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Aurora Kinase A proximity map reveals centriolar satellites as regulators of its ciliary function

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1st Editorial Decision 1st Dec 2020

Dear Elif,

Thank you for submitting your manuscript entitled 'Aurora Kinase A proximity interactome reveals centriolar satellites as regulators of its function during primary cilium biogenesis' to EMBO Reports. We have now received three referee reports, which are included below.

My apologies for this unusual delay in getting back to you, it took longer than anticipated to receive the full set of referee reports.

Referees find the proposed regulation of AURKA and ciliation by centriolar satellites in principle interesting. However, referees also raise largely overlapping concerns:

- More support showing the specificity of AURKA interactions should be provided (ref #1, points 1, 2 and 3, ref 2 point 2, ref #3, point 2, 3)
- Additional support is needed for the proposed regulation of AURKA by satellites (ref #1, point 4, ref#3 point 2, 4)
- Additional controls are required to rule out the contribution of cell cycle to the effect of AURKA inhibition on ciliation (ref #1, point 5, ref #3, standfirst, points 5, 6)
- Protein quantification and phosphosite determination are not robust (ref #2 points 2, 3, 4).
- The data do not conclusively support the presence of AURKA on the satellites (ref #3, point 1).
- Overall, the provided controls are insufficient throughout the work (all referees).

Given these comments from recognized experts in the field that are also experienced reviewers, and considering the amount of work required to address them, we cannot offer to publish your manuscript.

However, in case you feel that you can address the referee concerns in a timely and thorough manner, and can obtain data that would considerably strengthen the study as in the referee reports, we would have no objection to consider a revised manuscript (along with a point-by-point response to the referee concerns) in the future. Please note that if you were to send a new manuscript this would be assessed again with respect to the literature and the novelty of your findings at the time of resubmission and in case of a positive editorial evaluation, the manuscript would be sent back to the original referees. I would like to emphasize that we will be reluctant to approach the referees again in the absence of major revisions, and we need strong support from the referees to consider publication here.

Thank you in any case for the opportunity to consider this manuscript and my apologies once again for this unusual delay in the process. I am sorry that I cannot communicate more positive news, but nevertheless hope that you will find our referees' comments helpful.

Kind regards,

Deniz

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

Arslanhan, Firat-Karalar and colleagues here present an analysis of Aurora A kinase interactors and activities. The topic is of great general importance and interest. However, the current submission provides a relatively limited advance over what is already known. There are several technical issues that raise concern about how robust the findings presented here are. There are also several areas of the manuscript that could be consolidated/ condensed.

1. A key issue is the specificity of the interactions. Given the limited overlap with the BioGRID interactor list, some additional controls should be provided to ensure that the new dataset is not confounded by the overexpression of Aurora A. There is a substantial literature on the various impacts of Aurora A overexpression (e.g., Meraldi et al. EMBO J. 2002 21:483-92; Anand et al. Cancer Cell. 2003 3:51-62) that indicates potential concerns. What is the extent of the AURKA overexpression in the system here? The authors should provide some indication that the V5-BirA*-AURKA is fully functional, localises normally (beyond the centrosome localisation) and/ or that its expression as used here does not have dominant active effects.

2. While a potentially comprehensive list of interactors is of obvious value, the involvement of AURKA in the majority of the processes indicated in Figure 3B is known, to some extent. The novel aspects of the interactome indicated by the authors are not explored in detail and some additional experimental confirmation of the interactions with cell adhesion, DNA binding or RNA processing proteins should be provided for these conclusions to be convincing. This would also be of great interest to readers.

The level of potential overlap with Aurora B interactors should be considered; a BioGRID comparison might be useful in this respect.

3. The satellite interactions should be verified with the endogenous proteins and the reciprocal pulldowns, for at least some of them. An overexposure should be used to show the inputs with GFP-PCM1 in Figure 4D.

4. Does PCM1 deficiency in the authors' previously published IMCD3 model (Odabasi et al. (2019) EMBO Rep 20:e47723) alter AURKA stability? The provision of an additional, orthogonal experimental dataset would be helpful, particularly with the partial nature of the biological rescue effected by AURKA inhibition.

5. Cell cycle controls (flow cytometry or microscopy) should be provided for the serum restimulation experiments in Figure 7H, to determine whether changes in ciliation may be related to changes in cell cycle reentry through AURKA inhibition.

6. The introduction is rather lengthy and should be abbreviated. The articulation of additional questions on p.6 could be removed (from 'For example'...to '...transient ones.').

7. It is unclear what is meant by 'endogenous FLAG-BirA*-AURKA' and 'V5-BirA*AURKA' (p. 13). Figure 4E shows no evidence regarding endogenous AURKA, despite what is mentioned on p13. This should be clarified.

8. The experiment shown in Figure 4F is unclear. References should be cited to explain the approach used, of the non-satellite aggregates formed by PCM1 deletion mutants. It would be

useful to include a PCM1 full-length control for this experiment. The PCM1 construct '1-3600' should be referred to by its amino acid specification, not the nucleotide numbering; this should be made consistent throughout the MS.

9. The data in Figure 5D and 5E should be combined- the Table contains effectively all the information that the bar diagram conveys. It is unclear how the indicated S988 site (WVS) conforms to the R/K/N-R-X-S/T consensus, so this should be reviewed.

Minor points
10. Size markers should be included for Figure 6C; the Legend for this panel should be labelled correctly. Bar graphs are not required for these data; these results can be summarised on the immunoblot, not least as an s.e.m. that is determined on 2 repeats is not very meaningful.

11. The diagram in Figure 6D should indicate the timecourse followed- the inclusion of 24h is confusing.

12. There are some typos in the Figures that should be tidied up, e.g. Fig 5B 'Commasie'; 5C 'Phoshosite'; Fig 6C 'Quinescent'.

Referee #2:

This manuscript exploits the BioID mass spectrometry approach to identify protein interactors of the protein kinase Aurora A, which are then validated using over-expression, co-immunoprecipitation and co-localisation studies. While there appear to be some interesting findings from this study, including a number of proximity interactors that are localised to centriollar satellites, suggesting that AurA also has a role here, there are some significant issues with this manuscript that raises questions as to the validity of these findings.
In brief:
1. The paper lacks rigorous controls throughout - e.g. there are no loading controls for any of the immunoblots. There is no comparison of AurA localisation in parental cell lines with respect to the stable cells lines expressing BirA-AurA, and no indication of the relative levels of over-expression. I was unable to find any controls validating knockdown of PCM1 with the specific, but not the control siRNA.
2. I seriously question, based on the detail provided in the manuscript, how the protein quantification was performed. Only two replicates were performed, yet they appear to do some form of statistics to determine change. What do they mean by 'spectral counts equal to or greater than 2'. What controls do they have to check that their quantification method is valid? The rationale for selecting a NSAF value of 5.7 is really random, with the apparent justification that this is appropriate given that they see 'known' AurA interactors at this level. If the argument as presented is that the community have only touched the tip of the iceberg with respect to AurA interactors, then surely there will be many below this level that are true positives, but missed because of the way that the experiment/quantification was performed.
3. They appear to have compared their proximity interactors with 'high confidence' interactors in the STRING database (from methods), and then later it appears that they have compared all putative interactors from high and low-throughput strategies. Please be clear (and stringent) with how these comparisons are performed.
4. Having been through the methods carefully, it is somewhat surprising that they managed to identify any sites of phosphorylation given that they do not appear to have used any

kinase/phosphatase inhibitors in their lysis/extraction buffers. Please could they check?
Unfortunately, I also have serious concerns about the phosphosite information that are being reported in e.g. Fig. 5. They report Mascot scores, and highlight associated sites 'identified', but there is no indication of phosphosite identification confidence. Given that the Mascot scores are identical for the same peptide with different sites highlighted, it appears that this may be a case of ambiguous site localisation (possible even associated with the sasme MS/MS scan number) rather than that all of these sites have been defined, but it is impossible to know given the information presented. There are no spectra included to give confidence in any of the sites and therefore I would question the confidence of them all.
5. Could the authors please explain why they have used different concentration of MLN8237 in different experiments?
6. Unfortunately, the authors do not really do adequate justice in terms of referencing prior work, either in the AurA field, or the BioID/quantitative proteomics.

Referee #3:

This manuscript by Arslanhan et al. describes the proximity interactome of Aurora A and identifies centriolar satellites as regulators of AURKA function. As expected, they identify many proteins related to the centrosome. However, they uncover additional functional groups not reported in previous affinity purification systems. In particular, they identify an enrichment of proteins associated with centriole satellites. They further confirm that Aurora A physically interacts with potentially phosphorylates PCM1, a critical scaffolding protein for satellites. They further demonstrate that PCM1 depletion enhances Aurora A localization to and activity at the basal body in quiescent cells, where it disrupts cilium assembly. Notably, this effect is limited to cilium presence; it does not alter cilium length. While PCM1 knockdown decreases cilated cells, this effect is partly rescued with MLN8237. The manuscript concludes that centriolar satellites regulate AURKA protein levels to control its cilium assembly functions (abstract, introduction). Also it concludes that PCM1 phosphorylation may be important for its regulation.

This paper has a number of strengths as findings shed light on novel functions of Aurora A. First it employs a BioID approach and identifies a large library of proximate proteins-a valuable contribution to the literature. Second, it identifies PCM1 as an interactor and likely direct substrate of PCM1. Third, it finds an interesting ~2-fold increase of AURKA through protein stabilization upon PCM1 knockdown. Most of the approaches and many conclusions are well supported by the data. The manuscript appropriately reserved some conclusions, and described some of the possibilities in the discussion. Some significant weaknesses are there are potential confounders with cell-cycle effect of MLN8237, that the functional outcomes of AURKA loss on ciliagenesis appear to be synthetic only (only found upon PCM1 knockdown-which is fine, and interesting, but the conclusions should make this more clear). These and other issues to be addressed are outlined in more detail below.

Major:
(1) The manuscript appears to conclude that AURKA exists at the satellites even though it is not seen there in immunofluorescence. A more likely possibility not mentioned is that the interactions could simply occur at the centrosome (where many of these satellite proteins are also found), or through soluble pools of proteins. The data do not adequately support the claim that Aurora A is a novel part of satellites.
(2) Some basic controls appear to be missing, particularly with siPCM1.
a. There is no clear blot to show the degree of PCM1 knockdown achieved, nor whether this affected the centriolar satellites in these cells.

b. Only a single siRNA is used for PCM1 knockdown. Ideally a rescue experiment could be done, but at a minimum, 2 siRNAs are typical due to the known off-target effects of these reagents even if PCM1 knockdown is verified.
c. If the causal link to satellites regulating Aurora A (rather than just PCM1 per say) is claimed, then it would be important to similarly disrupt another protein that is required in satellites.
(3) Most experiments involve overexpressed proteins, which make it possible that some of the observed interactions are non-physiologic.
a. Ideally, coIP interactions such as Figure 4C-D would be reproduced with endogenous proteins.
b. Quantitative Western Blotting and Immunofluorescence should be performed to determine how the levels compare to Aurora A in the parental cell lines. Alternatively, it would be possible to knockdown the endogenous Aurora A. At a minimum, these limitations could be addressed more in the discussion, both in how it relates to the global Aurora A interactome (contributing to the minimal overlap observed in Fig. 2) and to their findings in particular. [For example, CEP63 was detected as a proximal interactor using stably expressed V5-BirA-AURKA, but not by transiently transfected Flag-BirA-AURKA.]
(4) The authors use an exogenously expressed PCM1 truncation mutant to create satellite aggregates and demonstrate the GFP-Aurora A localizes to these aggregates. Does this mutant completely disrupt the centrosome? Where does gamma tubulin or other centrosome-only proteins localize? Without this information, the findings are not very informative. Moreover, the authors detect exogenously expressed GFP-Aurora A, not endogenous Aurora A.
(5) MLN8237 doesn't affect cilium assembly unless PCM1 is depleted (Fig 7C-D); since AURKA is not required for cilium assembly, some of the conclusions around that could be moderated-it seems that AURKA activity restrains cilia assembly in the absence of certain synthetic conditions of satellite protein knockdown.
(6) MLN8237 causes cell-cycle arrest in mitosis, changing the cell cycle profile of the cell population. Even-in single cell analyses where interphase cells are quantified (such as acetylated-tubulin IF), the stage of G1 cells can differ. This is mitigated to an extent in serum starvation conditions, but still could be a concern, particularly with serum replete conditions (Figure 7G-I). It would be important to assess cell cycle profile in these conditions by PI/FACS, by quantifying mitotic index, or blotting for cell-cycle specific genes to determine which effects could be mediated indirectly by cell cycle states.

Minor:
(1) As the authors have phospho-T288 Aurora A antibody, it would be ideal to use it to verify that the degree by which MLN8237 inhibits its autocatalytic activity.
(2) Consider moderating conclusions in that MLN8237 doesn't affect ciliagenesis, but only has synthetic impacts upon PCM1 knockdown.
(3) The functional roles of the PCM1 phosphorylations is not described-I think that is OK, but the tangent in Figure 5 could be de-emphasized or explicitly described as being not linked to that which follows.
(4) Figure 1C; consider showing a shorter exposure for lanes 3-4 as it is overexposed and difficult to interpret.
(5) In Figure 1C, a minus biotin control would be helpful to identify the Aurora A-proximate bands, particularly in that the BirA-AurkA appears to be expressed higher than the BirA* control.
(6) I found Figures 2-3 relatively uninformative-consider condensing into one.
(7) Figure 4C-D: it would be helpful to label in the figure not just 'pulldown' but 'streptavidin pulldown' in C and 'GFP-trap pulldown' in D.
(8) Individual DAPI images would be helpful for all IF images, particularly when describing different mitotic stages. It also helps the reader evaluate DNA integrity. In Fig. 4, panel F, it is hard to appreciate where these aggregates are and if the overall integrity of the cell is disrupted.

(9) Consider insets in Fig. 4, panel E, and Fig. 7, panel B, to visually expand small structures.
(10) The word proximal, used throughout the test, suggests unidirectional upstream activity. I would suggest using 'proximate' to mean nearby, as there is no intention to refer discriminately to upstream (or root-directed) interactions.
(11) Minor typos:
a. "during when they localize" (p.4)
b. "interactions with its spatially and temporally binding partners" (p.6)
c. "at in control and" (p.15)
d. Figure 6C "Quinescent cells"
e. Figure 6D graph, Y-axis, "normalized to GAPDH", yet blot indicates "vinculin" as loading control
f. Figure 6 legend: "B" should be "C"

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1) ***Validation of the V5-BirA-AURKA stable cell line used:***
   - To quantitatively compare expression levels of V5-BirA*-AURKA relative to endogenous AURKA, we will perform immunoblotting with antibodies against AURKA and V5. We will also include new immunofluorescence data confirming the correct localization of V5-BirA*-AURKA relative to spindle microtubules and endogenous AURKA.

   - AURKA overexpression was shown to cause spindle multipolarity, multinucleation, apoptosis and centrosome amplification (e.g., Meraldi et al. EMBO J. 2002 21:483-92; Anand et al. Cancer Cell. 2003 3:51-62). To eliminate the possible artefacts that could be induced by overexpression and to validate the functionality of V5-BirA*-AURKA fusion, we will use immunoblotting and/or immunofluorescence to quantify the following phenotypes in control and V5-BirA*AURKA cells: 1) centrosome amplifications, 2) multinucleation, 3) mitotic index, 4) apoptosis.

   During the initial characterization of the cell line, we checked by microscopy to ensure that they are not defective in proliferation and mitotic progression. Therefore, I am confident that the quantitative analysis we will perform will validate that the cell line we used for BioID experiments reflects near physiological interactome of AURKA.

2) ***Quantitative analysis of the BioID data and its validation:***
   - While our paper was under review, we already reanalyzed the data by **NSAF** (normalized spectral abundance factor)-based analysis using different thresholds and **SAINT** (Significance Analysis of the Intearctome) analysis. The results of these two independent analysis with different thresholds both identified the same group of major functional clusters we reported in our manuscript and did not significantly change the composition of the AURKA interactome. Given that NSAF and SAINT analysis are the two most extensively used label-free quantitative proteomics approaches, these results further validates our approach and addresses the reviewer concerns raised regarding our datasets. In the revised manuscript, *we will include the results of this new analysis and clarify the filters and statistical analysis we used for NSAF analysis and STRING interacome comparisons*.

   - The AURKA proximity interactome identified various cellular compartments and biological processes (i.e. RNA binding/processing, cell adhesion, centriolar satellites) as novel relationships. In this manuscript, we extensively characterized the nature of the relationship between AURKA and centriolar satellites. While our paper was under review, a new BiorXiv paper by Damodaran et al. showed that AURKA regulates alternative splicing and reported physical interactions of AURKA with the splicing factors SRSF1, SRSF3, SRSF7, PCBP2. These validated interactions are present in the AURKA proximity interactome we identified, further confirming the new relationship with RNA biding/processing proteins revealed by our map (https://www.biorxiv.org/content/10.1101/2020.11.04.368498v1).

   - In addition to the interactions we validated in the submitted manuscript, we will perform immunoprecipitation experiments to test the interaction between AURKA and its new putative interactors as revealed by its proximity interactome. These include include CSPP1 (implicated in mitosis, cell adhesion ciliogenesis), TALPID3 (ciliogenesis factor), NEK1 (kinase mutated in ALS).

     I believe that the results of these new experiments and analysis, extensive characterization of the AURKA-centriolar satellite relationship and recent data in the field confirming interactions with RNA processing factors together strongly validate our approach and shows that the AURKA proximity interactome is a powerful resource for the field.

3) ***Validation of cellular localization and interaction of AURKA with centriolar satellites:***
   I would like to first highlight that PCM1 is the only protein that exclusively localizes to centriolar satellites and is essential for centriolar satellite integrity and assembly. While all satellite proteins defined so far interacts with PCM1, not all of them were verified to co-localize to satellite by immunofluorescence likely due to the low abundance and transient nature of interactions (Quarantotti et al, 2019, Gheiratmand et al. 2019). Therefore, we and others in the field use PCM1 as the golden standard for defining proteins as new centriolar satellite components.

   Our data on physical and proximity interaction of AURKA with PCM1 and other core satellite proteins (i.e. Cep131, Cep72) and its recruitment to granules formed by the N-terminus of PCM1 strongly demonstrates AURKA as a new satellite component. However, I also do understand the reviewer's concern on whether this is an overexpression artefact. To address their concerns and strengthen our conclusions, we will perform the following experiments:

- We will perform immunoprecipitation experiments with endogenous PCM1 and immunoblot the eluates with AURKA to test their interaction at the endogenous level.
- To test whether AURKA-PCM1 interact at the centrosome, we will perform co-immunoprecipitation experiments between PCM1 and AURKA in HeLa::SASS6-/- centriole-less cells and/or HEK293T cells treated with the PLK4 inhibitor centrione. We already have the KO cell lines to perform the proposed experiments.
- Given that GFP-PCM1-N induces formation of aggregates that recruit proteins that interact with PCM1, we will ectopically express GFP-PCM1 (control) and GFP-PCM1- to determine whether these aggregates recruit AURKA and other centrosome proteins (i.e gamma-tubulin, centrin) and how the centrosome integrity is affected.
- Given that satellite resident proteins that interact with PCM1 were shown to regulate cellular distribution of satellites, we will use quantitative immunofluorescence to determine the consequences of AURKA inhibition on centriolar satellite integrity and distribution.

The results of these interactions will together further validate the AURKA-centriolar satellite relationship. Finally, I would like to highlight that not all interactions detected by the BioID approach can be validated at the physical level by classical affinity purification methods, which is why BioID is superior in identifying weak, transient and insoluble interactions (i.e. Lamber et al. 2015). Therefore, orthogonal methods to confirm the new relationships revealed by BioID are required such as co-localization dependency and functional cooperation/competition, which we included in our manuscript and will further test with the proposed revision experiments.

4) ***Analysis and validation of the PCM1 phosphoproteomics data:***

We agree with the reviewer that the phosphophoproteomics data in the form we presented in Fig. 5 is not technically sound, which is mainly due to lack of inclusion of data and experimental details during our initial submission. We will address these concerns as follows:

- We performed two experimental replicates for phosphoproteomics analysis of AURKA with cells treated with DMSO (control) and AURKA inhibitor MLN8237. For preparation of the lysates for mass spectrometry, we did include protease and phosphatase inhibitors. Further clarification of this in the methods section will address the Reviewer#2's concern regarding the methodology.

- Regarding the Mascot scores and statistical analysis of the identified phosphopeptides, we have raw data in xls format that includes this information for the two experimental replicates. The file size was too big (>100 MB) and we were not able to include it in the initial submission. We are currently in the process of uploading this data in a public proteomics repository (ProteomeXChange), which will be accessible to reviewers when we resubmit. Additionally, we will also include details on the statistical analysis of the reported peptides in Fig. 5E to clarify how we selected these peptides and the what the p values and MASCOT scores are for control and MLN8237-treated samples. Taken together, these sets of data will address the technical concerns raised by the Reviewer#2.

- We will run Phos-tag SDS-PAGE gels with control and MLN8237-treated cell lysates. Given that Phostag causes a mobility shift in phosphorylated proteins, the results of these experiments will test the phosphorylation of PCM1 by AURKA and complement the phosphoproteomics data.

5) ***Validation of the functional assays regarding AURKA-PCM1 relationship during cilium biogenesis:***

- *Characterization of cell cycle states:* The reviewers raised a valid concern stating that cilium assembly and disassembly defects could be a consequence of possible cell cycle phenotypes associated with AURKA inhibition or PCM1 depletion (Fig. 7). To address this, we will quantify the cell cycle state of the control and PCM1-depleted cells, which were treated with DMSO control or MLN8237 using the following experiments: 1) quantification of mitotic index and proliferation state by immunofluorescence, 2) cell cycle profile analysis by flow cytometry analysis of Propidium Iodide-stained cells, 3) immunoblotting of samples with mitotic marker Cyclin B1.

- *Validation of PCM1 depletion and specificity of its associated phenotypes:* The PCM1 siRNA we used in this study was previously used and validated in previous work from my lab and others (i.e. Conkar et al. 2018, Dammermann et al. 2002). Consistent with previous literature, we validated the efficient depletion of PCM1 with the siRNA we used in the submitted manuscript, but did not include data. In the revised manuscript, we will include immunoblotting and immunofluorescence data for control and PCM1 siRNA-treated cells to validate the depletion efficiency of PCM1.

- To further test the specificity of the cilium assembly phenotypes associated with PCM1 depletion and AURKA inhibition, we will perform the following experiments as suggested by the reviewers:
    1) We will perform functional assays in Fig. 7 using cells transfected with previously published PCM1 shRNA targeting a different region on PCM1 than the one we used in the manuscript (Stowe et al. 2012) .
    2) We will perform functional assays in Fig. 7 using IMCD3::PCM1-/- satellite-less cells we previously characterized (Odabasi et al. 2018). Since IMCD3 cells ciliate using a different ciligoenesis pathway and the satellite-less cells are constitutively null, the results of these experiments will provide insight into the role of AURKA during cilium assembly in a different cell type and state.

- To further confirm that change in AURKA expression levels upon PCM1 depletion is specific to loss of centriolar satellites, we will quantify AURKA cellular levels in cells depleted for another satellite protein CCDC66, which unlike PCM1 is not required for satellite integrity.

6) Control experiments for the ones that are not covered above:

- We will include the loading controls for the immunoblots in Fig. 1.

- We will include immunoblots demonstrating the biotinylation state of the V5-BirA*-AURKA cell line with and without biotin in Fig. 1.

- We will include an immunoblot confirming the inhibition of AURKA activity by MLN8237 treatment.

- We performed AURKA inhibition with two different concentrations of MLN8237 that were used in literature. Using immunoblots, we validate that both concentrations inhibits AURKA activity. We will include cilium disassembly data in Fig. 7 that were done with 500 μm consistent with the cilium assembly experiments.

Editorial Decision 11th Dec 2020

Dear Elif,

Thank you for sending your revision strategy and my apologies for the delayed response.

I appreciate that you are willing to address the referee concerns. Having looked at everything, I would like to invite you to submit a revised manuscript. Please address all referee concerns in a complete point-by-point response.

I would like to emphasize that we need strong support from the referees to consider publication here. It is this aspect that is more difficult to assess at this stage, given the fact that the revisions would have to include substantive experimentation to be compelling.

We normally allow 3 months for revisions, but I can extend the deadline if needed (please see below my signature).

Thank you again for giving us the chance to consider your manuscript for EMBO Reports, I look forward to your revision.

Kind regards,

Deniz

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

*** Temporary update to EMBO Press scooping protection policy:
We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and have therefore extended our 'scooping protection policy' to cover the period required for a full revision to address the experimental issues highlighted in the editorial decision letter. Please contact the scientific editor handling your manuscript to discuss a revision plan should you need additional time, and also if you see a paper with related content published elsewhere.***

Referee #1:

Arslanhan, Firat-Karalar and colleagues here present an analysis of Aurora A kinase interactors and activities. The topic is of great general importance and interest.
We thank the reviewer for the constructive criticism of our manuscript. We are happy to see the reviewer found the topic of great general importance and interest.

However, the current submission provides a relatively limited advance over what is already known.
Our manuscript advances the field in two major ways: First, we report the first in vivo AURKA proximity map, which identified both known and previously undescribed relationships. Second, given the emerging functions of AURKA and satellites in primary cilium biogenesis, we extensively dissected this new relationship at the mechanistic and function level in our manuscript. Our results have two important implications:

1) Identification of a new mechanism for AURKA regulation
   Cellular and centrosomal localization, abundance and activity of AURKA is regulated by satellites in quiescent cells.
2) Defining the molecular pathways by which satellites regulate cilium biogenesis
   Centriolar satellites function during cilium assembly through regulating AURKA-mediated cilium disassembly and basal body maturation.

Despite its extensive characterization in mitosis, how AURKA mediates its diverse functions beyond mitosis and the mechanisms by which AURKA is regulated in these contexts remain largely unexplored. In this manuscript, we discovered that AURKA is regulated by satellites in quiescent cells and that this regulation is important for regulation of cilium biogenesis by centriolar satellites. In the revised manuscript, we also showed that AURKA and PCM1 play antagonistic roles during basal body maturation and thereby initiation of cilium assembly. Together, our results contributeto our understanding of spatiotemporal regulation of AURKA and as such, provides an important advance in defining full extent of AURKA functions and mechanisms affected in cancer.

There are several technical issues that raise concern about how robust the findings presented here are. There are also several areas of the manuscript that could be consolidated/ condensed.
- We agree with the reviewer that the manuscript could be condensed. To this end, we consolidated the mass spectrometry data and their GO and networking analysis in one figure (Fig. 2 and S2). Additionally, we significantly shortened the introduction and discussion to make them to the point.

-We agree with the technical concerns the reviewer raised regarding the analysis and validation of the BioID data. We addressed these concerns as detailed below in the point-by-point response. Together with the results of the experiments proposed by the other reviewers, we believe that the revised manuscript validates the power of the AURKA proximity interactome as a resource and strengthens our conclusions on AURKA-satellite relationship.

***1.** A key issue is the specificity of the interactions. Given the limited overlap with the BioGRID interactor list, some additional controls should be provided to ensure that the new dataset is not confounded by the overexpression of Aurora A.*
As previously, described, the low percentage of overlap between BioGRID and AURKA proximity interactome was expected due to the differences in the nature of interactions

monitored by traditional and proximity-based proteomic approaches (Lambert et al., 2014; Liu et al., 2018). This difference provides further support for the use of the proximity-based labeling approach to comprehensively map the AURKA interaction landscape. Given that our manuscript validates the power of the BioID approach in defining AURKA interactions, future studies in using this tool in a context-dependent manner will be important.

There is a substantial literature on the various impacts of Aurora A overexpression (e.g., Meraldi et al. EMBO J. 2002 21:483-92; Anand et al. Cancer Cell. 2003 3:51-62) that indicates potential concerns. What is the extent of the AURKA overexpression in the system here? The authors should provide some indication that the V5-BirA-AURKA is fully functional, localises normally (beyond the centrosome localisation) and/ or that its expression as used here does not have dominant active effects.*

- The reviewer raises an important concern regarding the physiological relevance of the AURKA proximity interactome. As the reviewer indicated, AURKA overexpression results in multiple phenotypes such as defects in mitotic progression, multinucleation, centrosome amplification and apoptosis. Therefore, we generated the AURKA interactome using cells that stably express V5-BirA*-AURKA at near endogenous levels. In the revised manuscript, we included the following sets of data to address the reviewer's concerns:

a) Using immunoblotting of cell lysates for AURKA and V5, we compared the expression level of V5-BirA*-AURKA relative to endogenous AURKA. These results confirmed that V5-BirA*-AURKA is expressed at near endogenous AURKA levels (Fig. 1B).

b) We included immunofluorescence data for staining the V5-BirA*-AURKA cell line with antibodies against AURKA and V5 (Fig. S1I). This result confirms the localization of AURKA to the centrosome in interphase cells and to the spindle poles and microtubules in mitotic cells. Additionally, it also shows that that the cell line is 100% positive for V5-BirA*-AURKA expression.

c) We showed that V5-BirA*-AURKA-expressing stable cell line behaves like the control cells in terms of its centrosome number, cell cycle profiles and mitotic defects by the following experiments:
Fig. S1A, B: centrosome number and percentage of multinucleation (immunofluorescence analysis for gamma-tubulin, DNA and microtubles)
Fig. S1D, E: their mitotic index (immunofluorescence analysis by DAPI)
Fig. S1D, F: spindle polarity (immunofluoerescence analysis for microtubules)
Fig. S1G: cell cycle profiles (flow cytometry and immunofluorescence)
Fig. S1H, apoptosis (immunoblotting for caspase 3)

Together, these results indicate that the V5-BirA*-AURKA-expressing stable line do not exhibit defects associated with AURKA overexpression.

***2.** While a potentially comprehensive list of interactors is of obvious value, the involvement of AURKA in the majority of the processes indicated in Figure 3B is known, to some extent. The novel aspects of the interactome indicated by the authors are not explored in detail and*

We generated the AURKA proximity map as a resource for this study and for future studies aimed at uncovering 1) new non-mitotic functions for AURKA, 2) molecular mechanism of action for known non-mitotic functions of AURKA, 3) new mechanisms for spatiotemporal regulation of AURKA. As indicated by the reviewer, the AURKA proximity interactome identified processes such as RNA binding/processing, cell adhesion, centriolar satellites as novel relationships.

Since investigating all novel relationships in one study is not possible, our approach for validating the proximity map was focusing on one novel relationship and extensively investigating it at the functional and mechanistic level. Given the emerging functions of AURKA and satellites during primary cilium biogenesis, we chose to dissect the AURKA-satellite proximity interaction and our results provided important insight into spatiotemporal regulation of AURKA in quiescent cells and into its functions during ciliogenesis as detailed above.

…some additional experimental confirmation of the interactions with cell adhesion, DNA binding or RNA processing proteins should be provided for these conclusions to be convincing. *This would also be of great interest to readers.* ***The level of potential overlap with Aurora B interactors*** *should be considered; a BioGRID comparison might be useful in this respect.*

To address the reviewer's comments on further validation of the AURKA proximity interactome, we performed the following analysis and experiments:

- We performed streptavidin pulldown experiments to test the interaction of AURKA with multiple proteins as predicted by its proximity interaction map. We chose proteins that were implicated across different cellular functions such as CSPP1 (implicated in mitosis, cell adhesion, ciliogenesis), TALPID3 (initiation of ciliogenesis) and CP110 (centriole cap required for centriole length control and initiation of ciliogenesis). To validate the specificity of AURKA interactions with satellites, we also tested interactions with satellite proteins that were not identified in the proximity map, which include SSX2IP (centriole duplication, microtubule anchoring) and BBS4 (component of the ciliary BBSome complex). AURKA interacted with CSPP1, Talpid3, CP110, but not with BBS4 and SSX2IP (Fig. 3C). These results provide further support for the specificity and physiological relevance of the AURKA proximity map. Additionally, they suggest mechanisms for AURKA functions during cilium assembly (removal of centriole cap CP110, basal body maturation via Talpid3) and centriole duplication (via SSX2IP).

- While our paper was under review, a new BiorXiv paper by Damodaran et al. came out. This study AURKA reported physical interactions of AURKA with the splicing factors and showed that AURKA regulates alternative splicing, Importantly, the new AURKA interactors they reported SRSF1, SRSF3, SRSF7, PCBP2, HNRNPC and NPM1 are present in the AURKA proximity map. Therefore, the result of this study confirms the proximity relationship of AURKA with RNA biding/processing proteins. We included results from this paper in the discussion section. (https://www.biorxiv.org/content/10.1101/2020.11.04.368498v1).

- In Fig. S2B, we compared the AURKA proximity map with the published Aurora B interactomes, which revealed overlapping and distinct interactions. 20% of the AURKA proximity interactome was shared with AURKB interactome.

Together, these results validate the use of the AURKA proximity interactome as a resource for the research community to study the multifaceted regulation and diverse functions of

3. *The satellite interactions should be verified with the endogenous proteins and the reciprocal pulldowns, for at least some of them. An overexposure should be used to show the inputs with GFP-PCM1 in Figure 4D.*

- PCM1 is essential for satellite assembly and is the molecular marker for satellites (Odabasi et al., 2019; Prosser and Pelletier, 2020; Wang et al., 2016). New satellite proteins are defined based on their interaction and/or co-localization with PCM1. Additionally, PCM1 is the only protein that exclusively localizes to satellites. Majority of other satellite residents also have centrosomal pools. Therefore, to test the endogenous interaction between satellites and

AURKA, we chose PCM1 as the prey protein. We performed endogenous pulldown of AURKA in asynchronous cells (+FBS) and quiescent ciliated cells (-FBS). PCM1 co-pelleted with AURKA, but not the IgG control, confirming that endogenous AURKA and PCM1 interact (Fig. 3E).

- We performed new set of co-immunoprecipiation experiments to test the interaction between GFP-PCM1 and FLAG-AURKA in control and centriole-less cells. In both conditions, AURKA interacted with PCM1, showing that their interaction does not depend on centrioles. We now included this data in Fig. 3D, which replaced the IP data presented in Fig. 4D of the original manuscript.

- We redistributed satellites to the cell periphery and showed that AURKA is specifically recruited to the peripheral satellite granules away from the centrosome (detailed below in point 8), suggesting that satellites sequester AURKA to regulate its localization and abundance.

Together, these new sets of experiments and data provide strong support to interaction between satellites and AURKA.

***4.** Does PCM1 deficiency in the authors' previously published IMCD3 model (Odabasi et al. (2019) EMBO Rep 20:e47723) alter AURKA stability? The provision of an additional, orthogonal experimental dataset would be helpful, particularly with the partial nature of the biological rescue effected by AURKA inhibition.*

The reviewer suggests an excellent experiment for testing our model in an orthogonal system as wells for comparing changes in AURKA regulation upon acute (RNAi) versus chronic (CRISPR/Cas9) loss of satellites by depleting and ablating PCM1, respectively. In the Odabasi et al. 2019 paper, we generated both RPE1::PCM1-/- and IMCD3::PCM1-/- cells and characterized them to elucidate satellite functions during intracellular (RPE1) and extracellular (IMCD3) ciliogenesis. While RPE1::PCM1-/- did not ciliate, IMCD3::PCM1-/- cells ciliated but less efficiently.

Since we used RPE1 cells to study cilium assembly in this manuscript, we performed the following experiments in RPE1::PCM1-/- cells for determining consequences of constitutive satellite loss on AURKA function and regulation.

- Using immunoblotting, we showed that AURKA cellular expression levels increased in RPE1 PCM1 KO cells relative to control cells (control: 1, RPE1 PCM1 KO: 1.3±5.7) (Fig. S4E). This increase is consistent with the increase associated with PCM1 depletion by RNAi.

- Using quantitative immunofluorescence, we showed that basal body levels of AURKA increased in RPE1 PCM1 KO cells as compared with control cells (Fig. S4G), which is consistent with the RNAi data. However, basal body levels of phospho-AURKA did not increase in RPE1 PCM1 KO cells, indicating that AURKA is not activated at the basal body in these cells.

- We and other showed that RPE1 PCM1 KO are almost inhibited for ciliation (Odabasi et al., 2019; Wang et al., 2016). Fig. S5B, shows that RPE1 PCM1 KO ciliated about 6.43% while the control cells ciliated about 87.52% upon 24 h serum starvation. Unlike its effect in cells depleted for PCM1 by RNAi, inhibition of AURKA activity in control and PCM1 KO cells did not have an effect on their ciliation efficiency. There might be two reasons for why AURKA inhibition did not restore ciliogenesis in PCM1 KO cells:
1) PCM1 KO did not result in an increase in phosphorylated AURKA at the basal body, which eliminates induced cilium disassembly induced by PCM1 depletion by RNAi.

2) Previous studies showed that acute and chronic loss of proteins might results in different phenotypes, in part due to compensatory mechanisms activated in knock-out cells (Hall et al., 2013).

5. *Cell cycle controls (flow cytometry or microscopy) should be provided for the serum restimulation experiments in Figure 7H, to determine whether changes in ciliation may be related to changes in cell cycle reentry through AURKA inhibition.*

The reviewer raises an excellent point on whether deciliation phenotypes upon restimulation might be due to cell cycle phenotypes associated with PCM1 depletion and/or AURKA inhibition. To address this, we quantified the mitotic index of the cells before and after serum restimulation (2 h and 24 h). Even 24 h after serum stimulation, there were few mitotic cells in both control and MLN8237-treated cells (Fig. S5E). RPE1 cells are immortalized by telomerase expression, have diploid genomes and respond to contact inhibition. Therefore, serum stimulation in confluent, ciliated RPE1 cultures were likely not sufficient to induce mitotic entry, suggesting that disassembly defects were not due to the difference between mitotic index.

6. *The introduction is rather lengthy and should be abbreviated. The articulation of additional questions on p.6 could be removed (from 'For example'...to '...transient ones.').*

We agree with the reviewer and shorted the introduction by summarizing some sections and removing the parts that are not directly related to the focus of our manuscript. Major changes are as follows:

- We removed the questions that we poised regarding mechanisms by which AURKA is regulated during cilium disassembly.
- We removed the detailed introduction on primary cilium and its biogenesis and rather focused on what is known about AURKA functions during cilium assembly and disassembly.
- We removed the part explaining the studies that compared traditional and proximity-based approaches as this part is also included in the results section.

7. *It is unclear what is meant by 'endogenous FLAG-BirA*-AURKA' and 'V5-BirA*AURKA' (p. 13). Figure 4E shows no evidence regarding endogenous AURKA, despite what is mentioned on p13. This should be clarified.*

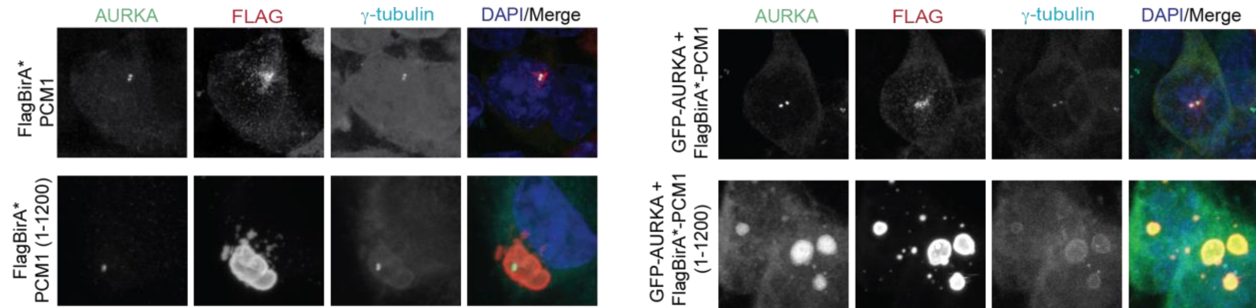
As the reviewer pointed out, cells were not stained for endogenous AURKA. For Fig. S3B of the revised manuscript, cells transfected with V5-BirA*-AURKA was stained for streptavidin and different markers of the centrosome and satellites to assess whether biotinylation of these structures can be determined by immunofluorescence. We corrected this textual error in the revised manuscript.

8. *The experiment shown in Figure 4F is unclear. References should be cited to explain the approach used, of the non-satellite aggregates formed by PCM1 deletion mutants. It would be useful to include a PCM1 full-length control for this experiment. The PCM1 construct '1-3600' should be referred to by its amino acid specification, not the nucleotide numbering; this should be made consistent throughout the MS.*

Rescue experiments in PCM1 KO cells with stable expression PCM1 truncation mutants showed that PCM1 (1-1200 a.a.) is required for satellite assembly (Odabasi et al., 2019; Wang et al., 2016). When overexpressed, PCM1(1-1200) N-terminal domain induces formation of large aggregates and sequesters a number of satellite proteins to these aggregates from their centrosome/satellite/cytoplasmic pools (i.e. PLK1 (Wang et al., 2013)). Therefore, expression of PCM1 (1-1200) has been used to test whether the proteins are sequestered at satellites.

- ***Figure below:*** We transfected cells with GFP-fusions of full length PCM1 and PCM1 (1-1200) and stained them for endogenous AURKA. Additionally, we co-transfected cells with FLAG-

AURKA and GFP-fusions of full length PCM1 and PCM1 (1-1200). Neither FLAG-AURKA or endogenous AURKA co-localized with full-length PCM1 at the satellites. While FLAG-AURKA co-localized with PCM1 N-term granules, endogenous AURKA did not. This difference might be due to the abundance of AURKA in cells and differences in its binding affinity to satellites and centrosomes.



- Given that this approach relies on overexpression of N-terminal region of PCM1, it is possible that it might induce artifacts that do not recapitulate the physiological conditions. As an alternative approach for testing the specificity of AURKA-satellite interaction and for determining determine whether AURKA is stored at satellites, we made use of the inducible trafficking assay we developed for investigating satellite interactions and functions (Aydin et al., 2020). Rapamycin-inducible dimerization of PCM1 to the kinesin motor domain results in redistribution of satellites to the cell periphery. We previously showed that the satellite clusters at the cell periphery sequesters low abundance satellite interactors like gamma-tubulin and as such, these assays is a powerful tool to distinguish satellite and centrosome interactions. We stained cells expressing GFP-PCM1 and HA-Kif5b (motor domain) before and after rapamycin treatment for AURKA and gamma-tubulin. While gamma-tubulin and AURKA localization was restricted to the centrosome before rapamycin treatment, both proteins were recruited to the satellite clusters at the periphery (Fig. 3F). These results show that endogenous AURKA is sequestered to satellites and is potentially regulated by satellites.

***9.** The data in Figure 5D and 5E should be combined- the Table contains effectively all the information that the bar diagram conveys. It is unclear how the indicated S988 site (WVS) conforms to the R/K/N-R-X-S/T consensus, so this should be reviewed.*

- In the original manuscript, we included all phosphosites depleted upon MLN8237 treatment independent on whether they conform to the AURKA consensus site or not. For the revised manuscript, we performed an additional set of experiments to test whether PCM1 is phosphorylated. As summarized below, the results of these experiments did not support PCM1 as a putative substrate for AURKA.

-To quantitatively analyze PCM1 phosphorylation status upon MLN8237 treatment, we performed one more phosphoproteomics experiment in control and MLN8237-treated cells expressing FLAG-PCM1. MaxQuant analysis of the phosphorylated peptides identified across three experimental replicates did not identify any phosphosites significantly depleted or absent in MLN8237-treated cells. Additionally, we did not observe a difference in the migratory behavior of PCM1 in control and MLN8237-treated cells (Fig. S3F).

Minor points

***10.** Size markers should be included for Figure 6C; the Legend for this panel should be labelled correctly. Bar graphs are not required for these data; these results can be summarized on the immunoblot, not least as an s.e.m. that is determined on 2 repeats is not very meaningful.*
We included the size markers for the immunoblotting data in Fig. 6C (now Fig. 4C) and corrected the labeling in its legend. We removed the bar graph and wrote the fold change of AURKA and p-AURKA below the immunoblots

11. The diagram in Figure 6D should indicate the timecourse followed- the inclusion of 24h is confusing.
We mislabeled the diagram associated in Fig. 6D (now Fig. 4D). In the revised manuscript, we removed the diagrams in this figure (now Fig. 4) and instead explained the experimental methodology in the results and methods sections.

***12.** There are some typos in the Figures that should be tidied up, e.g. Fig 5B 'Commasie'; 5C 'Phoshosite'; Fig 6C 'Quinescent'.*
We corrected the typos in Fig. 5B, 5C and 6C.

Referee #2:

This manuscript exploits the BioID mass spectrometry approach to identify protein interactors of the protein kinase Aurora A, which are then validated using over-expression, co-immunoprecipitation and co-localisation studies. While there appear to be some interesting findings from this study, including a number of proximity interactors that are localised to centriollar satellites, suggesting that AurA also has a role here, there are some significant issues with this manuscript that raises questions as to the validity of these findings.
We thank the reviewer for the constructive criticism of our manuscript. We were encouraged to see the reviewer found the findings of our manuscript interesting. We agree with the reviewer's concern on the lack of controls, quantitative analysis of the BioID data and its validation, which together weakens the future use of the AURKA proximity data as a resource in the field. We addressed these concerns in the revised manuscript by reanalyzing the BioID data and performing new experiments as suggested by the reviewers. The results of these new set of experiments collectively validated the power of the AURKA proximity map as a resource and strengthened our conclusions on the role of centriolar satellites on AURKA function and regulation. In addition to identifying the AURKA proximity map, we would like to highlight that our manuscript advances the field by uncovering new mechanisms for spatiotemporal regulation of AURKA function and mechanism in quiescent cells.

Major concerns:

***1.** The paper lacks rigorous controls throughout - e.g. there are no loading controls for any of the immunoblots. There is no comparison of AurA localisation in parental cell lines with respect to the stable cells lines expressing BirA-AurA, and no indication of the relative levels of over-expression. I was unable to find any controls validating knockdown of PCM1 with the specific, but not the control siRNA.*
We agree with the reviewer that essential controls are missing in several experiments throughout the paper. In the revised manuscript, we included the following controls and performed additional experiments to strengthen our conclusions:

- We performed immunoblotting for vinculin as a loading control in Fig. 1D, which reflects the differences in the quantity of different cellular extracts analyzed for validating expression and biotinylation V5-BirA* and V5-BirA*-AURKA.

- To deplete PCM1 in human cells, we used two different PCM1 siRNAs, which were validated used for functional experiments by us and others (Conkar et al., 2019; Dammermann and Merdes, 2002). In the revised manuscript, we performed immunoblotting and immunofluorescence to quantify the efficiency of PCM1 depletion and loss of centriolar satellites. The immunoblots in Fig. S4A shows that PCM1 is more efficiently depleted by siRNA#1 than siRNA#2. The immunofluorescence data in Fig. S4A confirms that satellites are lost upon depletion of PCM1 and that the efficiency of the two siRNAs is different from each other.

- To ensure that PCM1 phenotypes we reported were not due to off target effects, we performed cilium assembly experiments in control and PCM1-depleted cells with a second siRNA that targets 1053-1071 bp of PCM1. Although this siRNA was less efficient in depletion of PCM1 (Fig. S4A), the associated ciliogenesis phenotypes in DMSO and MLN8237-treated cells were similar to the ones we reported for the first PCM1 we used (Fig. S5A). Additionally, we quantified changes in basal body levels of AURKA and p-AURKA in cells depleted with the PCM1 siRNA#2 and the results were similar to PCM1 siRNA#1 (Fig. 4A, B)

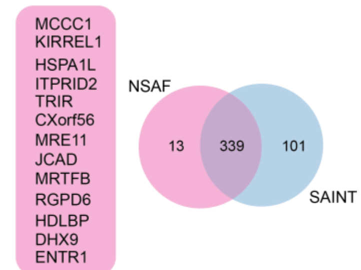
- We performed immunoblotting of lysates from cells stably expressing V5-BirA*-AURKA using antibodies against AURKA and V5. These results confirmed that V5-BirA*-AURKA is expressed at near endogenous AURKA levels (Fig. 1B). Additionally, we quantified and compared the phenotypes of control and V5-BirA*-AURKA stable cells for centrosome amplification, multinucleation, mitotic index, apoptosis and cell cycle profiles (Fig. S1). Together, these results indicated that the V5-BirA*-AURKA-expressing stable line do not exhibit defects associated with AURKA overexpression.

***2**. I seriously question, based on the detail provided in the manuscript, how the protein quantification was performed. Only two replicates were performed, yet they appear to do some form of statistics to determine change. What do they mean by 'spectral counts equal to or greater than 2'. What controls do they have to check that their quantification method is valid? The rationale for selecting a NSAF value of 5.7 is really random, with the apparent justification that this is appropriate given that they see 'known' AurA interactors at this level. If the argument as presented is that the community have only touched the tip of the iceberg with respect to AurA interactors, then surely there will be many below this level that are true positives, but missed because of the way that the experiment/quantification was performed.*
We agree with the reviewer that selecting an NSAF value of 5.7 based on validated interactors might impose a bias in analysis of the BioID data and can result in filtering out previously undescribed interactors relevant for AURKA function and regulation.

NSAF (normalized spectral abundance factor) and **SAINT** (Significance Analysis of the Intearctome) are the two label-free quantitative proteomics approaches extensively used for analysis of the BioID data. In the original manuscript, we analyzed the mass spectrometry data using the **NSAF** method and set the confidence thresholds to include the validated AURKA interactors. To address the reviewer's concerns on the bias these thresholds might impose, we reanalyzed the mass spectrometry data from 2 biological replicated for V5-BirA*-AURKA and 4 biological replicates for V5-BirA* using **SAINT** analysis, which generates a probability score for each protein interaction identified in affinity purification-MS datasets. For defining specific

AURKA interactions, we used a SAINT score cut-off of >0.95, which returned 440 high confidence interactors.

Out of the 352 AURKA interactors defined by NSAF analysis, 339 of them was returned by SAINT analysis, showing that the composition of the AURKA interactome did not change significantly (see figure). Additionally, the interactors identified by these approaches revealed the same functional clusters (Fig. 2, S2). Of note, SAINT analysis returned 101 more interactors, indicating that the thresholds we set for NSAF analysis were more stringent. To sum up, these results validate our analysis approach in generating the AURKA interactome. In order to make benchmarking with other BioID studies easier and setting thresholds independent of the validated interactors, we replaced the NSAF analysis data with SAINT analysis data in the revised manuscript and reanalyzed the data for BioGRID overlap (Fig. 2A), GO enrichment (Fig. 2B), networking analysis (Fig. 2C, Fig. S2A) and comparative analysis with published datasets (Fig. S2B, 3A).



***3.** They appear to have compared their proximity interactors with 'high confidence' interactors in the STRING database (from methods), and then later it appears that they have compared all putative interactors from high and low-throughput strategies. Please be clear (and stringent) with how these comparisons are performed.*

We agree with the reviewer that comparative analysis of the AURKA proximity interactome with validated interactors (papers that experimentally validates the interactions) and BioGRID data (low and high throughput studies that predicted putative interactors) resulted in lack of clarity in our analysis. In fact, when we compared the validated interactors with the BioGRID data, we found that BioGRID data was comprehensive and included all validated AURKA interactors.

For the revised manuscript, we performed the following analysis and clarified them in the methods section:

Fig. 2A: We included comparative analysis of the AURKA proximity map with the AURKA interactors included in the BioGRID repository (Fig. 2A). About 12% of the high-confidence AURKA interactors (52 proteins) overlapped with BioGRID, identifying the remaining 338 proteins as previously undescribed AURKA interactors. The low percentage of overlap is consistent with the literature and highlights differences in the nature of interactions probed by the BioID approach and traditional approaches.

Fig. S2B: We organized the top 200 AURKA proximity interactors into an interaction network by combining STRING database and ClusterONE plug-in on Cytoscape. This analysis grouped proteins based on their interconnection and identified the functional clusters representing subnetworks and potential multiprotein complexes (Fig. S2B).

Fig. 2C: To gain insight into the molecular mechanism of AURKA function and regulation, we generated sub-interaction interaction networks by assigning proteins based on enriched subcellular compartments (centriolar satellite and centrosome) and biological processes (cell division, centriole duplication, primary cilium biogenesis, microtubule organization, cell adhesion).

4. Having been through the methods carefully, it is somewhat surprising that they managed to identify any sites of phosphorylation given that they do not appear to have used any kinase/phosphatase inhibitors in their lysis/extraction buffers. Please could they check?

The reviewer raises a very important concern, which was due to a textual error from our side. We actually included phosphatase inhibitors (Sigma PhosSTOP-blend of phosphatase inhibitors) and protease inhibitors (Leupeptin, Pepstatin and Chymostatin, PMSF) to the lysis buffer we used for pulldown experiments. We revised the methods section to include this important technical detail.

Unfortunately, I also have serious concerns about the phosphosite information that are being reported in e.g. Fig. 5. They report Mascot scores, and highlight associated sites 'identified', but there is no indication of phosphosite identification confidence. Given that the Mascot scores are identical for the same peptide with different sites highlighted, it appears that this may be a case of ambiguous site localisation (possible even associated with the sasme MS/MS scan number) rather than that all of these sites have been defined, but it is impossible to know given the information presented. There are no spectra included to give confidence in any of the sites and therefore I would question the confidence of them all.

- We agree with the reviewer that the way we presented and analyzed the data were not quantitative and resulted in unclarity. In the original manuscript, we made a list of all peptides depleted or absent in the mass spectrometry data generated from MLN8237-treated cells compared to DMSO control across 2 independent experiments. Since most of these peptides were only identified in one experiment, we repeated the experiment for the revised manuscript and used **3** independent replicates to analyze how MLN8237 changes the phosphorylation profile of PCM1.

- In collaboration with EMBL proteomics facility, we analyzed all three experimental replicates together using MaxQuant and included the results of this analysis in Table 4. The table includes a localization probability for the phosphosite(s) (column: "Localization prob "), addressing the reviewer's comment on the "phosphosite identification confidence". Further, table includes the signal intensities of the individual peptides (e.g. column: "Intensity DMSO_rep1___1" for single phosphorylated, and "…_2" for double phosphorylated and "…_3" for triple phosphorylated peptides; highlighted in different shades of green)), as well as a label free quantification (LFQ) of the individual samples (Table 4). Together, this allowed us to look for consistent changes throughout 3 replicates and provided numbers for the probability of a phosphosite and its fold-change towards its control. For visualization of the data for PCM1, the signal intensities of the different conditions were plotted in bar graphs next to the respective columns for the signal intensities (Table 4, Tab 2). Based on the comparison of the bar graphs across 3 replicates, we concluded that MLN8237 treatment did not result in reproducible depletion or absence of the identified phsophopeptides. Together, these results do not support PCM1 as a putative substrate for AURKA. We revised the manuscript accordingly to include this conclusion.

- We compared the migratory profile of PCM1 in control and MLN8237-treated cells using Phos-tag gel. The size of PCM1 in control and MLN8237-treated quiescent cells were similar in the Phos-tag gel, suggesting that PCM1 is not phosphorylated by AURKA (Fig. S3F).

- To test regulation of PCM1 by AURKA in a different approach, we examined the consequences of AURKA inhibition on satellite integrity and distribution. Satellites were still present in MLN8237-treated cells (Fig. S3G). Moreover, pericentrosomal PCM1 levels did not change upon MLN8237 treatment as compared to control cells (Fig S3G). Together, these results show that AURKA activity is not required for satellite integrity and distribution.

5. Could the authors please explain why they have used different concentration of MLN8237 in different experiments?
- We used 0.5 µM MLN8237 for cilium assembly and disassembly experiments, which we chose based on previous studies utilizing similar assays and validated by immunoblotting. As for cilium and assembly disassembly experiments, we chose 0.5 µM MLN8237. However, we mislabeled the concentration we used for cilium disassembly as 1 µM instead of 0.5 µM. We apologize for this textual error and corrected it in the revised manuscript.

- As for phosphoproteomics experiments, we chose 1 µM MLN8237 in order to make benchmarking with published AURKA mitotic phosphoproteomics analysis (Kettenbach et al., 2011).

- We immunoblotted extracts from cells treated with 0.5 µM to 1 µM MLN8237 and DMSO with an antibody against AURKA and phospho-T288 AURKA and confirmed that both concentrations of MLN8237 inhibited the kinase activity of AURKA and included this data to Fig. S3D.

6. Unfortunately, the authors do not really do adequate justice in terms of referencing prior work, either in the AurA field, or the BioID/quantitative proteomics.
We now included more citations for AURKA and BioID throughout the manuscript. Since these there are many research articles that pertain to key discoveries for BioID and AURKA, we chose to cite review articles in some cases in order to be comprehensive. We would appreciate feedback on whether we missed any key references.

Referee #3:

This manuscript by Arslanhan et al. describes the proximity interactome of Aurora A and identifies centriolar satellites as regulators of AURKA function. As expected, they identify many proteins related to the centrosome. However, they uncover additional functional groups not reported in previous affinity purification systems. In particular, they identify an enrichment of proteins associated with centriole satellites. They further confirm that Aurora A physically interacts with potentially phosphorylates PCM1, a critical scaffolding protein for satellites. They further demonstrate that PCM1 depletion enhances Aurora A localization to and activity at the basal body in quiescent cells, where it disrupts cilium assembly. Notably, this effect is limited to cilium presence; it does not alter cilium length. While PCM1 knockdown decreases cilated cells, this effect is partly rescued with MLN8237. The manuscript concludes that centriolar satellites regulate AURKA protein levels to control its cilium assembly functions (abstract, introduction). Also it concludes that PCM1 phosphorylation may be important for its regulation.

This paper has a number of strengths as findings shed light on novel functions of Aurora A. First it employs a BioID approach and identifies a large library of proximate proteins-a valuable contribution to the literature. Second, it identifies PCM1 as an interactor and likely direct substrate of PCM1. Third, it finds an interesting ~2-fold increase of AURKA through protein stabilization upon PCM1 knockdown. Most of the approaches and many conclusions are well supported by the data. The manuscript appropriately reserved some conclusions, and described some of the possibilities in the discussion. Some significant weaknesses are there are potential confounders with cell-cycle effect of MLN8237, that the functional outcomes of AURKA loss on ciliagenesis appear to be synthetic only (only found upon PCM1 knockdown-which is fine, and interesting, but the conclusions should make this more clear). These and other issues to be addressed are outlined in more detail below.

We thank the reviewer for the accurate summary of our findings and for the constructive criticism of our manuscript. We were encouraged to see the reviewer found the data presented in the manuscript as technically sound and highlighted its contribution to the field by providing a resource and uncovering a new relationship between AURKA and satellites. We agree with the concerns raised by the reviewer and appreciate the extensive suggestions on addressing these concerns. The revision experiments significantly strengthened our conclusions as discussed. Moreover, they revealed that AURKA and PCM1 have antagonistic functions during basal body maturation (IFT88 recruitment) and this in part explains their ciliogenesis phenotypes (Fig. 5F, G)

Major points:

(1) *The manuscript appears to conclude that AURKA exists at the satellites even though it is not seen there in immunofluorescence. A more likely possibility not mentioned is that the interactions could simply occur at the centrosome (where many of these satellite proteins are also found), or through soluble pools of proteins. The data do not adequately support the claim that Aurora A is a novel part of satellites.*
Centriolar satellites are macromolecular protein complexes scaffolded by PCM1, which is essential for satellite integrity and is defined as the molecular marker for satellites. Therefore, satellite residents are defined by their interaction and/or co-localization with PCM1. As the reviewer noted, majority of satellite proteins also localize to the centrosome. In fact, PCM1 is the **only protein** that exclusively localizes to the centrosomes.

The satellite proteome consists of over 200 proteins and a significant portion of their interactions are low abundance, weak and/or transient. This is why the functional and regulatory links between centrosomes and satellites have prominently emerged through application of proximity-based proteomic profiling of satellites. **Therefore, our results on identification of AURKA as a satellite component through proximity mapping but not cellular localization was not unexpected.** Although all satellite proteins reported so far interacts with PCM1, not all of them localizes to satellites due to the transient and low abundance nature of satellite interactions. Examples are PLK4, gamma-tubulin and CEP63. These proteins were defined as satellite residents by functional assays, proximity interactions, displacement experiments and/or co-IPs. To provide further evidence for the identification of AURKA as a new satellite component, we performed the following experiments:

-The reviewer raises excellent points regarding where AURKA-PCM1 interact in cells. While we proposed satellites as sites of interactions, they could also interact at the centrosome and/or cytosol, which are not mutually exclusive. To address whether this interaction is dependent on centrosome, we performed co-IP experiments between AURKA and PCM1 in HEK293T cells treated with the PLK4 inhibitor centrinone, which results in centriole-less cells by inhibiting centriole duplication and depleting centrioles. In both control and centriole-less cells, GFP-PCM1 co-precipitated with FLAG-AURKA, indicating that their interaction does not depend on centrosomes (Fig. 3D).

- We performed chemically-inducible satellite trafficking assay to further test whether AURKA is sequestered at the satellites. As explained below in our response to point 4, the results of this assay provide further support for the specificity of the interaction between satellites and AURKA (Fig 3F).

- We performed streptavidin pulldown experiments to test the interaction of AURKA with multiple centrosome and satellite proteins as predicted by its proximity interaction map. We chose

proteins that were implicated across different cellular functions such as CSPP1 (implicated in mitosis, cell adhesion, ciliogenesis), TALPID3 (initiation of ciliogenesis) and CP110 (centriole cap required for centriole length control and initiation of ciliogenesis). To validate the specificity of AURKA interactions with satellites, we also tested interactions with satellite proteins that were not identified in the proximity map, which include SSX2IP (centriole duplication, microtubule anchoring) and BBS4 (component of the ciliary BBSome complex). AURKA interacted with CSPP1, Talpid3, CP110, but not with BBS4 and SSX2IP (Fig. 3C). Together with the interactions of AURKA with PCM1, CEP131 and CEP72, these results provide further support for the specificity and physiological relevance of the AURKA proximity map. Additionally, they suggest mechanisms for AURKA functions during cilium assembly and centriole duplication.

Together, these new sets of experiments and data provide strong support to interaction between satellites and AURKA. In the revised manuscript, we included a discussion on why endogenous AURKA were not detected at the satellites by immunofluorescence.

***(2)** Some basic controls appear to be missing, particularly with siPCM1.*
a. There is no clear blot to show the degree of PCM1 knockdown achieved, nor whether this affected the centriolar satellites in these cells.
In previous studies, we and others used the PCM1 siRNA (siRNA#1) for functional assays and validated its depletion efficiency by immunofluorescence and immunoblotting (Conkar et al., 2019; Dammermann and Merdes, 2002). In the revised manuscript, we used an additional PCM1 siRNA for depletion experiments, which were also validated before (Dammermann and Merdes, 2002). We performed immunoblotting and immunofluorescence to quantify the efficiency of PCM1 depletion and loss of centriolar satellites using these two siRNAs. The immunoblots in Fig. S4A shows that PCM1 is more efficiently depleted by siRNA#1 than siRNA#2. The immunofluorescence data in Fig. S4A confirms that satellites are lost upon depletion of PCM1 and that the efficiency of the two siRNAs is different from each other.

b. Only a single siRNA is used for PCM1 knockdown. Ideally a rescue experiment could be done, but at a minimum, 2 siRNAs are typical due to the known off-target effects of these reagents even if PCM1 knockdown is verified.
- To validate the specificity of the ciliogenesis phenotypes associated with PCM1 depletion, we ordered a second PCM1 siRNA (siRNA#2) targeting a different region in human PCM1 (1053-1071 bp). In Fig. S4A, we quantified the depletion efficiency of this siRNA using immunoblotting and immunofluorescence. This siRNA was less effective and resulted in partial depletion. RPE1 cells transfected with this siRNA were defective in ciliation, which was partially rescued by MLN8237 treatment like its effects in PCM1-depleted cells by siRNA#1 (Fig. S5A). Additionally, we quantified changes in basal body levels of AURKA and p-AURKA in cells depleted with the PCM1 siRNA#2 and the results were similar to PCM1 siRNA#1 (Fig. 4A, B). Together, these results eliminate the possibility of off-target effects.

c. If the causal link to satellites regulating Aurora A (rather than just PCM1 per say) is claimed, then it would be important to similarly disrupt another protein that is required in satellites.
PCM1 is the only satellite protein that exclusively localizes to satellites and is essential for satellite integrity and assembly. Therefore, it is defined as the molecular marker and scaffolding protein for satellites. PCM1 depletion or deletion results in loss of satellites (Fig. S4B) and restrictive localization of satellite residents at the centrosome. However, depletion of other satellite proteins does not interfere with satellite assembly, and only affect satellite distribution profile in cells (Fig. S4D) (Gupta et al., 2015).

Given that depletion/deletion of PCM1 is the only condition that results in satellite-less cells, we do not anticipate that depletion of satellite residents other than PCM1 to result in similar phenotypes for AURKA. To test this for a previously characterized satellite protein, we quantified AURKA levels in control and CCDC66-depleted cells and showed that they did not change (Fig. S4C).

***(3)** Most experiments involve overexpressed proteins, which make it possible that some of the observed interactions are non-physiologic.*

a. Ideally, coIP interactions such as Figure 4C-D would be reproduced with endogenous proteins.
We performed endogenous pulldown of AURKA in asynchronous cells (+FBS) and quiescent ciliated cells (-FBS). PCM1 co-pelleted with AURKA, but not the IgG control, confirming that endogenous AURKA and PCM1 interact (Fig. 3D).

b. Quantitative Western Blotting and Immunofluorescence should be performed to determine how the levels compare to Aurora A in the parental cell lines. Alternatively, it would be possible to knockdown the endogenous Aurora A. At a minimum, these limitations could be addressed more in the discussion, both in how it relates to the global Aurora A interactome (contributing to the minimal overlap observed in Fig. 2) and to their findings in particular. [For example, CEP63 was detected as a proximal interactor using stably expressed V5-BirA-AURKA, but not by transiently transfected Flag-BirA-AURKA.]
- The reviewer raises an important concern regarding the physiological relevance of the AURKA proximity interactome. As the reviewer indicated, AURKA overexpression results in multiple phenotypes such as defects in mitotic progression, multinucleation, centrosome amplification and apoptosis. Therefore, we generated the AURKA interactome using cells that stably express V5-BirA*-AURKA at near endogenous levels. In the revised manuscript, we included the following sets of data to address the reviewer's concerns:

a) Using immunoblotting of cell lysates for AURKA and V5, we compared the expression level of V5-BirA*-AURKA relative to endogenous AURKA. These results confirmed that V5-BirA*-AURKA is expressed at near endogenous AURKA levels (Fig. 1B).

b) We included immunofluorescence data for staining the V5-BirA*-AURKA cell line with antibodies against AURKA and V5 (Fig. S1I). This result confirms the localization of AURKA to the centrosome in interphase cells and to the spindle poles and microtubules in mitotic cells. Additionally, it also shows that that the cell line is 100% positive for V5-BirA*-AURKA expression.

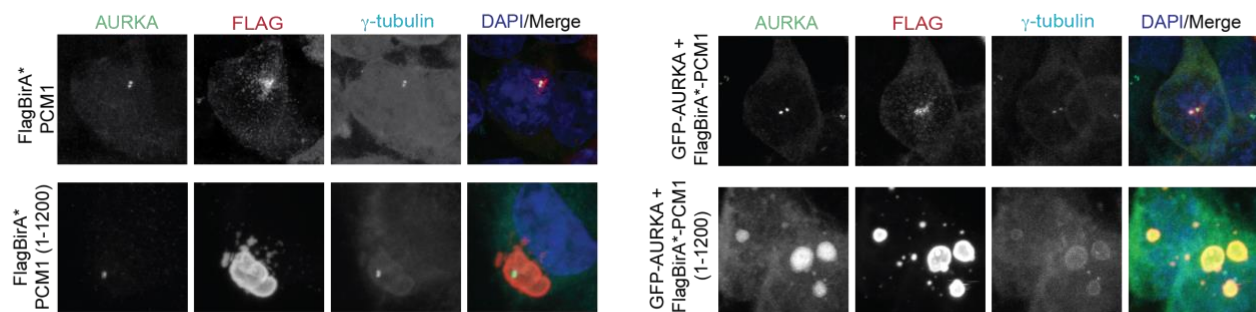
c) We showed that V5-BirA*-AURKA-expressing stable cell line behaves like the control cells in terms of its centrosome number, cell cycle profiles and mitotic defects by the following experiments:
Fig. S1A, B, C: centrosome number and percentage of multinucleation (immunofluorescence analysis for gamma-tubulin, DNA and microtubles)
Fig. S1D, E: their mitotic index (immunofluorescence analysis by DAPI)
Fig. S1D, F: spindle polarity (immunofluoerescence analysis for microtubules)
Fig. S1G: cell cycle profiles (flow cytometry and immunofluorescence)
Fig. S1H, apoptosis (immunoblotting for caspase 3)

Together, these results indicate that the V5-BirA*-AURKA-expressing stable line do not exhibit defects associated with AURKA overexpression.

- In Fig. 3C, we used streptavidin pulldowns to validate the interaction between AURKA and different satellite proteins as predicted by the AURKA proximity map. AURKA interacted with all 6 proteins predicted by the proximity map but CEP63. This might either be due to the weak nature of this interaction or ectopic expression conditions or that this interaction might be unspecific, which is inherent to mass spectrometry-based approaches to some extent. Of note, the fact that majority of the proteins we tested interacted with AURKA validates the high confidence levels of the AURKA proximity map. We now included these points in the discussion section.

(4) The authors use an exogenously expressed PCM1 truncation mutant to create satellite aggregates and demonstrate the GFP-Aurora A localizes to these aggregates. Does this mutant completely disrupt the centrosome? Where does gamma tubulin or other centrosome-only proteins localize? Without this information, the findings are not very informative. Moreover, the authors detect exogenously expressed GFP-Aurora A, not endogenous Aurora A.
Rescue experiments in PCM1 KO cells with stable expression PCM1 truncation mutants showed that PCM1 (1-1200 a.a.) is required for satellite assembly (Odabasi et al., 2019; Wang et al., 2016). When overexpressed, PCM1(1-1200) N-terminal domain induces formation of large aggregates and sequesters a number of satellite proteins to these aggregates from their centrosome/satellite/cytoplasmic pools (i.e. PLK1 (Wang et al., 2013)). Therefore, expression of PCM1 (1-1200) has been used to test whether the proteins are sequestered at satellites.

- ***Figure below:*** We transfected cells with GFP-fusions of full length PCM1 and PCM1 (1-1200) and stained them for endogenous AURKA. Additionally, we co-transfected cells with FLAG-AURKA and GFP-fusions of full length PCM1 and PCM1 (1-1200). Neither FLAG-AURKA or endogenous AURKA co-localized with full-length PCM1 at the satellites. While FLAG-AURKA co-localized with PCM1 N-term granules, endogenous AURKA did not. In all cases, gamma-tubulin localized to the centrosome, showing that PCM1 (1-1200) expression does not disrupt the centrosome. This difference might be due to the abundance of AURKA in cells and differences in its binding affinity to satellites and centrosomes.



- Given that this approach relies on overexpression of N-terminal region of PCM1, it is possible that it might induce artifacts that do not recapitulate the physiological conditions. As an alternative approach for testing the specificity of AURKA-satellite interaction and for determining determine whether AURKA is stored at satellites, we made use of the inducible trafficking assay we developed for investigating satellite interactions and functions (Aydin et al., 2020). Rapamycin-inducible dimerization of PCM1 to the kinesin motor domain results in redistribution of satellites to the cell periphery. We previously showed that the satellite clusters at the cell periphery sequesters low abundance satellite interactors like gamma-tubulin and as such, this assays is a powerful tool to distinguish satellite and centrosome interactions. We stained cells expressing GFP-PCM1 and HA-Kif5b (motor domain) before and after rapamycin treatment for

AURKA and gamma-tubulin. While gamma-tubulin and AURKA localization was restricted to the centrosome before rapamycin treatment, both proteins were recruited to the satellite clusters at the periphery (Fig. 3F). These results show that endogenous AURKA is sequestered to satellites and is potentially regulated by satellites.

***(5)** MLN8237 doesn't affect cilium assembly unless PCM1 is depleted (Fig 7C-D); since AURKA is not required for cilium assembly, some of the conclusions around that could be moderated-it seems that AURKA activity restrains cilia assembly in the absence of certain synthetic conditions of satellite protein knockdown.*

As the reviewer explained, AURKA activity restrains cilium assembly only in the absence of satellites. Based on our results, AURKA levels increase at basal body and is activated upon PCM1 depletion, which then activates the cilium disassembly pathway. In control cells, AURKA is not activated at the basal body so there is not induction of cilium disassembly and as such, AURKA inhibition does not affect the percentage of ciliated cells relative to DMSO-treated controls. As the reviewer suggested, we moderated our conclusions and discussed this in the "discussion section" in pg. 21.

***(6)** MLN8237 causes cell-cycle arrest in mitosis, changing the cell cycle profile of the cell population. Even in single cell analyses where interphase cells are quantified (such as acetylated-tubulin IF), the stage of G1 cells can differ. This is mitigated to an extent in serum starvation conditions, but still could be a concern, particularly with serum replete conditions (Figure 7G-I). It would be important to assess cell cycle profile in these conditions by PI/FACS, by quantifying mitotic index, or blotting for cell-cycle specific genes to determine which effects could be mediated indirectly by cell cycle states.*

The reviewer raises a valid concern on the possibility that cilium assembly and disassembly defects could be due to cell cycle phenotypes associated with AURKA inhibition or PCM1 depletion (Fig. 5). We thank the reviewer for suggesting experiments to address this. In the revised manuscript, we quantified the cell cycle state of the control and PCM1-depleted cells, which were treated with DMSO control or MLN8237 using the following experiments:

- We compared the percentage of quiescent cells after serum starvation in 4 conditions: 1) siControl + DMSO, 2) siControl + MLN8237, 3) siPCM1 + DMSO, 4) siPCM1 + MLN8237. To this end, we stained cells with the proliferation marker Ki67. There were no significant differences in the percentage of quiescent cells after serum starvation across all 4 conditions (Fig. S5C). Additionally, the ciliogenesis phenotypes across these four conditions remained the same when we quantified the percentage of ciliation in Ki67- quiescent cells (Fig. S5C). These results show that the ciliogenesis phenotypes we report in Fig. 5B are direct consequences of defects in biogenesis rather than indirect consequences of cell cycle defects.

- To address deciliation phenotypes upon serum stimulation might be due to cell cycle phenotypes associated with PCM1 depletion and/or AURKA inhibition, we quantified the mitotic index of the cells before and after serum restimulation (2 h and 24 h). Even 24 h after serum stimulation, there were few mitotic cells in both control and MLN8237-treated cells (Fig. S5E). RPE1 cells are immortalized by telomerase expression, have diploid genomes and respond to contact inhibition. Therefore, serum stimulation in confluent, ciliated RPE1 cultures were likely not sufficient to induce mitotic entry, suggesting that disassembly defects were not due to the difference between mitotic index.

Minor:

***(1)** As the authors have phospho-T288 Aurora A antibody, it would be ideal to use it to verify that the degree by which MLN8237 inhibits its autocatalytic activity.*
We used 0.5 µM MLN8237 for cilium assembly and disassembly experiments and 1 µM for phosphoproteomics experiments. In Fig. S5A, we immunoblotted extracts from cells treated with 0.5 µM to 1 µM MLN8237 or DMSO with an antibody against AURKA and phospho-T288 AURKA. Reduction in the phospho-AURKA signal confirms the inhibition of the autocatalytic activity of AURKA at both concentrations.

***(2)** Consider moderating conclusions in that MLN8237 doesn't affect ciliagenesis, but only has synthetic impacts upon PCM1 knockdown.*
We moderated our conclusions by including discussion on the synthetic effects in pg. 21 in the discussion section.

***(3)** The functional roles of the PCM1 phosphorylations is not described-I think that is OK, but the tangent in Figure 5 could be de-emphasized or explicitly described as being not linked to that which follows.*
As we described extensively in our response to Reviewer 2 (point 4), we performed an additional phosphoproteomic experiment to quantitatively determine whether PCM1 is phosphorylated by AURKA and if so, which sites are putative candidates for phosphorylation. MaxQuant analysis of mass spectrometry data from 3 experimental replicates did not identify any phosphosites that were reproducibly depleted upon MLN8237 treatment. Moreover, the migratory behavior of PCM1 did not change between control and MLN8237-treated cell extracts ran on Phos-tag gels. These results do not support PCM1 as a putative substrate for AURKA and our results in the manuscript instead shows regulation of AURKA by satellites. We revised manuscript to include and discuss this conclusion.

***(4)** Figure 1C; consider showing a shorter exposure for lanes 3-4 as it is overexposed and difficult to interpret.*
For detection of proteins by immunoblotting, we used the Licor Odyssey infrared imaging system, which allows quantitative comparison without the limitations associated with chemiluminescence. To allow visualization of individual bands in lysates prepared from biotin-treated V5-BirA*-AURKA-expressing cells, we modified the image settings and replaced the blot in Fig. 1D.

***(5)** In Figure 1C, a minus biotin control would be helpful to identify the Aurora A-proximate bands, particularly in that the BirA-AurkA appears to be expressed higher than the BirA* control.*
We immunoblotted lysates from control (-biotin) and biotin-treated V5-BirA* and V5-BirA*-AURKA-expressing cells. The blot included in Fig. S1J confirms induction of biotinylation upon incubation of cells with biotin and shows the differences in the biotinylation profile of V5-BirA* and V5-BirA*-AURKA-expressing cells.

***(6)** I found Figures 2-3 relatively uninformative-consider condensing into one.*
We consolidated results from Fig. 2-3 in one figure (Fig. 2, S2) and revised the manuscript accordingly to condense these parts.

***(7)** Figure 4C-D: it would be helpful to label in the figure not just 'pulldown' but 'streptavidin pulldown' in C and 'GFP-trap pulldown' in D.*
We thank the reviewer for the suggestion. We revised the manuscript to specify what type of pulldown experiment was performed in Fig. 3 and Fig. S3.

(8) Individual DAPI images would be helpful for all IF images, particularly when describing different mitotic stages. It also helps the reader evaluate DNA integrity. In Fig. 4, panel F, it is hard to appreciate where these aggregates are and if the overall integrity of the cell is disrupted.
We agree with the reviewer that including DAPI staining as individual channels will be helpful in some figures to indicate the cell cycle stage of the presented cells and to identify apoptotic cells, when these phenotypes are relevant to the conclusions. To address these concerns, we revised the following figures as detailed below:
- included individual DAPI channels for characterization of the V5-BirA*-AURKA stable line in Fig. S2
- we increased the intensity of the DAPI channels in some of the merged images where DAPI was not resolvable

(9) Consider insets in Fig. 4, panel E, and Fig. 7, panel B, to visually expand small structures.
As suggested by the reviewer, we included insets in Fig. S3B and Fig. 5A.

(10) The word proximal, used throughout the test, suggests unidirectional upstream activity. I would suggest using 'proximate' to mean nearby, as there is no intention to refer discriminately to upstream (or root-directed) interactions.
We thank the reviewer for the suggestion. We replaced “proximal” with “proximate”.

(11) Minor typos:
a. "during when they localize" (p.4)
b. "interactions with its spatially and temporally binding partners" (p.6)
c. "at in control and" (p.15)
d. Figure 6C "Quinescent cells"
e. Figure 6D graph, Y-axis, "normalized to GAPDH", yet blot indicates "vinculin" as loading control
f. Figure 6 legend: "B" should be "C"
We thank the reviewer for indicating these typos. We corrected them in the revised manuscript. Since we condensed the introduction and discussion in the revised manuscript, we removed some of these sentences from the manuscript.

1st Revision - Editorial Decision 7th May 2021

Dear Elif,

Thank you for submitting your revised manuscript. It has now been seen by all of the original referees.

As you can see, the referees find that the study is significantly improved during revision and recommend publication. However, referee #2 has significant outstanding concerns. I have discussed these concerns further with referees #1 and #3. We have decided that an expansion of the analysis to validate additional substrates from the proximity map is beyond the scope of this paper. Please address the concerns of referee #2 regarding the presentation of Table 4 Tab 2. It is not required to add the positive control for the Phos-Tag (the last paragraph of referee #2).

Please address all of the remaining referee concerns and provide a point-by-point response. Please let me know if you would like to discuss any of the points further.

In addition, I need you to address the editorial points below:

• There are currently 6 keywords. For technical reasons, we cannot accommodate more that 5 keywords. Thus, please remove one of the keywords.
• As per our guidelines, please add a 'Data Availability Section', where you give information about the primary datasets produced in this study that are deposited in an appropriate public database (see <http://embor.embopress.org/authorguide#dataavailability>). If it is not applicable, make a statement that no data were deposited in a public database.
• Please rename Competing Interests section as Conflict of Interests.
• Please rename Experimental Procedures section as Materials and Methods.
• The title is currently too long for our format requirements. It should be max 100 characters (including spaces).
• We notice that there are 5 supplementary figures which need the correct nomenclature and style, either:
a) Figure EV# should be individually uploaded with the legends in the word Article file; or
b) Figures and legends should be uploaded in one pdf file as an Appendix file, with a Table of Contents as a cover page.
Either way, please remember to update the callouts accordingly.
• Please separate the figures as one file per figure.
• As per our format requirements, in the reference list, citations should be listed in alphabetical order and then chronologically, with the authors' surnames and initials inverted; where there are more than 10 authors on a paper, 10 will be listed, followed by 'et al.'. Please see https://www.embopress.org/page/journal/14693178/authorguide#referencesformat
• We note the following regarding the figure callouts: Fig 3F callout is missing. Fig S5 has callouts for panels F-H, which don't exist. There is a callout to "Supplementary Figure 2".
• There are 4 tables which should be renamed as Dataset EV1-EV4. Their legends need to be included in the tables.
• Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.

• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The revised submission from Arslanhan, Firat-Karalar and their colleagues has tackled the issues that were raised by the reviewers. I note that there was good agreement between the reviewers on the principal concerns. Reviewer 2 provided some particularly useful technical comments. The manuscript now presents a clear advance in our knowledge of the cellular roles of Aurora A kinase, a topic of general interest, with new findings on how centriolar satellites contribute to its regulation. I am supportive of its publication in EMBO Reports.

There remain some clarifications that I consider important, however- while mostly textual in nature, there are some additions or corrections to Figures that should be made in a final version.

1. p.7: The controls presented in Fig. S1 help to address the concerns I raised about dominant-positive impacts of this overexpression, but the description of V5-BirA*-AURKA as 'expressed at near endogenous levels' is misleading, as the endogenous protein remains fully expressed. It is more correct in my view to state that the transgene product is 'overexpressed to a level equivalent to the endogenous protein' (or similar). I emphasize this point because it remains a formal issue with the study, even though I appreciate that the authors have made a strong effort to resolve it.
2. It would be useful to alphabeticise the list of interactors in the Venn diagram shown in Fig 2A.
3. Size markers should be included for all gels/ blots and scale bars for all micrographs, including those in the Supplemental data.
4. It would be clearer to label the control experiments throughout as 'V5-BirA*', rather than just 'control'. This would be useful for Fig. S1 in particular, where it is not explicit what the controls were until panel J.
5. The CCDC66 experiment in Fig. S4 is not quantitated (as is indicated in the legend to Fig. S4C). This should be included and the relevant description of the findings on p.15 reviewed. As written, it is unclear why the CCDC66 knockdown should not affect AURKA levels. The authors should clarify how CCDC66 knockdown impacts satellites differently from PCM1 knockdown so that this experiment can be interpreted fully by the reader (assuming the quantitation establishes that there

is no effect on AURKA levels).
6. The ciliary signals in Fig. S5D are very difficult to make out in the image shown; an improved image should be provided to indicate what is being quantitated in the disassembly experiment.

Minor points
p.5: The main paragraph should begin 'The primary cilium...'. The spelling of 'von Hippel-Lindau' should be checked
p.6: 'The mechanisms...remain'...
The legend to Figure S1I should be reviewed for clarity.
p.12 (line 11): Fig. 4D is incorrectly cited for Fig. 3D.
p.13 (line 9): Fig. 4F is incorrectly cited for Fig. 3F.
p.17 (final sentence): 'centrosomal levels...were reduced...'.

Referee #2:

The authors have on the whole done a decent job of addressing the reviewers' comments. However, given the system that they have established and the data that was presented in the previous submission, I find it strange that they have not evaluated which of their interaction partners in the identified network are potential substrates of the protein kinase which they are investigating. I am also not convinced based on the data presented that PCM1 is actually NOT a substrate of AurA.
(It was actually hard to define how much was changed in this revision given that there was not a description of what those changes were.)
Failure to explore this proximity network for putative AurA substrates (bearing in mind that the one target they evaluated, PCM1, they determine not to be phosphorylated by AurA, although see comments below), is a missed opportunity; the question thus remains open as to the functional role of the AurA protein kinase in this network at centriolar satellites.
The fact that the proximity interaction between PCM1 and AURKA was abolished upon inhibition of AURKA activity, suggests that there is another regulatory phosphorylation event, yet this crucial intermediary has not been identified. However, given the way that they have analysed the data from this experiment (Table 4, Fig. 3), I'm still not sure that they can absolutely rule out PCM1 phosphorylation by AurA - while they treat cells with MLN8237, they do not appear to compare phosphorylation levels in untreated cells to evaluate differences in PCM1 phosphorylation, instead appearing to look for AurA consensus? If they are in actual fact looking at phosphopeptide ratios as a function of treatment with MLN8237, this data is not presented (no fold changes reported, or indication of data normalisation as a function of in-gel digestion-based sample processing). The part of the manuscript describing this aspect is unclear (bottom pg 13)
Looking at the data in Table 4 - Tab2 presented the signal intensity data from the two conditions, 2 replicates, (as bar plots in column BV) - the data as presented here does not suggest any trends in the data as a function of treatment, with the major effect being the replicate. It would be more useful to present this information as a heat map to avoid misinterpretation of this experiment. Please remove column BV and equivalents.
Also the data as represented in BW-CC and CD-CI do not appears to make sense. I can't see that the peptides in CD-CI contain 3 phosphate groups, and, if they did, the data should also not be represented in either BP-BU (single phosphosite) or BW-CB (two phosphosites). This raises concerns that the (corresponding) author does not actually understand these data, or how to interpret them. Again, as represented, this data is misleading.
Rather than presenting all the phosphoproteomics data, it would be more useful to extract the data

of interest pertaining to PCM1 and present the ratio of phosphopeptides (phosphosites) as a function of MLN8237.
The Phos-Tag data presented in Fig. S3F shows no change in gel-shift of PCM1 as a function of cell treatment in MLN8237. Yet, again, there is no positive control for this assay - given that there is a change in (at least) Thr288 phosphorylation of AurA under these conditions, why has this not been used as a control to demonstrate functionality of this assay?

Referee #3:

In the revised manuscript, Aurora Kinase A proximity interactome reveals centriolar satellites as regulators of its function during primary cilium biogenesis (EMBOR-2020-51902V3), Arslanhan et al. make a significant effort to address each major and minor concern of ours and are candid about addressing discrepancies and/or limitations to their findings. To that extent, they have satisfied many of the issues we raised with the initial submission. There were, however, a few oversights in the revised manuscript that we feel would be in the authors' best interests to address them prior to publication.

#1 In the rapamycin-induced forced trafficking experiments (Fig. 3F and in Results), the authors claim that both AURKA and gamma tubulin relocalize to the cell periphery in response to rapamycin exposure. The images presented in the revised manuscript support AURKA, but not gamma tubulin, relocalization to the cell periphery. This was likely unintended image selection as recent work from their group (Aydin et al., 2020, PLoS Biol) indicates that gamma tubulin can relocalize to the periphery (Fig.1) as well as other satellite proteins (Fig. 2). It appears that the effects from rapamycin-induced trafficking is not binary (at the centrosome or at the periphery), which is fine. Given that other methods (IF, PCM1 truncation mutant) do not clearly demonstrate AURKA association with satellites, we request quantitation of peripheral AURKA and gamma tubulin in the rapamycin-induced trafficking experiments. This could be done in a similar fashion as Aydin et al.

#2 The Fig. S5E panel, its figure legend, and a callout in the Results section was excluded from the manuscript, despite the authors mentioning the experiment in their response. These should be included with the final submission as it addresses our concern about cell cycle profile in the MLN8237 experiments.
Minor points
1. We recommend the authors carefully review all of their figure callouts as we noted reference to Fig. S5F-G, which are not included in this submission. Likely they meant Fig. 5F & G.
2. Given the larger field of view in Fig. S4B, it would be helpful if the authors highlight a cell where PCM1 is depleted and one where it isn't.

Referee #1:

The revised submission from Arslanhan, Firat-Karalar and their colleagues has tackled the issues that were raised by the reviewers. I note that there was good agreement between the reviewers on the principal concerns. Reviewer 2 provided some particularly useful technical comments. The manuscript now presents a clear advance in our knowledge of the cellular roles of Aurora A kinase, a topic of general interest, with new findings on how centriolar satellites contribute to its regulation. I am supportive of its publication in EMBO Reports.
We thank the reviewer for the positive comments on our revised manuscript.

There remain some clarifications that I consider important, however- while mostly textual in nature, there are some additions or corrections to Figures that should be made in a final version.

1. p.7: The controls presented in Fig. S1 help to address the concerns I raised about dominant-positive impacts of this overexpression, but the description of V5-BirA*-AURKA as 'expressed at near endogenous levels' is misleading, as the endogenous protein remains fully expressed. It is more correct in my view to state that the transgene product is 'overexpressed to a level equivalent to the endogenous protein' (or similar). I emphasize this point because it remains a formal issue with the study, even though I appreciate that the authors have made a strong effort to resolve it.
We now revised this sentence as "…. stable cells that overexpress V5-BirA*-AURKA fusion to a level similar to the endogenous protein"

2. It would be useful to alphabeticise the list of interactors in the Venn diagram shown in Fig 2A.
We alphabetically ordered the list of interactors in Fig. 2A.

3. Size markers should be included for all gels/ blots and scale bars for all micrographs, including those in the Supplemental data.
We included size markers for all gels/blots except for Fig. EV3E, which represents the band we cut out for mass spectrometry analysis of FLAG-PCM1.

4. It would be clearer to label the control experiments throughout as 'V5-BirA*', rather than just 'control'. This would be useful for Fig. S1 in particular, where it is not explicit what the controls were until panel J.
We labeled the control experiments representing cells expressing V5-BirA* as "V5-BirA*" instead of control.

5. The CCDC66 experiment in Fig. S4 is not quantitated (as is indicated in the legend to Fig. S4C). This should be included and the relevant description of the findings on p.15 reviewed. As written, it is unclear why the CCDC66 knockdown should not affect AURKA levels. The authors should clarify how CCDC66 knockdown impacts satellites differently from PCM1 knockdown so that this experiment can be interpreted fully by the reader (assuming the quantitation establishes that there is no effect on AURKA levels).
- We agree with the reviewer that results of the CCDC66 loss-of-function experiments were not well-described. We clarified this by revising that part of the manuscript as follows:
"Finally, to investigate whether these phenotypes are specific to loss of satellites associated with PCM1 depletion, we performed similar experiments in cells depleted for another satellite protein CCDC66, which is required for pericentrosomal satellite clustering but not for cilium assembly (Fig. EV4C, D) (Conkar et al, 2017)."

- We did not quantify the pericentrosomal PCM1 levels in CCDC66-depleted cells as we previously quantified and reported this phenotype in Conkar et al, 2017 paper. Instead, we cited this paper and included a representative picture for the satellite redistribution phenotype of CCDC66-depleted cells.

6. The ciliary signals in Fig. S5D are very difficult to make out in the image shown; an improved image should be provided to indicate what is being quantitated in the disassembly experiment.
We revised the representative micrographs to highlight the cilia marked by acetylated tubulin.

Minor points
p.5: The main paragraph should begin 'The primary cilium...'. The spelling of 'von Hippel-Lindau' should be checked
p.6: 'The mechanisms...remain'...
The legend to Figure S1I should be reviewed for clarity.
p.12 (line 11): Fig. 4D is incorrectly cited for Fig. 3D.
p.13 (line 9): Fig. 4F is incorrectly cited for Fig. 3F.
p.17 (final sentence): 'centrosomal levels...were reduced...'.
We thank the reviewer for careful reading of our manuscript. We corrected all the typos, grammar errors and figure miscalling as suggested by the reviewer.

Referee #2:

The authors have on the whole done a decent job of addressing the reviewers' comments. However, given the system that they have established and the data that was presented in the previous submission, I find it strange that they have not evaluated which of their interaction partners in the identified network are potential substrates of the protein kinase which they are investigating. I am also not convinced based on the data presented that PCM1 is actually NOT a substrate of AurA. (It was actually hard to define how much was changed in this revision given that there was not a description of what those changes were.)

Failure to explore this proximity network for putative AurA substrates (bearing in mind that the one target they evaluated, PCM1, they determine not to be phosphorylated by AurA, although see comments below), is a missed opportunity; the question thus remains open as to the functional role of the AurA protein kinase in this network at centriolar satellites.
The fact that the proximity interaction between PCM1 and AURKA was abolished upon inhibition of AURKA activity, suggests that there is another regulatory phosphorylation event, yet this crucial intermediary has not been identified. However, given the way that they have analysed the data from this experiment (Table 4, Fig. 3), I'm still not sure that they can absolutely rule out PCM1 phosphorylation by AurA - while they treat cells with MLN8237, they do not appear to compare phosphorylation levels in untreated cells to evaluate differences in PCM1 phosphorylation, instead appearing to look for AurA consensus? If they are in actual fact looking at phosphopeptide ratios as a function of treatment with MLN8237, this data is not presented (no fold changes reported, or indication of data normalisation as a function of in-gel digestion-based sample processing). The part of the manuscript describing this aspect is unclear (bottom pg 13)

Looking at the data in Table 4 - Tab2 presented the signal intensity data from the two

conditions, 2 replicates, (as bar plots in column BV) - the data as presented here does not suggest any trends in the data as a function of treatment, with the major effect being the replicate. It would be more useful to present this information as a heat map to avoid misinterpretation of this experiment. Please remove column BV and equivalents. Also the data as represented in BW-CC and CD-CI do not appears to make sense. I can't see that the peptides in CD-CI contain 3 phosphate groups, and, if they did, the data should also not be represented in either BP-BU (single phosphosite) or BW-CB (two phosphosites). This raises concerns that the (corresponding) author does not actually understand these data, or how to interpret them. Again, as represented, this data is misleading.

Rather than presenting all the phosphoproteomics data, it would be more useful to extract the data of interest pertaining to PCM1 and present the ratio of phosphopeptides (phosphosites) as a function of MLN8237.

The Phos-Tag data presented in Fig. S3F shows no change in gel-shift of PCM1 as a function of cell treatment in MLN8237. Yet, again, there is no positive control for this assay - given that there is a change in (at least) Thr288 phosphorylation of AurA under these conditions, why has this not been used as a control to demonstrate functionality of this assay?

To address the reviewer's concerns on data presentation,

- **we applied a color scheme with the lowest value in white and the highest in red (Table 4, Tab2).**
- **Table4, Tab1 presents all the phosphoproteomics data, which provides a resource for future studies. To highlight potential phosphorylation sites on PCM1, we included the PCM1-specific phosphopeptides in Table4, Tab2.**

Referee #3:

In the revised manuscript, Aurora Kinase A proximity interactome reveals centriolar satellites as regulators of its function during primary cilium biogenesis (EMBOR-2020-51902V3), Arslanhan et al. make a significant effort to address each major and minor concern of ours and are candid about addressing discrepancies and/or limitations to their findings. To that extent, they have satisfied many of the issues we raised with the initial submission. There were, however, a few oversights in the revised manuscript that we feel would be in the authors' best interests to address them prior to publication.
We thank the reviewer for the positive comments on our revised manuscript.

#1 In the rapamycin-induced forced trafficking experiments (Fig. 3F and in Results), the authors claim that both AURKA and gamma tubulin relocalize to the cell periphery in response to rapamycin exposure. The images presented in the revised manuscript support AURKA, but not gamma tubulin, relocalization to the cell periphery. This was likely unintended image selection as recent work from their group (Aydin et al., 2020, PLoS Biol) indicates that gamma tubulin can relocalize to the periphery (Fig.1) as well as other satellite proteins (Fig. 2). It appears that the effects from rapamycin-induced trafficking is not binary (at the centrosome or at the periphery), which is fine. Given that other methods (IF, PCM1 truncation mutant) do not clearly demonstrate AURKA association with satellites, we request quantitation of peripheral AURKA and gamma tubulin in the rapamycin-induced trafficking experiments. This could be done in a similar fashion as Aydin et al.
We agree with the points raised by the reviewer. As the reviewer noted, rapamycin treatment did not result in a binary effect for proteins we tested previously (Aydin et al.

2020 PLOS Biology). To quantitatively describe how peripheral satellite redistribution affects AURKA localization, we quantified the centrosomal levels of AURKA before and after rapamycin treatment. As a positive control, we used gamma-tubulin. Centrosomal levels of both AURKA and gamma-tubulin were reduced in rapamycin-treated cells as compared to control cells (Fig. 3G). To better represent recruitment of AURKA and gamma-tubulin to peripheral satellite clusters, we included a new fluorescent micrograph for rapamycin-treated cells in Fig. 3F.

#2 The Fig. S5E panel, its figure legend, and a callout in the Results section was excluded from the manuscript, despite the authors mentioning the experiment in their response. These should be included with the final submission as it addresses our concern about cell cycle profile in the MLN8237 experiments.
The fluorescent micrographs presented in Fig. EV5D represent our result that serum stimulation and MLN8237 treatment did not induce cell cycle entry in ciliated, confluent RPE1 cultures. We included the following sentence and figure callout in the revised manuscript:
" This phenotypic difference was not due to cell cycle defects as the mitotic index of the confluent, ciliated RPE1 cells treated with DMSO or MLN8237 and stimulated with serum were similar (Fig. EV5D)."

<u>*Minor points*</u>
1. We recommend the authors carefully review all of their figure callouts as we noted reference to Fig. S5F-G, which are not included in this submission. Likely they meant Fig. 5F & G.
We carefully reviewed figure callouts and corrected the mistaken ones.

2. Given the larger field of view in Fig. S4B, it would be helpful if the authors highlight a cell where PCM1 is depleted and one where it isn't.
We included insets to highlight cells where PCM1 was present or depleted.

2nd Revision - Editorial Decision 26th May 2021

Dear Elif,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Kind regards,

Deniz
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

--

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For generating the AURKA proximity map, two experimental replicates for V5-BirA*-AURKA and four experimental replicates for V5-BirA* were analyzed by SAINT analysis. For quantitative mass spectrometry experiments, three experimental replicates for each condition was analyzed by MaxQuant. No statistical test is used for determining sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Exclusion criteria was not applied except when techinical quality was too low to obtain reliable data, in particular in antibody stainings in immunofluorescnece experiments.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	To avoid sujective or biased pipetting of drugs and DNA Mixes, everthing is prepared as master mixes when applicable.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Microscopy-based quantifications, both at the imaging and quantitation part, were done in a blinded manner. All microscopy data was compared with each other and was taken using the same exposure settings.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes, statistical tests used for quantification are described in the methods section and the p values are included in figure legends and the figures.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We performed Student's t-test or ANOVA for normal distribution data. We performed SAINT analysis for label free quantitation of the mass spectrometry data.

Is there an estimate of variation within each group of data?	Yes, SD and SEM are shown in the graphs and figure legends.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	This is provided in the "Antibodies" part of the "Materials and Methods".
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	This is provided in the "Cell Culture and Transfection" part of the "Materials and Methods".

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
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10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The raw mass spectrometry data was provided in the Table format with the manuscript.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Underlying quantitative data is provided in figures and associated legends.
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