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Centrosome defects cause microcephaly by activating the 53BP1-USP28-TP53 mitotic surveillance pathway

Thao Phan, Aubrey Maryniak, Christina Boatwright, Junsu Lee, Alisa Atkins, Andrea Tijhuis, Diana Spierings, Hisham Bazzi, Floris Foijer, Philip Jordan, Travis Stracker, and Andrew Holland
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Corresponding author(s): Andrew Holland (aholland@jhmi.edu)

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1st Editorial Decision 30th Jul 2020

Thank you again for submitting your manuscript on the roles of the mitotic surveillance pathway in centrosome defect-associated microcephaly for our consideration. I have now heard back from three expert referees, whose comments are copied below. As you will see, the reviewers all appreciate your characterization of the involved molecular players in relevant model systems, but remain somewhat divided regarding the overall significance and conceptual advance conveyed by the derived conclusions. Furthermore, they also raise a number of major and minor experimental concerns that would require satisfactory addressing.

Following further discussions with the referees and within our editorial team, we concluded that we would be interested in considering a revised manuscript further despite referee 2's conceptual concerns, as long as you should be able to decisively address all the more specific issues raised in the reports. Regarding the further-reaching question by referee 2 whether USP28/53BP1 directly act in response to increased duration of mitosis or some other aspect of cell division perturbation, I realize that definitively working out such mechanisms may exceed the scope of the present revision; nevertheless, any data you may be able to add to back up the presented model or to make some of the posed alternative explanations less likely, should clearly strengthen the impact of this study.

As indicated on previous occasions, we would in light of the unpredictable situation around the COVID-19 pandemic be happy to discuss extensions of the revision period, as well as any particular questions/comments you may have related to the reviewers' reports or your revision plans - so please do not hesitate to contact me at any time in such case. Please also remember that it is our policy to allow only a single round of major revision, making it important to carefully answer to all referee points during this revision.

Referee #1:

Prior work has shown that normal cells measure the amount of time that they spend in mitosis. with

cells that spend longer than a threshold time activating a mitotic surveillance pathway. The mitotic surveillance pathway consists of the ubiquitin protease USP28 and the p53-binding protein 53BP1, and its activation leads to stabilization of the transcriptional activator p53, resulting in cell senescence or death. One way that the mitotic duration sensor can be activated is via centrosome loss, which prolongs mitosis. Centrosomes catalyze microtubule assembly for spindle formation and in their absence spindle assembly takes significantly longer.

Mutations in different centrosome proteins have been shown to cause loss of neural progenitor cells (NPCs) during brain development leading to microcephaly. Yet how this occurs, and whether loss of NPCs is due to activation of the mitotic surveillance pathway downstream of prolonged mitosis, is not known. In this manuscript, the authors examine the role of the mitotic surveillance pathway in two previously described mouse models of centrosome-related microcephaly: (1) mice homozygous for a gene-trap insertion in the Cep63 gene (Cep63T/T) and (2) mice in which the gene encoding Sas4/CPAP is conditionally deleted using Nestin-Cre (Sas4cKO). The authors show that neural progenitor cells from mice with both centrosomal perturbations exhibit increased mitotic duration that leads to p53 activation and cell death in an USP28 & 53BP1 dependent manner. They also show that microcephaly induced by either of these centrosome-based perturbations, but not DNA damage induced microcephaly caused by inactivation of SMC5, can be rescued by inactivation of the mitotic surveillance pathway.
The presented work is very well executed and clearly explained. The authors convincingly translate and confirm the previous findings in RPE1 cells to mouse models and show that centrosome-associated microcephaly results from activation of the mitotic surveillance pathway. I recommend publication of this work in EMBO. I have only a few minor comments that the authors may wish to consider prior to publication.

Minor Comments:
1. For the in vitro analysis of dissociated cells, how is arrest defined? Based on the text it seems to be failure to re-enter mitosis within the "time frame of our movies". What is the time frame of the movies? Shouldn't defining a cell as arrested mean that it has failed to enter mitosis even though it has been filmed following mitosis for at least a set number of hours? What if a cell enters its first mitosis relatively late in the filming session and then doesn't have time to enter again before the end of the filming session-in this case, the cell should not be considered to have arrested. Please clarify how this is being analyzed.
2. The mitotic index of Smc5cKO looks different to control in Figure 6E. Even though the difference is not significant, the value of the t-test should be shown rather than "N.S" to allow the reader to compare the difference to other experiments.
3. What causes NPC delamination? It seems like the function of mitotic surveillance pathway is to prevent disorganization of the brain by cells that have experienced cell division defects. Can the authors comment on this?

Referee #2:

The manuscript by Phan et al. builds on previous in vitro data reporting a p53-dependent pathway, referred to as the mitotic surveillance pathway, which monitors duration of mitosis preventing division of unfit cells. This pathway was previously reported to monitor duration of mitosis preventing the proliferation of cells in which mitosis lasted longer in the previous cell division. By using two models of primary recessive microcephaly (MCPH), the authors show that neural progenitors with defects in Cep63 and Sas4 display increased duration of mitosis which correlates

with increased cell death. In vivo, ablation of two MSP components, Usp28 and 53BP1, rescues microcephaly similar to that reported previously with p53. None of these components rescue microcephaly resulting from mutations in the unrelated model caused by SMC5 mutation.

Overall, the manuscript contains a very rich characterization of these models and neural progenitors generated from the developing brain. However, the enthusiasm is mostly limited by two factors.

First, The individual models and the crosses with p53 were already described in previous publications. Prolonged mitosis and the fact that prolonged mitosis triggers a p53 response was also shown in these publications (e.g. Bazzi PNAS 2014). Prolonged mitosis induced by nocodazole was also used in the PNAS 2014 publication (Fig. 7) to propose a prolonged mitosis-p53-cell death model. So the novelty results from the crosses with Usp28 and 53BP1 as well as SMC5. I would not consider SMC5 as a relevant model here as it is not related to MCPH but to microcephaly accompanying primordial dwarfism.

Second, whereas the data generated in Usp28 and 53BP1 double knockouts are interesting, the question is to what extent the fact that other p53-pathway components mimic the effect of p53 is novel or unexpected. The authors claim that the novelty arises from the fact that this is a specific pathway that monitors the duration of mitosis (as they reported previously using cell lines in vitro). So the relevant question at the end is: is the effect of Usp28/53BP1 something unique or specific of the duration of mitosis (what authors repeatedly call as mitotic surveillance pathway? I am not convinced that is the clearly shown in the paper. The authors provide evidence of increased pH3/Ki67 ratio in vivo and increased DOM in vitro (as previously shown in the PNAS 2014 paper). These observations correlate with p53 response and death but it is very possible that aberrant centrosome dynamics, aberrant spindle dynamics, subsequent changes in mRNA/protein levels etc. (and not only DOM) may trigger the p53 response. At this moment, this is simply a correlation and no real experiment in the article demonstrate that DOM is the critical event triggering the Usp28/53BP1/p53 pathway in MCPH. I do agree that nocodazole induces the same effect (as shown in the PNAS 2014 paper) but this does not demonstrate that DOM (or the mitotic surveillance pathway) is the trigger for these effects in primary microcephaly. For instance, Usp28 KO also reduced phH2AX, suggesting effects of the Usp28-p53 pathway in additional aberrations (independent from DOM) that may contribute to the observed effect.

For instance, in Fig. 2D-E: WT cells display a significant reduced death compared to microcephaly models even when considering the same duration of mitosis: compare for instance 2nd column (35-60 min) in Fig. 2D versus 2E. Overall, it is very likely that the centrosomal defects caused by Cep63 and Sas4 mutations induce several aberrations in centrosome dynamics, spindle dynamics and downstream processes that cannot be simply explained by the duration of mitosis. No problem about the Usp28-53P1-p53 pathway (which is clearly validated using the genetic models in this manuscript and previous publications with p53), but data are not convincing enough to claim that is the increased DOM (used to define the mitotic surveillance pathway) what causes primary microcephaly.

Referee #3:

This study by Holland and colleagues investigates the role of 53BP1/USP28/TP53 surveillance pathway in activating p53 signaling to induce microcephaly. This pathway was previously shown by

the authors to be activated following delayed mitosis of immortalized cells. The authors study two previously characterized mouse mutants in the centrosomal proteins, Cep63 and Sas4, known to cause microcephaly and affect progenitor mitosis. The authors first show in their own hands that both mutants exhibit microcephaly. Further they use live imaging of neural progenitor cells (NPCs) to demonstrate an average longer mitotic duration, which is an outcome previously linked to microcephaly. Longer mitosis correlates with increased cell death of the daughter cell in both mutants. They then ask if these phenotypes are rescued by eliminating 53BP1 or Usp28; This shows rescue of cortical size, density of laminar markers, Pax6+ NPC number, and as expected cell death. Interestingly, live imaging shows that loss of the surveillance pathway does not rescue mitotic duration but does appear to rescue apoptosis linked to mitosis. Finally, the authors contrast this with another non-centrosomal mutant which is not rescued in the same fashion. From this the authors infer that mutations specifically in centrosomal proteins delay mitosis resulting in activation of this pathway and microcephaly.

Many microcephaly mutants show increased p53 signaling and many of these phenotypes have been shown to rescued in the absence of p53. The findings in this paper addresses a gap in the field: what activates p53 following prolonged mitosis and how is this linked to microcephaly? Overall the findings are interesting and the use of several genetic models comprehensive. The findings will be of interest to researchers investigating mechanisms of mitosis and brain development. However, I have several concerns with some of the experiments which need to be addressed.

1. The quality of live imaging panel images in Figure 1J (and to some extent Figure 5E) is poor and should be improved. The cells appear to be very dim and possibly out of focus. Thus, it is almost impossible to appreciate any delay in mitosis, and raises questions about how the authors quantify mitosis. The methods don't make clear how mitosis is measured (presumably from morphology of histone signal?).

2. There are significant issues with statistics in the paper. First, several figures lack statistics to support the conclusions. Figure 1F, 5F: Are there significant differences in mitotic categories across phenotypes? Figures 2D-F; Figure 5G,I: The authors conclude that the probability of producing non-viable progeny increases proportionally with mitotic duration but this correlation would be better supported with statistics (such as Chi square). Statistics would also help to evaluate differences in arrest and growth across different durations (by applying post-hoc analyses).

Second, when comparing several genotypes, including rescue, the authors only used unpaired t tests. However, a test such as Anova is more appropriate to assess first if there are significant differences across all genotypes, and second applying post-hoc comparison between two genotypes (eg. Cep63;53bp versus WT and Cep63 alone, etc). This applies to many figures but is especially relevant for interpreting rescue experiments in Figure 3, 4, 5. In this regard, it may be best to not put both centrosome mutants in the same graph as this increases the number of statistical tests needed.

3. The rescue experiments suggest differences in rates of cell death across mitotic durations (Figure 5G, I). But it would be valuable to see a direct comparison between rates of cell death in double knockout versus single knockout. All that is shown is distributions for each. This would better support the notion on p.17 that USP28 is required to initiate cell death in progeny of cells that delay in mitosis. The nocodazole experiment helps but is indirect and again here no comparisons are made between genotypes.

4. The authors claim on p. 12 that ablation of the mitotic surveillance pathway restores brain size

and production of the correct number of neurons. However, the latter is not substantiated by data (simply showing more NPCs is insufficient). To support this, they should verify that the rescue actually results in production of more newborn neurons (fir this they could quantify cell cycle exit). It may be that the laminar neuron number rescue is simply a rescue of apoptosis. Both mechanisms could also contribute.

5. Further clarification of the cell death mechanisms would also be valuable. On p. 14 the authors specify that cell death happens in NPCs but they don't show that. In fact, based upon the patterns in Figure 4D it seems it is not simply limited to NPCs. This mechanism should be clarified.

6. Primary NPC cultures were quantified for Pax6 but a substantial number of intermediate progenitors are also present at E14.5, and this should be quantified. The mitotic defects could be occurring in these cells as well, which the authors should discuss.

Minor:

1. The introduction implies MCPH is only caused by depletion of NPCs, but it may also be caused by excessive apoptosis of neurons.

2. For figures comparing nestin, please specify what the wild-type control is. NestinCre has known background issues so it is important to compare to Cre alone.

3. P 9 the authors should not state that cell death partially explains microcephaly since these are in vitro experiments. Suggest to soften wording in this location.

4. The model in Figure 6J should be adjusted to reflect that radial glia mitosis occurs adjacent to the ventricle, at the apical surface, as opposed to more basally.

5. Figure 1G: The analysis of aneuploidy examines so few cells so it is not clear if this difference in moderate aneuploidy in Figure 1G is meaningful and belongs in the main figure.

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Dissociated NPC were filmed for 48 hours and cells that enter mitosis within the first 24 hour period were selected for fate tracing. This timeframe was chosen as < 7% of cells we analyzed had a > 24 hour cell cycle (Figure EV1M). Therefore, it is highly likely that cells that fail to divide again within the remaining time of the movie have undergone a

cell cycle arrest. In the revised manuscript we have updated the materials and methods section to clarify this point.

2. The mitotic index of Smc5cKO looks different to control in Figure 6E. Even though the difference is not significant, the value of the t-test should be shown rather than "N.S" to allow the reader to compare the difference to other experiments.

To address this point, we have also analyzed two additional *Smc5^{cKO}* animals and added these 2 data points to our graph (Figure 6E). This new data confirms that the mitotic index of the *Smc5^{cKO}* animals are similar to that of wildtype animals. In addition, we have replaced the N.S annotations with a numerical P value for this figure and all of the other figures throughout the manuscript wherever there is space to do so. We have also provided an excel file with all the data plotted in the paper and a separate file reporting the statistical analysis that was performed (Table EV1 and EV2).

3. What causes NPC delamination? It seems like the function of mitotic surveillance pathway is to prevent disorganization of the brain by cells that have experienced cell division defects. Can the authors comment on this?

We don't think that the mitotic surveillance pathway is functioning to prevent neural progenitor delamination. First, we observed delamination of NPCs in *Cep63$^{T/T}$* and *Sas4^{cKO}* cortices whether or not the mitotic surveillance pathway is functional (Figure 4A). In the case of *Cep63$^{T/T}$; Usp28$^{-/-}$* or *Sas4^{cKO}; Usp28^{cKO}* animals, we observed more mislocalized NPCs compared to animals with intact USP28 function. However, this is likely a result of delaminated NPCs continuing to proliferate rather than an increased frequency of delamination *per se*. Second, in the absence of centrosome defects, disabling the mitotic surveillance pathway does not result in NPC delamination. Based on what has been observed in other models of microcephaly, it is likely that centrosome loss leads to delamination because centrosomal microtubules help stabilize adherens junctions that are required for anchoring NPCs at the ventricular surface.

Referee #2:

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progenitors generated from the developing brain. However, the enthusiasm is mostly limited by two factors.

First, The individual models and the crosses with p53 were already described in previous publications. Prolonged mitosis and the fact that prolonged mitosis triggers a p53 response was also shown in these publications (e.g. Bazzi PNAS 2014). Prolonged mitosis induced by nocodazole was also used in the PNAS 2014 publication (Fig. 7) to propose a prolonged mitosis-p53-cell death model. So the novelty results from the crosses with Usp28 and 53BP1 as well as SMC5. I would not consider SMC5 as a relevant model here as it is not related to MCPH but to microcephaly accompanying primordial dwarfism.

Prior work from Bazzi and Anderson (Bazzi et al., PNAS 2014) examined the impact of centrosome loss in *Sas4*$^{-/-}$ mouse embryos. *Sas4*$^{-/-}$ embryos were shown to survive until mid-gestation before undergoing widespread, P53-dependent apoptosis. This study linked the cell death that occurred in the *Sas4*$^{-/-}$ embryos to an increased mitotic duration. However, it remained unclear what activates P53 in response to an increased mitotic duration. In our paper, we built on this work by showing that P53 activation in the brain of microcephaly models with centrosome defects occurs as a result of activation of the mitotic surveillance pathway. Moreover, disabling the mitotic surveillance pathway, through knockout of the deubiquitinase *Usp28*, suppresses P53 activation and restores the expansion of the NPC pool and brain size in two microcephaly mouse models with centrosome defects. Our study provides the first evidence that the mitotic surveillance pathway has physiological relevance *in vivo* and that pathological activation of this pathway could be linked to human disease.

The reviewer correctly points out that hypomorphic mutations in SMC5 complex components are linked to microcephalic primordial dwarfism. Importantly, primary microcephaly and microcephalic primordial dwarfism can be caused by different mutations in the same gene, suggesting these diseases represent a phenotypic spectrum with overlap in the underlying pathological mechanisms. Indeed, different mutations in SAS4 (as well as other centrosome proteins) have been linked to primary microcephaly and Seckel syndrome, a type of microcephalic primordial dwarfism (Bond et al., Nat Gen, 2005; Al-Dosari et al., J Med Genet, 2010). Moreover, mutations in *CEP63* cause Seckel syndrome, and consistently the *Cep63*$^{T/T}$ mouse models we examined exhibits microcephaly and a growth delay (Marjanovic et al., Nat Comms, 2015). Thus, the mouse models used in our study are relevant to understanding microcephaly and the related syndrome of microcephalic primordial dwarfism. In this regard, it is interesting to note that although inactivation of the mitotic surveillance pathway rescued brain size in *Cep63*$^{T/T}$ mice, it did not rescue growth retardation. This highlights tissue-specific differences in the sensitivity and/or function of the mitotic surveillance pathway and suggests that centrosome defects cause whole-body growth retardation and microcephaly via distinct mechanisms.

Second, whereas the data generated in Usp28 and 53BP1 double knockouts are interesting, the question is to what extent the fact that other p53-pathway components

mimic the effect of p53 is novel or unexpected. The authors claim that the novelty arises from the fact that this is a specific pathway that monitors the duration of mitosis (as they reported previously using cell lines in vitro). So the relevant question at the end is: is the effect of Usp28/53BP1 something unique or specific of the duration of mitosis (what authors repeatedly call as mitotic surveillance pathway? I am not convinced that is the clearly shown in the paper.

Although USP28 and 53BP1 act upstream to trigger P53 activation in response to a mitotic delay, these proteins are clearly not required for all P53-dependent responses *in vitro* or *in vivo*. Indeed, multiple lines of evidence from our lab and others have shown that the mitotic surveillance pathway is mechanistically distinct from the DNA damage response. In addition, aneuploidy, oxidative stress and centrosome amplification all activate P53 independent of USP28 and 53BP1. Thus, although we cannot claim that the mitotic surveillance pathway *exclusively* monitors the duration of mitosis, at present we lack evidence for alternative functions of this pathway.

Further to this, our manuscript provides genetic evidence to show that USP28 is only required to activate P53 in a limited set of circumstances. We show that loss of the non-centrosome protein SMC5 leads to P53-dependent microcephaly that is not rescued by the inactivation of the mitotic surveillance pathway but is suppressed following *Chk2* knockout (Atkins et al., eLife in revision, 2020). By contrast, the microcephaly phenotype in *Cep63*$^{T/T}$ mice is not rescued by knockout of *Chk2* or *Atm* (Marjanovic et al., Nat Comms, 2015), but is suppressed by inactivation of the mitotic surveillance pathway. Taken together, these data argue that the microcephaly phenotype in *Smc5* knockout mice arises from activation of the DNA damage response, while defects in centrosome proteins lead to mitotic delays that activate the mitotic surveillance pathway. Therefore, although the loss of SMC5 and centrosome proteins both activate P53, the upstream pathways are genetically distinct. Since most of the genes mutated in primary microcephaly encode spindle/centrosome proteins or DNA damage response factors, our work suggests that mutations in these two classes of genes lead to microcephaly by activating one of two different pathways.

The authors provide evidence of increased pH3/Ki67 ratio in vivo and increased DOM in vitro (as previously shown in the PNAS 2014 paper). These observations correlate with p53 response and death but it is very possible that aberrant centrosome dynamics, aberrant spindle dynamics, subsequent changes in mRNA/protein levels etc. (and not only DOM) may trigger the p53 response. At this moment, this is simply a correlation and no real experiment in the article demonstrate that DOM is the critical event triggering the Usp28/53BP1/p53 pathway in MCPH. I do agree that nocodazole induces the same effect (as shown in the PNAS 2014 paper) but this does not demonstrate that DOM (or the mitotic surveillance pathway) is the trigger for these effects in primary microcephaly.

Experiments in cultured cells have shown that the ability of daughter cells to proliferate or arrest is very tightly correlated with the time of mitosis in the mother cell (Uetake et al., Curr Bio, 2011; Lambrus et al., JCB, 2015). Since no other alteration was observed to track with daughter cell fate, a reasonable interpretation is that the duration of mitosis

in the mother cell is being actively surveilled. We acknowledge, however, that this remains to be definitively proven. Demonstrating that the duration of mitosis is the sole event that triggers the mitotic surveillance pathway is not trivial; at present we do not fully understand how the pathway functions at a molecular level (although we are making progress) and we know of no way to shorten mitotic duration without inducing unwanted defects on the fidelity of chromosome segregation.

In any cellular clock there must be a timekeeping element to serve as a physical manifestation of elapsed time. In principle, a timekeeper can be anything with the ability to predictably change over time. Ions, metabolites, miRNA, and promoter elements have all been proposed as timekeepers, but biology primarily assigns timekeeping duties to proteins. In the case of the mitotic surveillance pathway our current data suggest the timekeeper is a protein that is degraded during mitosis. Therefore, the reviewer is correct that an increased duration of mitosis is directly readout from a change in the abundance of another factor, in this case a protein. Manipulation of timekeeper abundance followed by careful tracking of cell fate would offer additional evidence that mitotic duration is being monitored. These are experiments that are ongoing in our laboratory, but it is important to note that they are performed in cultured cells and we have much to learn before we can translate the findings into mouse models.

Nocodazole is a microtubule poison that we and others have used to reversibly delay cells in mitosis and monitor daughter cell fate. We have obtained very similar results with the kinesin inhibitor dimethylenastron, which delays cells in mitosis without altering microtubule dynamics. Washout of nocodazole or dimethylenastron allows normal spindle assembly and continued mitotic progression. Nocodazole washout doesn't induce long-lasting effects, since cells that enter mitosis after the drug is removed complete mitosis with the normal timing and exhibit cell fates that are indistinguishable from untreated cells. By delaying cells in mitosis using pharmacological agents without impacting centrosome function, we provide causative evidence that the extended mitosis of mother cells and not centrosome defects *per se,* are responsible for eliciting *Usp28*-dependent death in daughter cells.

For instance, Usp28 KO also reduced phH2AX, suggesting effects of the Usp28-p53 pathway in additional aberrations (independent from DOM) that may contribute to the observed effect.

DNA damage occurs in dying cells as a result of caspase-activated DNase activity (Enari, Sakahira et al., 1998). In fact, the increase in γH2A.X+ cells observed in the cortices of *Sas4*^{cKO} and *Cep63*$^{T/T}$ animals nearly all occurs in apoptotic cells (Figure EV4E,F). Thus, the reduction in the number of γ-H2AX^{+} cells following *Usp28* knockout is an indirect result of suppressing cell death and does not argue for a distinct role of the mitotic surveillance pathway. We have now clarified this point in the revised version of our manuscript.

For instance, in Fig. 2D-E: WT cells display a significant reduced death compared to

microcephaly models even when considering the same duration of mitosis: compare for instance 2nd column (35-60 min) in Fig. 2D versus 2E. Overall, it is very likely that the centrosomal defects caused by Cep63 and Sas4 mutations induce several aberrations in centrosome dynamics, spindle dynamics and downstream processes that cannot be simply explained by the duration of mitosis. No problem about the Usp28-53P1-p53 pathway (which is clearly validated using the genetic models in this manuscript and previous publications with p53), but data are not convincing enough to claim that is the increased DOM (used to define the mitotic surveillance pathway) what causes primary microcephaly.

The reviewer points out that in the control condition 15.6% of the daughter cells that are generated by mothers that spend 31-60 mins in mitosis undergo cell death, compared with 33.4% in *Sas4*^{cKO} and 42.7% in *Cep63*$^{T/T}$ NPCs. This distribution of cell fates across the 3 different genotypes is not statistically significant (chi square test P=0.3124).

Indeed, it is difficult to draw any solid conclusion by comparing to the 31-60 mins category in wildtype cells, as the *n* for the group is low since only 7% (24/330 cells) of the control cells spend > 30 minutes in mitosis. To directly examine how an increased mitotic duration impacts the fate of wild type NPCs, we performed the nocodazole washout experiment that allows us to reversibly delay mitosis in wildtype NPCs without impacting centrosome function. In these experiments, 47% of the daughter cells that are generated by mothers that spend 31-60 mins in mitosis undergo cell death, a value that is comparable to that observed in *Cep63*$^{T/T}$ and *Sas4*^{cKO} NPCs. Overall, we feel our data is consistent with the proposal that an increased mitotic duration is the major determinant that defines the fate of *Cep63*$^{T/T}$ and *Sas4*^{cKO} NPCs.

Referee #3:

This study by Holland and colleagues investigates the role of 53BP1/USP28/TP53 surveillance pathway in activating p53 signaling to induce microcephaly. This pathway was previously shown by the authors to be activated following delayed mitosis of immortalized cells. The authors study two previously characterized mouse mutants in the centrosomal proteins, Cep63 and Sas4, known to cause microcephaly and affect progenitor mitosis. The authors first show in their own hands that both mutants exhibit microcephaly. Further they use live imaging of neural progenitor cells (NPCs) to demonstrate an average longer mitotic duration, which is an outcome previously linked to microcephaly. Longer mitosis correlates with increased cell death of the daughter cell in both mutants. They then ask if these phenotypes are rescued by eliminating 53BP1 or Usp28; This shows rescue of cortical size, density of laminar markers, Pax6+ NPC number, and as expected cell death. Interestingly, live imaging shows that loss of the surveillance pathway does not rescue mitotic duration but does appear to rescue apoptosis linked to mitosis. Finally, the authors contrast this with another non-centrosomal mutant which is not rescued in the same fashion. From this the authors infer that mutations specifically in centrosomal proteins delay mitosis resulting in activation of this pathway and microcephaly.

Many microcephaly mutants show increased p53 signaling and many of these phenotypes have been shown to rescued in the absence of p53. The findings in this paper addresses a gap in the field: what activates p53 following prolonged mitosis and how is this linked to microcephaly? Overall the findings are interesting and the use of several genetic models comprehensive. The findings will be of interest to researchers investigating mechanisms of mitosis and brain development. However, I have several concerns with some of the experiments which need to be addressed.

1. The quality of live imaging panel images in Figure 1J (and to some extent Figure 5E) is poor and should be improved. The cells appear to be very dim and possibly out of focus. Thus, it is almost impossible to appreciate any delay in mitosis, and raises questions about how the authors quantify mitosis. The methods don't make clear how mitosis is measured (presumably from morphology of histone signal?).

Our original intention was to use H2B-mCherry to track chromosomes in mitosis so that we could monitor mitotic duration and identify mitotic errors. However, after purchasing the H2B-mCherry mice from Jax and crossing them with our animals, we discovered that the tagged H2B-mCherry protein in these animals does not localize to the chromosomes! Rather, this fusion protein provides a nuclear signal that disperses at the beginning of mitosis and reappears in late anaphase when nuclear envelope reformation occurs. While we were disappointed that the H2B-mCherry protein did not track chromosomes, we were able to make use of this line to monitor mitotic duration by determining the time taken from NEBD to nuclear envelope reformation. To spare other investigators this same fate, we contacted Jax and asked them to modify the description to reflect the fact that this mouse line provides a nuclear and not chromosomal signal (https://www.jax.org/strain/023139).

We recognize that in our original version of the manuscript we failed to highlight this important distinction and we apologize for the confusion this may have caused. We have now changed our text and figure labels from “H2B-mCherry” to “Nuclear-mCherry” and added an explanation for this to our materials and methods. We have also replaced the original movie montage with higher quality images (Figure 1J) and added arrows to indicate the daughter cells that arise following cell division (Figure 1J and Figure 5D). Finally, we have also included two representitive movies (Movie EV1 and EV2) to highlight the events we were tracking.

2. There are significant issues with statistics in the paper. First, several figures lack statistics to support the conclusions. Figure 1F, 5F: Are there significant differences in mitotic categories across phenotypes? Figures 2D-F; Figure 5G,I: The authors conclude that the probability of producing non-viable progeny increases proportionally with mitotic duration but this correlation would be better supported with statistics (such as Chi square). Statistics would also help to evaluate differences in arrest and growth across different durations (by applying post-hoc analyses).

The reviewer correctly pointed out that our original manuscript failed to include statistical analyses for our stacked bar graphs. We appreciate the constructive suggestions on the statistical analysis that would be appropriate for these types of data. In the revised version of the manuscript, we have:

- Replotted all of the graphs of cell fate per mitotic category to show the SEM (Figure 2D-F, Figure 5G,I).
- Added the *P*-value from chi-square tests with post-hoc analysis (multiple comparison with Bonferroni corrections) comparing each delayed mitotic category (31-60min, 60-90min, and >90min) to the non-delayed (0-30mins).

Second, when comparing several genotypes, including rescue, the authors only used unpaired t tests. However, a test such as Anova is more appropriate to assess first if there are significant differences across all genotypes, and second applying post-hoc comparison between two genotypes (eg. Cep63;53bp versus WT and Cep63 alone, etc). This applies to many figures but is especially relevant for interpreting rescue experiments in Figure 3, 4, 5. In this regard, it may be best to not put both centrosome mutants in the same graph as this increases the number of statistical tests needed.

Upon reflection, we agree with the reviewer that individual unpaired t-tests were not suitable to for the assessment of many of our datasets, especially those in Figure 3-6. We have now re-analyzed out data using a one-way Anova test followed by a post-hoc analysis to compare the all data within each graph in pairs. The *P*-values from our post-hoc analysis have been added to all the relevant graphs in Figure 3-6 and Figure EV3-5. Finally, we have provided an excel file with all the data plotted in the paper and a separate file reporting the statistical analysis that was performed (Table EV1 and EV2).

3. The rescue experiments suggest differences in rates of cell death across mitotic durations (Figure 5G, I). But it would be valuable to see a direct comparison between rates of cell death in double knockout versus single knockout. All that is shown is distributions for each. This would better support the notion on p.17 that USP28 is required to initiate cell death in progeny of cells that delay in mitosis. The nocodazole experiment helps but is indirect and again here no comparisons are made between genotypes.

We have added plots to compare the frequency of cell death, arrest and growth between different genotypes (Figure EV2A and Figure EV5D,H). These graphs help support the claim that inactivation of the mitotic surveillance pathway (through knockout of *Usp28*) suppresses cell death to restore cell proliferation.

4. The authors claim on p. 12 that ablation of the mitotic surveillance pathway restores brain size and production of the correct number of neurons. However, the latter is not substantiated by data (simply showing more NPCs is insufficient). To support this, they should verify that the rescue actually results in production of more newborn neurons (fir this they could quantify cell cycle exit). It may be that the laminar neuron number rescue is simply a rescue of apoptosis. Both mechanisms could also contribute.

Although we showed that the number of NPCs is restored following the knock-out of the mitotic surveillance pathway in *Cep63*$^{T/T}$ and *Sas4*^{cKO} brains, we acknowledge that this is not sufficient evidence to claim that neuronal production was also fully restored. The standard experiment to support this claim would be to perform a pulse labeling of NPCs to measure cell cycle exit and the rate of neuron production. Unfortunately, we had to dramatically reduce the size of our animal colony during COVID-19 lockdown and generating new embryonic samples for this particular experiment would fall outside the timeframe of this revision. Given we cannot show that neurons are produced at the same rate across all genotypes we have removed this claim from revised text.

5. Further clarification of the cell death mechanisms would also be valuable. On p. 14 the authors specify that cell death happens in NPCs but they don't show that. In fact, based upon the patterns in Figure 4D it seems it is not simply limited to NPCs. This mechanism should be clarified.

We agree with the reviewer that cell death is likely to be occurring in both NPCs and neurons since cleaved caspase-3 (CC3) staining is not only limited to the VZ and SVZ of the developing cortex. To carefully assess this, we co-stained E14.5 cortices with CC3 and PAX6 and TBR2 (to stain NPCs) or TBR1 (to stain neurons). This revealed that 55% of the CC3+ cells in *Cep63*$^{T/T}$ brains and 57% of the CC3+ cells in *Sas4*^{cKO} brains are PAX6+ or TBR2+, showing that extensive apoptosis is occurring in NPCs. In addition, we observed that the remaining apoptotic cells (45% in *Cep63*$^{T/T}$ brains and 43% in Sas4^{cKO} brains) co-stained with TBR1, indicating that death also occurs in neurons. This is consistent with a model in which cell death takes place after the completion of mitosis in NPCs with centrosome defects. As some daughter cells differentiate into neurons while others retain their PAX6+ or TBR2+ fate, we expect apoptosis to be observed in both populations. We have added this quantification along with images of representative staining to Figure EV4C,D, and modified our text to highlight the fact that both NPCs and neurons undergo cell death in the developing brains of *Cep63*$^{T/T}$ *and Sas4*^{cKO} mice.

6. Primary NPC cultures were quantified for Pax6 but a substantial number of intermediate progenitors are also present at E14.5, and this should be quantified. The mitotic defects could be occurring in these cells as well, which the authors should discuss.

Primary cultures at E14.5 include both Pax6+ radial glial cells as well as Tbr2+ intermediate progenitor cells. In our analysis, we see Tbr2+ cells make up ~5-10% of the cells in culture at the start of the 48 hours of imaging (Figure EV1J). Our live imaging experiments follow the fate of both radial glial cells and intermediate progenitors. Thus, it is likely that both radial glial cells and intermediate progenitors delay in mitosis and activate the mitotic surveillance pathway. This was not clearly specified in our original manuscript and we have now modified the text to clarify this point.

Minor:

1. The introduction implies MCPH is only caused by depletion of NPCs, but it may also be caused by excessive apoptosis of neurons.

We apologize for the oversight. We have now added this to the introduction.

2. For figures comparing nestin, please specify what the wild-type control is. NestinCre has known background issues so it is important to compare to Cre alone.

We included both Nestin-Cre+ and Cre negative controls within our wildtype samples. In the revised manuscript, we make this distinction clear by highlighting the data points within the wildtype datasets that are from Nestin-Cre+ animals (Figure 1D, Figure 3B,C and Figure EV1D). To minimize any differences in the level of Cre expression in our Nestin-Cre+ animals, we have also made sure to only maintain and analyze animals with a single copy of the Cre transgene. In the limited number of Nestin-Cre+ mice we have analyzed, we do not observe obvious differences between Nestin-Cre+ and Cre negative mice.

3. P 9 the authors should not state that cell death partially explains microcephaly since these are in vitro experiments. Suggest to soften wording in this location.
We agree with the reviewer and have modified the text to remove this claim.

4. The model in Figure 6J should be adjusted to reflect that radial glia mitosis occurs adjacent to the ventricle, at the apical surface, as opposed to more basally.
We have made adjustment to the model in Figure 6J so that the division of radial glia cells are adjacent to the ventricle.

5. Figure 1G: The analysis of aneuploidy examines so few cells so it is not clear if this difference in moderate aneuploidy in Figure 1G is meaningful and belongs in the main figure.
Chromosome missegregation has been observed to occur in cultured cells dividing without centrosomes (Lambrus et al., JCB 2015). Since this could trigger P53 activation, we felt it was important to rule out the possibility that loss of CEP63 or SAS4 cause cell death because of an accumulation of aneuploid cells. The absence of detectable aneuploidy in *Cep63*$^{T/T}$ neural progenitors and the low level of aneuploidy of both *Sas4*^{cKO} and *Sas4*^{cKO}*;Usp28*^{cKO} are important observations that support our hypothesis that P53 is activated by the mitotic surveillance pathway and not aneuploidy.

We decided to use scDNAseq to analyze the degree of aneuploidy in our samples because this technique provides an unambiguous representation of the complete karyotype of each interphase cell sequenced. Unfortunately, scDNAseq is also expensive and thus we had to compromise on sample size (~20 cells/ culture) and the number of biological replicates (2 per genotypes) that would fit within a reasnoble budget. It is worth noting that analyzing 20 cells by scDNAseq provides information equivalent to a sample size of 400 cells analyzed with a FISH probe to a single chromosome (20 cells x 20 chromosome pairs). In addition, scDNAseq allows us to

observe gains and losses of regions of chromosomes that could be missed using other approaches.

Thank you for submitting your revised microcephaly manuscript for our consideration. It has now been assessed once more by the original reviewer 3, who found their key concerns well-addressed and the study in principle suitable for publication. We would therefore be happy to proceed with acceptance for The EMBO Journal, after a few minor concerns related to statistics analyses/presentation raised in the referee's comments copied below, as well as a number of important editorial points, have been addressed:

Referee #3:

In this revised manuscript by Holland and colleagues, the authors have been very attentive to concerns raised. They have tempered conclusions as necessary and importantly, performed new statistical analyses. In all, I find the study to be well carried out, interesting and important for the field.

One remaining concern is the labeling of statistics in figures. In several panels there are p values or NS noted above bar graphs but the comparisons made for these are unclear. This is clear in the supplementary table included but needs to be clear in both figures and figure legends. 1) For color coded ones (Eg Figure 2D), one can infer this is between a mutant and control (but inferring is dangerous). Another way to indicate these comparisons is to move the pink stars between the 2 pink categories (spread out the bars to have space in figure). But is it really the case that the comparison of growth conditions in figure 2F is not significant, as only the pink color is shown? Please clarify. 2) The p values/NS shown in Figure 3, 4 are very confusing and the authors need to clarify what these values represent when no lines are indicated.

Response to Referee #3's Comments

In this revised manuscript by Holland and colleagues, the authors have been very attentive to concerns raised. They have tempered conclusions as necessary and importantly, performed new statistical analyses. In all, I find the study to be well carried out, interesting and important for the field.

One remaining concern is the labeling of statistics in figures. In several panels there are p values or NS noted above bar graphs but the comparisons made for these are unclear. This is clear in the supplementary table included but needs to be clear in both figures and figure legends. 1) For color coded ones (Eg Figure 2D), one can infer this is between a mutant and control (but inferring is dangerous). Another way to indicate these comparisons is to move the pink stars between the 2 pink categories (spread out the bars to have space in figure).

We highly appreciate the reviewer's helpful suggestions. For all of our graphs containing stacked bar graphs, we have added to the figure legends information on the groups that are used for statistical comparisons.

Regarding changes to the statistics labels in our figures, we decided not to adopt the recommended strategy of putting the stars for significance between the respective categories because we felt that it may cause further confusion. For example, in Figure 2F, putting stars in between the 61-90min and >90min category may cause readers to think we are comparing these 2 groups instead of comparing them to the 0-30min group. Instead, we have optimized the ways we display the chi-square statistics and aligned the stars for significance immediately below (Figures: 1F, 2D-F, 5B, 5G, EV1F-G, EV1J, EV2A, EV2E, EV4C, EV5A-B, EV5D, and EV5H). We hope that this new way of displaying the statistics and the clarifications added to the figure legends will be sufficient to avoid any confusion.

But is it really the case that the comparison of growth conditions in figure 2F is not significant, as only the pink color is shown? Please clarify.
The reviewer is correct that the percentage of growth in the different mitotic duration groups in Figure 2F is significantly different. We had left out these comparisons due to space limitations, but have now added them to the figures (Figure 2D-F and Figure 5G) and updated our supplementary Table EV2 file accordingly.

2) The p values/NS shown in Figure 3, 4 are very confusing and the authors need to clarify what these values represent when no lines are indicated.
We agree that in trying to display all the relevant statistical information, some of the P values and significance signs in our Figure 3-6 and Figure EV3-EV6 were not clearly represented. We have now added lines and color coded these values/star symbols to clearly indicate the type of test and comparisons made for each figure.

2nd Revision - Editorial Decision 22nd Oct 2020

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Andrew J Holland
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2020-106118R

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 $\chi 2$ 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?	Experiments were performed in at least 3 biological replicates (3 different mice/embryos) where ever possible.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	At least 3 animals per genotypes were used for each analysis. Controls includes wildtype littermates.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data was excluded from the analysis.
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.	Animals or all genotypes/cells derived from these animals were treated with the same conditions within any sets of experiments.
For animal studies, include a statement about randomization even if no randomization was used.	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.	Multiple quantifications (e.g. live-cell imaging quantification, cortical thickness measurement, single cell DNA seq analysis) were performed blinded by different people.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Certain sample processing steps and quantifications were performed by people blinded to the genotypes.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Only data sets with measurements of 100 cells or more were assumed to have normal distribution. All other data sets are assess without assuming normal distribution.
Is there an estimate of variation within each group of data?	No.

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http://www.selectagents.gov/

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).	Rabbit-PAX6 (Covance, PRB-278P, 1:500), Chicken-TBR2 (EMD Millipore, AB15894, 1:250), Rat-a-Tubulin (Pierce Antibodies, MA1-80017, 1:500), Goat-γ-Tubulin (homemade, polyclonal, raised against the peptide CDEYHAATRPDYISWGTQEQ, 1:500), Rabbit-Ki67 (D3B5) (Cell Signaling, 9129, 1:500), Mouse-pH3 (Cell Signaling, 9701, 1:100), Rabbit-CC3 (Asp175) (Cell Signaling, 9661, 1:500), Rabbit-Centrin (in house, raised against human Centrin2 (a.a. 1-172) ;1:500), Rabbit-γ-H2AX (p-Ser139) (Cell Signaling, 2577, 1:250), Mouse-TP53 (1C12) (Cell Signaling, 2524S, 1:250), Mouse-TBR1 (Proteintech, 66564-1-Ig, 1:250), Mouse-PAX6 (Thermo Fisher Scientific, MA1-109, 1:250), Rat-CTIP2 (Abcam, ab18465, 1:1000) and Rabbit-CUX1 (ProteinTech, 11733-1-AP, 1:1000), Mouse-TUJ1 (Covance, MMS-435P, 1:1000).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	N/A.

* 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.	Analysis of Smc5cKO and Cep63T/T mice and associated genotypes was performed on a congenic C57BL6 background, while analysis of Sas4cKO mice and associated genotypes was performed on a mixed FVB/NJ and C57BL6 background. Genotyping was carried out using standard PCR protocols. Embryos and adults from both genders were included in our analysis. Mice were housed and cared for in an ¬AAALAC-accredited facility, and all animal experiments were conducted in accordance with Institute Animal Care and Use Committee approved protocols.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
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 manuscript complies with the ARRIVE guidelines on reporting relevant aspects of animal studies.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	All animal experiments were conducted in accordance with Institute Animal Care and Use Committee (Johns Hopkins University School of Medicine) approved protocols.
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
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.	N/A
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	N/A
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).	N/A
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).	N/A
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.	N/A

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.	N/A