EMBO reports

Gradual centriole maturation associates with the mitotic surveillance pathway in mouse development

Cally Xiao, Marta Grzonka, Charlotte Meyer-Gerards, Miriam Mack, Rebecca Figge, and Hisham Bazzi **DOI: 10.15252/embr.202051127**

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1st Editorial Decision 6th Aug 2020

Dear Dr. Bazzi,

Thank you for the submission of your research manuscript to our journal, which was now seen by two referees, whose reports are copied below.

I apologize for this unusual delay in getting back to you, which was caused by the current high rate of new submissions to our office, affecting our usually much shorter editorial handling time.

Referees appreciate the analysis, however, they also raise concerns that need to be addressed to consider publication here. In particular,

- further analysis on the centrosome maturation is required (both referees)
- (rescue) phenotypes need to be quantified (referee #2)
- p53 levels need to be assessed to confirm that their levels are restored in rescued double ko embryos (referee #3, point 1).

Regarding point 2 of referee #3, co-depletion of Sas4 and 53bp1 or Usp28 in mESCs is not required as this rescue was already shown in vivo.

Moreover, regarding point 3 of referee #3, if you have the TRIM37-/- Sas4-/- double ko already at hand, this would strengthen the manuscript. If not, please address this point by an alternative approach (to see whether p53 suppression independent of a prolonged mitotic duration can rescue the phenotypes of Sas4 depletion). Please let me know if you would like to discuss this point further.

I find the reports informed and constructive, and believe that addressing the concerns raised will significantly strengthen the manuscript.

Should you be able to address all criticisms in full, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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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.***

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1. A data availability section providing access to data deposited in public databases is missing (where applicable).
2. Your manuscript contains statistics and error bars based on n=2. Please use scatter plots in these cases.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

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When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14693178/authorguide#transparentprocess
You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

4) a complete author checklist, which you can download from our author guidelines (<http://embor.embopress.org/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<http://embor.embopress.org/authorguide>).

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <http://embor.embopress.org/authorguide#expandedview>.

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) We would also encourage you to include the source data for figure panels that show essential data.

Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available <http://embor.embopress.org/authorguide#sourcedata>.

8) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <http://embor.embopress.org/authorguide#datacitation>.

9) Please make sure to include a Data Availability Section before submitting your revision - if it is not applicable, make a statement that no data were deposited in a public database. Primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see <http://embor.embopress.org/authorguide#dataavailability>).

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Method) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

10) Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates.
Please also include scale bars in all microscopy images.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #2:

This study by Xiao and colleagues investigate the timing of activation of the mitotic surveillance pathway upon centriole loss during mouse development. The authors make use of a centriole-loss conditional model (Sas-4−/−), deletion of which causes centrosome loss, prolonged mitosis and p53-dependent cell death during early embryogenesis. By crossing null alleles of the p53 regulators 53BP1 and USP28 into this mutant strain, they confirm that the mitotic surveillance pathway is conserved in mice in vivo, and that 53BP1 and USP28 are essential for its conduction upstream of p53. They also show that the mitotic surveillance pathway is activated around E7 of embryonic development. Finally, they discover that centriole maturation correlates temporally with the establishment of the mitotic surveillance pathway in vivo, and suggest that only when the embryonic cells start to depend on centrosomes as MTOCs in mitosis and ciliogenesis, that the loss of centrioles and activation of the p53-dependent mitotic surveillance pathway occurs.

Overall, the work is well performed, the data are clear and strong, and the results are fairly convincing. The inclusion of a cilia mutant (IFT88) was a particularly nice approach, to compare and contrast between cilia loss and centrosome + cilia loss. However, certain parts of the story are too preliminary/under-developed, and further experiments are needed to strengthen/support the conclusions and make it suitable for publication in EMBO Reports. Specifically, the final section regarding centrosome maturation needs further exploration.

Comments and concerns:

- Fig 1A - The authors show that deletion of 53bp1 and Usp28 in the Sas4-null rescues the embryonic developmental defect at E9.5. However, no quantitation of this is shown: how many embryos showed a rescue, versus not? What was deemed as a "rescue"? Similarly, loss of p53 in these embryos (Fig1B) was not quantified. How prevalent/penetrant was this phenotype? This also applies to CASP3 analysis in Fig1C. Some quantitative measure of these observations should be included.

- Again, quantification for the phenotypes shown in Fig 2C and D are lacking, and should be added.

- Importantly, the final section regarding centriole maturation as a trigger for allowing cells to sense loss of centrosomes, and temporal activation of the surveillance mechanism (Fig4C and D) needs to be further tested. First, centriole maturation can be defined in many ways including: recruitment of distal and subdistal appendages, accumulation of PCM on the immature centrioles, and the ability to assemble a functional cilium. The authors only looked at recruitment of one distal appendage marker as a surrogate for centriole maturation, and the data presented is merely a correlation between the timing of Cep164 recruitment and when the mitotic surveillance pathway is active. To this reviewer, this is the most interesting part of this manuscript - since the rest of it mostly confirms the observations regarding the role of p53, 53bp1 and Usp28 that were previously defined in vitro. Therefore, I believe further dissection of "centriole maturation" is needed to support the conclusions in the study. One way to do this is to utilize the in vitro mESC isolated from these mutant mice (as in Fig 3A-D). The authors can systematically manipulate genes needed for distal appendage assembly, subdistal appendage assembly, PCM recruitment, and ciliogenesis - then test the consequences with regards to activation of the surveillance pathway. This will help to narrow down which aspects of "centriole maturation" are the key triggers for this activation.

- Fig 5 is mentioned in the text but is missing from the Figure panels.

Referee #3:

Review:

The authors report that the mitotic surveillance pathway is activated upon loss of Sas4 in vivo and in derived mouse embryonic stem cells. The findings are supportive evidence of previously published data (Lambrus et al., 2016, Fong et al., 2016, and Meitinger et al. 2016) done in cell culture. They provide evidence that genetic ablation of p53 or its upstream regulators, 53bp1 and Usp28 can resolve p53-dependent growth retardation and cell death in Sas4 mutant embryos. They further present immunohistochemical data for the appearance of matured centrioles after embryonic day 6.5 at which time the mitotic surveillance system becomes active. They conclude that the cell death

in embryos lacking centrioles is due to the activation of the mitotic surveillance pathway leading to a p53-dependent cell cycle arrest. Overall this is a straightforward study which recapitulates a pathway previously demonstrated in cells. The strength of the paper is the development of double mutants that rescue the development of embryos lacking centrioles. The major weakness of this paper concerns the lack of mechanistic insights that would answer why 53bp1 and Usp28 regulate p53 stabilization upon loss of centrioles. Below are points that the authors should consider:

Major concerns:
1. The authors demonstrated that p53 is stabilized in Sas4 mESC (Figure EV3), the authors should investigate the levels of p53 in WT, Sas4 -/-, Sas4 -/- 53bp1 -/- and Sas4 -/- Usp28 -/- embryos to confirm that p53 levels are restored.
2. In mESC, the authors demonstrate that upon Sas4 loss there is an increase in nuclear p53 and mitotic index; the authors should attempt to rescue the phenotype by co-depletion of Sas4 and 53bp1 or Usp28. An additional control of co-depletion of p21 and Sas4 would further solidify the claim that the mitotic surveillance pathway is activated in these mutants similar to the loss of PLK4.
3. As TRIM37 depletion suppresses mitotic defects in PLK4-inhibited cells, the authors should generate a Sas4 -/- TRIM37 -/-, double knockout to determine whether p53 suppression independent of a prolonged mitotic duration can rescue the developmental phenotypes found in Sas4 mutants.
4. In Figure 4C, the authors states centrioles begin to generate distal appendages (CEP164), given the significance of this finding, the authors should perform EM of E3.5-6.5 to demonstrate the appearance of these mother centriolar structures.

Minor concerns:
1. The author should clarify how they define n =, was the fluorescence intensity quantified in 4 embryos or 4 cells (ex. Fig 2C, 3C, etc)?
2. Figure 4A, previous published data should be removed or put in supplemental.
3. Fig EV2C should be moved into the main figure.
4. Figure 5 is missing.
5. Quantifications should be provided for Fig EV3.

Referee #2:

This study by Xiao and colleagues investigate the timing of activation of the mitotic surveillance pathway upon centriole loss during mouse development. The authors make use of a centriole-loss conditional model (Sas-4−/−), deletion of which causes centrosome loss, prolonged mitosis and p53-dependent cell death during early embryogenesis. By crossing null alleles of the p53 regulators 53BP1 and USP28 into this mutant strain, they confirm that the mitotic surveillance pathway is conserved in mice in vivo, and that 53BP1 and USP28 are essential for its conduction upstream of p53. They also show that the mitotic surveillance pathway is activated around E7 of embryonic development. Finally, they discover that centriole maturation correlates temporally with the establishment of the mitotic surveillance pathway in vivo, and suggest that only when the embryonic cells start to depend on centrosomes as MTOCs in mitosis and ciliogenesis, that the loss of centrioles and activation of the p53-dependent mitotic surveillance pathway occurs.
Overall, the work is well performed, the data are clear and strong, and the results are fairly convincing. The inclusion of a cilia mutant (IFT88) was a particularly nice approach, to compare and contrast between cilia loss and centrosome + cilia loss. However, certain parts of the story are too preliminary/under-developed, and further experiments are needed to strengthen/support the conclusions and make it suitable for publication in EMBO Reports. Specifically, the final section regarding centrosome maturation needs further exploration.

We thank the reviewer for positively evaluating our work and we have further analyzed the centriole maturation phenotype as described below

Comments and concerns:
- Fig 1A - The authors show that deletion of 53bp1 and Usp28 in the Sas4-null rescues the embryonic developmental defect at E9.5. However, no quantitation of this is shown: how many embryos showed a rescue, versus not? What was deemed as a "rescue"? Similarly, loss of p53 in these embryos (Fig1B) was not quantified. How prevalent/penetrant was this phenotype? This also applies to CASP3 analysis in Fig1C. Some quantitative measure of these observations should be included.

The phenotypic rescue criteria are defined in the text, for example, embryo size and morphology, turning, the presence of somites. We provided quantitative data: at least five embryos at E9.5 for each double mutant with all of the phenotypic criteria met in every embryo. We also added quantitative measures for p53 and Cl-CASP3 (Fig. 1 D and E), which were blunted in the double mutants compared to *Sas-4*$^{-/-}$ mutants.

- Again, quantification for the phenotypes shown in Fig 2C and D are lacking, and should be added.

The quantification of 53BP1 and USP28 at E6.5 and E5.5 were provided (Fig. 2 C and D) to highlight the difference in expression between the epiblast and visceral endoderm.

- Importantly, the final section regarding centriole maturation as a trigger for allowing cells to sense loss of centrosomes, and temporal activation of the surveillance mechanism (Fig4C and D) needs to be further tested. First, centriole maturation can be defined in many ways including: recruitment of distal and subdistal appendages, accumulation of PCM on the immature centrioles, and the ability to assemble a functional cilium. The authors only looked at recruitment of one distal appendage marker as a surrogate for centriole maturation, and the data presented is merely a correlation between the timing of Cep164 recruitment and when the mitotic surveillance pathway is active. To this reviewer, this is the most interesting part of this manuscript - since the rest of it mostly confirms the observations regarding the role of p53, 53bp1 and Usp28 that were previously defined in vitro. Therefore, I believe further dissection of "centriole maturation" is needed to support the conclusions in the study. One way to do this is to utilize the in vitro mESC isolated from these mutant mice (as in Fig 3A-D). The authors can systematically manipulate genes needed for distal appendage assembly, subdistal appendage assembly, PCM recruitment, and ciliogenesis - then test the consequences with regards to activation of the surveillance pathway. This will help to narrow down which aspects of "centriole maturation" are the key triggers for this activation.

We agree with the reviewer's assessment and have added two more data sets (Fig. 4C and D) to support our hypothesis on the correlation between centriole maturation, centrosome functions and the establishment of the mitotic surveillance pathway. In particular, we tested several antibodies against distal or subdistal appendages of centrioles, and found that the anti-ODF2 subdistal appendage showed specific staining in mouse embryos. We quantified the percentage of centrosomes containing ODF2 and the data showed a similar correlation to CEP164, where more centrosomes contained ODF2 as the embryos progressed in development from E3.5 to E6.5. Moreover, we also quantified the ability of centrioles to recruit the PCM proteins TUBG and PCNT as another measure of centriole maturation, and indeed centrioles in the epiblast cells of more developed embryos at E6.5 had more of these PCM proteins on their centrosomes compared to those at E5.5 embryos. We also attempted to quantify MTOC activity of centrosomes *in vivo* using the Nocodazole and washout method, but the detection of the asters was not technically feasible in 3D embryos at E5.5 and E6.5.

In addition, our work in mESCs has revealed that this *in vitro* system does not fully recapitulate centrosome biology during mouse embryonic development *in vivo*. First, mESCs already have centrosomes but, in our hands, only 3% of the cells are ciliated, a percentage that increases to a mere 6% upon partial differentiation. In our opinion, a full characterization of centriolar appendages in mESCs during pluripotency and differentiation requires an extensive set of biochemical experiments that are beyond the scope of our current manuscript.

Combined with the correlation with the functional readouts of mature centrioles, namely as MTOCs in mitosis and as basal bodies in cilia formation, our new data *in vivo* support our conclusion that centrioles mature during mouse development (E3.5-E6.5) and this is associated with their capacity to participate in mitosis and ciliogenesis.

- Fig 5 is mentioned in the text but is missing from the Figure panels.

We apologize for this mistake and Fig. 5 depicting our model is now included.

Referee #3:

The authors report that the mitotic surveillance pathway is activated upon loss of Sas4 in vivo and in derived mouse embryonic stem cells. The findings are supportive evidence of previously published data (Lambrus et al., 2016, Fong et al., 2016, and Meitinger et al. 2016) done in cell culture. They provide evidence that genetic ablation of p53 or its upstream regulators, 53bp1 and Usp28 can resolve p53-dependent growth retardation and cell death in Sas4 mutant embryos. They further present immunohistochemical data for the appearance of matured centrioles after embryonic day 6.5 at which time the mitotic surveillance system becomes active. They conclude that the cell death in embryos lacking centrioles is due to the activation of the mitotic surveillance pathway leading to a p53-dependent cell cycle arrest. Overall this is a straightforward study which recapitulates a pathway previously demonstrated in cells. The strength of the paper is the development of double mutants that rescue the development of embryos lacking centrioles. The major weakness of this paper concerns the lack of mechanistic insights that would answer why 53bp1 and Usp28 regulate p53 stabilization upon loss of centrioles. Below are points that the authors should consider:

We thank the reviewer for evaluating our work and agree that the major open question is how 53BP1 and USP28 regulate p53 upon centriole loss. In our humble opinion, cellular mechanistic studies are much harder to elucidate in an *in vivo* setting like E3.5-E6.5 mouse embryos.

Major concerns:

1. The authors demonstrated that p53 is stabilized in Sas4 mESC (Figure EV3), the authors should investigate the levels of p53 in WT, Sas4 -/-, Sas4 -/- 53bp1 -/- and Sas4 -/- Usp28 -/- embryos to confirm that p53 levels are restored.

The quantification data are now added (Fig. 1D and E) and they showed that the phenotypic rescue correlates with p53 downregulation in the double mutant embryos compared to centrosome mutants, similar to what has been shown *in vitro*.

2. In mESC, the authors demonstrate that upon Sas4 loss there is an increase in nuclear p53 and mitotic index; the authors should attempt to rescue the phenotype by co-depletion of Sas4 and 53bp1 or Usp28. An additional control of co-depletion of p21 and Sas4 would further solidify the claim that the mitotic surveillance pathway is activated in these mutants similar to the loss of PLK4.

We have provided the rescues *in vivo* and have indeed conducted these rescue experiments in mESCs. However, mESCs that are mutant for *53bp1* or *Usp28* show growth defects independent of centriole loss. We currently do not have an explanation for some of these observations and have opted instead to share our *in vivo* work because *in vitro* experiments have been performed in other mammalian cell lines. The mESC system is not fully characterized yet to assess whether the growth defect is due to the cell cycle (p21) or cell death.

3. As TRIM37 depletion suppresses mitotic defects in PLK4-inhibited cells, the authors should generate a Sas4 -/- TRIM37 -/-, double knockout to determine whether p53 suppression independent of a prolonged mitotic duration can rescue the developmental phenotypes found in Sas4 mutants.

As the reviewer correctly pointed out, *Trim37* mutations accelerate mitosis in acentriolar cells and *bypass* the activation of the mitotic surveillance pathway. However, *Trim37* mutations do *not* rescue cells that were artificially arrested in mitosis and may not act *within* the mitotic surveillance pathway. Because our study is focused on the mitotic surveillance pathway, we did not generate the double mutants with *Trim37*; however, we do agree that it would be interesting to perform the experiment *in vivo* in future studies.

4. In Figure 4C, the authors states centrioles begin to generate distal appendages (CEP164), given the significance of this finding, the authors should perform EM of E3.5-6.5 to demonstrate the appearance of these mother centriolar structures.

Given the importance of this finding to support our conclusions of centriole maturation, we have now added another marker of subdistal appendages ODF2 that showed similar kinetics (Fig. 4C). Because super-resolution microscopy provides further details about centriolar appendages, we provide high resolution images of the distal (CEP164) and subdistal appendages (ODF2) using this technique (Fig. 4B and C). In addition, our new data showed that the PCM proteins TUBG and PCNT increase in the area around the centrioles from E5.5 to E6.5 (Fig. 4D), where the recruitment of PCM proteins is another measure of centriole maturation.

Minor concerns:

1. The author should clarify how they define n =, was the fluorescence intensity quantified in 4 embryos or 4 cells (ex. Fig 2C, 3C, etc)?

The data points refer to either embryos (n) or experiments (N, in the case of mESCs). This is now clarified in the figure legends and all the numbers are included.

2. Figure 4A, previous published data should be removed or put in supplemental.

This panel is now part of supplemental Fig. EV3 along with the other panels of the mitotic index quantifications.

3. Fig EV2C should be moved into the main figure.

The mitotic surveillance pathway activation has not been shown to regulate the levels of 53BP1 or USP28. Our aim was to show that these proteins are present in WT embryos even before the activation of the pathway in *Sas-4* mutants. This supplemental figure was removed and an additional quantification of the proteins in the two germ layers that are differentially affected by pathway activation is now provided (Fig. 2C and D).

4. Figure 5 is missing.

We apologize for this mistake and the missing figure is now provided in the updated version.

5. Quantifications should be provided for Fig EV3.

Fig. EV3B showed that the p53 mutant mESCs had no detectable p53. Instead, the quantification of nuclear p53 by IF is provided in (Fig. 3B) because the p53 antibody is more sensitive using IF.

1st Revision - Editorial Decision 12th Nov 2020

Dear Hisham,

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

As you can see, the referees find that the study is significantly improved during revision and recommend publication. However, they have some remaining concerns that need to be addressed before publication.

- Please acknowledge in the Results & Discussions section that as it stands a causality between centriole maturation and activation of mitotic surveillance pathway is elusive, as noted by ref #2 as well.

- Regarding point 1 of referee #3, since you provided quantifications on p53 stainings of Ctrl, Sas-4-/-, Sas-4-/- 53bp1-/- and Sas-4-/- Usp28-/- embryos (Fig 1D), showing it with western blotting as well is not required for publication here.

- Regarding points 2 and 3 of referee #3, please discuss these points in the Results & Discussion section but reducing the significance of mESC Sas4 data is not required.

- Please include the data showing percentage of ciliated cells in non-differentiated and differentiated cells that you refer to in the point-by-point response, as suggested by ref #3 in point 4.

Moreover, I need you to address some editorial points below:

• We notice that Figs EV1+2 panels are not called out in the text.
• 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 #2:

The authors have addressed the majority of my concerns, and have added additional data to help solidify their conclusions. These include quantitative measures of p53, CL-CASP3, 53BP1 and

USP28 in the mutant embryos (which are represented by new graphs). Moreover, they performed further immunofluorescence-based analysis of centriole and centrosome maturation, which again supports they theory that centrosome maturation correlates with the timing of the mitotic surveillance mechanism. I was disappointed to see that they were unable to directly test the role of centriole/centrosome maturation in their mESC system, which would have provided direct proof of this requirement. This leaves open the question of causality, but this can be tested in the future using an amenable cell culture system.

Overall, I believe the revisions have addressed the weaknesses I identified and the manuscript is now suitable for publication.

Referee #3:

Xiao and colleagues have revised their manuscript "Gradual centriole maturation associates with the mitotic surveillance pathway in mouse development". The in vivo data supports the previous cell culture works by the Oegama, Tsou and Holland labs; confirming that the mitotic surveillance pathway exists in an animal model. In the revision, the authors have added quantitation for Figures 1 and 4 and added IF of the subdistal/distal protein ODF2 and PCM components Pericentrin and gamma-tubulin. While this data provides evidence of their claim that centriole maturation coincides with the emergence of the surveillance pathway components, the evidence is not concrete. The remaining concerns are below:

Remaining concerns:
1. The request of this reviewer was to have the authors measure the levels of P53 by western blot in the WT, Sas4 -/-, Sas4 -/- 53bp1 -/- and Sas4 -/- Usp28 -/- embryos. If a revision were to be considered, this would be a necessary experiment.
2. In response to: "The mESC system is not fully characterized yet to assess whether the growth defect is due to the cell cycle (p21) or cell death." As this study is largely the recapitulation of findings in cell culture, this study requires innovation in the form of expanding our understanding of the surveillance pathway. The current working hypothesis is that P21 acts downstream of P53 in the mitotic surveillance pathway. The major advance to these studies being done in vivo would be to demonstrate that P21 ablation can overcome the P53 upregulation in response to centriole loss. Without this piece of data the study is largely demonstrative that the surveillance pathway exists in mice. This is not to say the paper needs to include this data for consideration for publication.
3. The statement "mESCs that are mutant for 53bp1 or Usp28 show growth defects independent of centriole loss" raises some concern due to the assertion in Figure 3 that loss of Sas4 in mESC is sufficient to evoke the mitotic surveillance pathway. The reasons behind the growth defects in 53bp1 and Usp28 should be explained as this doesn't occur in the knockout mice and the authors should consider reducing the significance of the Sas4 data in mESC as the model may not be representative of what occurs in vivo.
4. Reviewer 2 and myself asked the authors to demonstrate that the centrioles that mature over development are indeed mother centrioles, we asked whether centrioles can ciliate and appendage structures be imaged by EM. The use of antibodies to ODF2 and PCM markers was not sufficient to demonstrate the presence of mature centrioles during development. If a revision is considered showing the 3% and 6% of ciliated cells in non-differentiated and differentiated cells, respectively, should be quantitated and included.

Referee #2:

The authors have addressed the majority of my concerns, and have added additional data to help solidify their conclusions. These include quantitative measures of p53, CL-CASP3, 53BP1 and USP28 in the mutant embryos (which are represented by new graphs). Moreover, they performed further immunofluorescence-based analysis of centriole and centrosome maturation, which again supports they theory that centrosome maturation correlates with the timing of the mitotic surveillance mechanism. I was disappointed to see that they were unable to directly test the role of centriole/centrosome maturation in their mESC system, which would have provided direct proof of this requirement. This leaves open the question of causality, but this can be tested in the future using an amenable cell culture system.

Overall, I believe the revisions have addressed the weaknesses I identified and the manuscript is now suitable for publication.

We thank the reviewer for positively evaluating our work and recommending it for publication.

Referee #3:

Xiao and colleagues have revised their manuscript "Gradual centriole maturation associates with the mitotic surveillance pathway in mouse development". The in vivo data supports the previous cell culture works by the Oegama, Tsou and Holland labs; confirming that the mitotic surveillance pathway exists in an animal model. In the revision, the authors have added quantitation for Figures 1 and 4 and added IF of the subdistal/distal protein ODF2 and PCM components Pericentrin and gamma-tubulin. While this data provides evidence of their claim that centriole maturation coincides with the emergence of the surveillance pathway components, the evidence is not concrete. The remaining concerns are below:
Remaining concerns:
1. The request of this reviewer was to have the authors measure the levels of P53 by western blot in the WT, Sas4 -/-, Sas4 -/- 53bp1 -/- and Sas4 -/- Usp28 -/- embryos. If a revision were to be considered, this would be a necessary experiment.
2. In response to: "The mESC system is not fully characterized yet to assess whether the growth defect is due to the cell cycle (p21) or cell death." As this study is largely the recapitulation of findings in cell culture, this study requires innovation in the form of expanding our understanding of the surveillance pathway. The current working hypothesis is that P21 acts downstream of P53 in the mitotic surveillance pathway. The major advance to these studies being done in vivo would be to demonstrate that P21 ablation can overcome the P53 upregulation in response to centriole loss. Without this piece of data the study is largely demonstrative that the surveillance pathway exists in mice. This is not to say the paper needs to include this data for consideration for publication.
3. The statement "mESCs that are mutant for 53bp1 or Usp28 show growth defects independent of centriole loss" raises some concern due to the assertion in Figure 3 that loss of Sas4 in mESC is sufficient to evoke the mitotic surveillance pathway. The reasons behind the growth defects in 53bp1 and Usp28 should be explained as this doesn't occur in the knockout mice and the authors should consider reducing the significance of the Sas4 data in mESC as the model may not be representative of what occurs in vivo.

We have acknowledged in the manuscript that we currently do not know the pathways operating upstream or downstream of p53 in *Sas-4* mutant mESCs.

4. Reviewer 2 and myself asked the authors to demonstrate that the centrioles that mature over development are indeed mother centrioles, we asked whether centrioles can ciliate and appendage structures be imaged by EM. The use of antibodies to ODF2 and PCM markers was not sufficient to demonstrate the presence of mature centrioles during development. If a revision is considered showing the 3% and 6% of ciliated cells in non-differentiated and differentiated cells, respectively, should be quantitated and included.

The data on cilia was included and briefly discussed in the manuscript. More directed lineage differentiation methods, and proper controls including p53 and cilia mutants, are required to follow up on our findings in this study.

2nd Revision - Editorial Decision 20th Nov 2020

Dear Hisham,

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 study!

Kind regards,

Deniz
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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 iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

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

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample size was determiend as three or more embryos per genotype as is the standard in the field. No statistical method was employed to select sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The R's of Replacement, Reduction and Refinement were followed to minimize the use of animals. The mimimum number of animals to give a statistically reproducible outcome was chosen. Three or more embryos per genotype was chosen as an acceptable standard.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All technically successful experiments were used in the analyses and none were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No randomization was performed and the samples were grouped by genotype.
For animal studies, include a statement about randomization even if no randomization was used.	The phenotype of the embryo refelected its genotype and no randomization was used.
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.	Blinding was not performed durng analyses.
4.b. For animal studies, include a statement about blinding even if no blinding was done	The phenotype of the embryos revealed their genotype and blinding was not performed.
5. For every figure, are statistical tests justified as appropriate?	Yes, the appropriate statistical tests were employed.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The data were assumed to fit a normal distribution but no methods were used to assess this assumption.
Is there an estimate of variation within each group of data?	The standard deviation was used as an estimate of variation.

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

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Is the variance similar between the groups that are being statistically compared?	

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).	The antibodies used in this study were all veriefied for use in the intended application, and they were specified in a table in 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.	No established cell lines were used in this study, but the primary mESCs used were tested for mycoplasma contamination and gave a negative result.

* 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.	We used early mouse embryos, with no sex specified, from FVB/N background. The mice were housed in IVC cages in the CECAD animal facility under standard conditions and in accordance with animal welfare approvals. The genetic modifications were described in this or previous published studies.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The breeding experiments described were approved by the LANUV authority in NRW, Germany.
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.	Our work complies with the ARRIVE guidelines on animal reporting.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable
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.	Not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable
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.	Not applicable

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	A statement was added that data Availability is not applicable for this study
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).	Not applicable
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Not applicable