical and genomic datasets deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
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