Growth disadvantage associated with centrosome amplification drives population-level centriole number homeostasis

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Review Timeline:

| Event                  | Date       |
|------------------------|------------|
| Submission Date        | 2019-04-04 |
| Editorial Decision     | 2019-05-05 |
| Revision Received      | 2020-09-08 |
| Accepted               | 2020-09-09 |

Editor-in-Chief: David Drubin

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
RE: E19-04-0195  
TITLE: Mechanisms of centriole number homeostasis in populations of human cells

Dear authors,
the two reviewers who examined your article did a great job. They read it very carefully and made numerous excellent suggestions to improve the manuscript and strengthen the conclusions. Considering that the added value of this work lies in the strength of the investigation, rather than the novelty of the conclusions, it is important to address ALL these comments. We hope you will be able to address them and look forward to receive the revised version of your manuscript.
Sincerely
Manuel

------------------------------------------------------------------------

Dear Dr. Stearns:

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made. Any specific areas to be addressed are outlined in the reviewer comments included below.

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When submitting your revision online please use the link below, and include a cover letter that details, point-by-point, how the Monitoring Editor's and reviewers comments have been addressed. When entering the author names online, enter them exactly as they appear on the manuscript title page. Please send only the latest revised manuscript. DO NOT resend any previous versions. Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

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Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
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Reviewer #1 (Remarks to the Author):

Summary
Here, the authors study how wild-type centriole numbers are recovered after induction of centrosome amplification in populations of human cells. They consider four different possibilities that would account for this phenomenon: first, extra centrioles are incapable of duplication and thus, progressively lost during cell division; second, extra centrioles are eliminated; third, extra centrioles suffer asymmetric segregation, such that one daughter cell inherits wild-type numbers; and fourth, extra centrioles impart negative consequences on cell proliferation. Using fixed and live cell imaging methods, and proliferation assays, the authors find support for the fourth hypothesis and conclude that selection against centrosome amplification allows the population to return to wild-type centriole numbers. Finally, they discuss the implications of their findings in the context of cancer, where centrosome amplification is widely observed.

General remarks

By using a structured and straightforward approach, the authors provide important insights regarding centriole number dynamics in human cell population. The paper makes up for an interesting and easy-to-follow read.

However, the generalizability of the proposed mechanism is questionable. Furthermore, there are several aspects we consider the authors should address prior to publication.

Major remarks

Results and discussion

Figure 1: the authors investigate if extra centrioles undergo precise duplication, using a procentriole marker, and perform live cell imaging to see if the number of centrioles in the daughter cells corresponds to that of the mother, before mitosis (to further confirm if there are defects in duplication or if extra centrioles are eliminated), after inducing Plk4 overexpression with doxycycline. The authors conclude neither seem to be playing a role in the return to number equilibrium. We have two major remarks regarding this data.

1) first, if it is true that extra centrioles are able to duplicate efficiently, it is an important finding. However, we do not think this conclusion can be drawn from the experimental results. It could be that excess Plk4 still resides at the centrosome after doxycycline washout for a couple of cell cycles. This could be addressed by doing a western blot in the days following drug washout, or alternatively, a qPCR. A mutually illuminating approach would be to collect cells near the end of the experiment (for example, at 8 or 9 days), repeat the SAS-6 staining, and score duplication events associated to extra centrioles.

2) second (fig. 1C), the authors state that they image cells live for three days and score centrioles at the beginning and at the end of the experiment. Then, they compare centriole numbers between mother and daughter cells. However, given the doubling time of RPE-1, it is possible that cells divide more than once during that period. Can the authors present the results in a different way, indicating division events along time and centriole duplication/segregation events.

3) third, the sample size for the live-cell imaging setup is substantially low. However, although it
does not seem centrioles are being lost along cell division, it does seem there are daughter cells with higher numbers than the mother (that can be ascribed by the fact that they account for centriole duplication in the daughter cells), suggesting ongoing centrosome amplification. We acknowledge it may be time-consuming to repeat the experiment so we suggest the address this issue using statistics. For example:

H0: the number of daughter cells, after centriole duplication, containing different number of centrioles compared to their mother (before mitosis) is equal to zero (p=0) - i.e. extra centrioles duplicate normally and there is no centriole elimination

H1: the number of daughter cells, after centriole duplication, containing different number of centrioles compared to their mother (before mitosis) is greater than zero (p>0) - i.e. extra centrioles duplicate have impaired duplication and/or there is centriole elimination

And assuming a binomial distribution under the null, the authors can then determine the p-value and statistical power of their test.

Sample sizes and replicates: although we could find sample sizes and number of replicates for all experiments, the authors do not report them consistently. For example, in the legend corresponding to figure 3C, it reads "The numbers with each bar indicate n numbers". After reading the text, it becomes clear that this corresponds to 32 cells in total, collected from 4 experiments. We suggest that the authors go through all figures and ensure that they report samples sizes per experiment and number of experiments such that it is immediately readable.

Figure 3C: the authors compare centriole numbers in the two daughter cells after cell division and observe that most of the daughter cells (70%) differ by at most one centriole. The authors conclude centriole segregation is symmetric. However, it should be noted that this result likely depends on absolute centriole numbers. In other words, if there is only one extra centriole, it is expected that the cell would fall under this category. We recommend that the authors show the distribution of centriole numbers per cell and, within each category, make reference to the percentage of cells in the 0-1 category, 2-3, etc. Nevertheless, as it is, it can be assumed that centriole segregation is not grossly asymmetric.

Figure 4F: the authors define a simple mathematical model, using experimentally measured parameters (division rates, probability of cell cycle arrest or cell death, etc.) as inputs, and compare it to experimental data. Although the authors claim that the model "accurately recapitulates the population dynamics of the experimental cultures", we consider this is impossible to assess without any quantitative measure (such as the coefficient of determination). In fact, visually, the predicted curves differ substantially from the experimental results. On the other hand, their approach may not be ideal. Instead of plugging-in experimentally measured parameter values, we recommend that the authors fit the model (for example using a maximum-likelihood-based approach) and, posteriorly, compared the estimated parameter values with their measurements. We would also like to stress that while the models does capture the trend in the experiments, in a strict modelling perspective, it is not possible to ascribe why this model outperforms other models based on any other of the competing hypotheses.

Minor remarks

Abstract
"However, extra centrioles in these cells cause an increase in the duration of the cell cycle, resulting in a proliferative disadvantage relative to normal cells in the population. The extent of this disadvantage is sufficient to account for the observed rate of return to normal centriole number. (l. 14-17)"

It is not clear if "increase in the duration of the cell cycle" also includes cell cycle arrest, which by our interpretation seems to play a significant role in determining the population (cf. figure 4C - approximately, 40% of cells do not divide in the time window of observation"). If that is the case, we suggest the authors should rewrite these sentences.

Introduction

Comparison to Wong et al. 2015: the authors seek to understand the mechanisms underlying the return to centriole number equilibrium, which was first described by this paper: https://www.ncbi.nlm.nih.gov/pubmed/25931445. In that study, the authors treat the cells with a drug (centrinone) that abrogates centriole duplication, in order to deplete centrioles from the population. They find that, in the absence of p53, cells continue cycling and assemble the centrioles de novo, which results in transient centrosome amplification followed by return to wild-type numbers.

Here, the authors induce Plk4 overexpression directly, which is known to induce canonical and de novo amplification. Therefore, the source of centrosome amplification in the two systems is not exactly identical. Furthermore, centriole loss constitutes a potential selective step (by inducing cell cycle arrest) that is absent in this work.

In our perspective, the authors should stress the differences between the two systems, and we consider that the observation that centriole numbers return to their original distribution after transient Plk4 overexpression is novel. In other words, the observation is the same, but the system is different.

Results and discussion

Figure 1C: for completeness purposes, the authors should have provided negative controls for every day of the experiment (i.e. percent of DMSO-treated cells with and without centrosome amplification). This is but a minor concern, as we expect the results would not differ significantly from the control the authors present.

Figure 1B: the authors label the cells with EdU to score cycling cells and observe a relative decrease in the proportion of EdU-positive cells with centrosome amplification with respect to wild-type numbers. Intriguingly, the effect only becomes apparent after 48h. Can this be attributed to variation within the experiments or is it possible that the proliferative disadvantage imparted by extra centrosomes is delayed?

Segregation bias (l. 183-4): clarification is required in what the authors mean by "segregation bias".

Proposed mechanism: we consider that the authors are overly assertive regarding the contribution of cell cycle delay to their observations, and in general. Their results indicate that approximately 40% of the cells with centrosome amplification undergo cell cycle arrest, suggesting a considerable
effect in the population dynamics. Furthermore, as previously mentioned, we are not entirely convinced that the other hypothetical mechanisms (impaired duplication, centriole elimination and asymmetric segregation) are so negligible. Finally, the authors should refer to Baudoin et al. (https://www.biorxiv.org/content/biorxiv/early/2019/01/21/526731.full.pdf), where they investigate the return to wild-type centriole numbers after tetraploidization, and propose a different mechanism from the one in this study, despite sharing a form of negative selection against centrosome amplification.

Generalizability to cancer: the authors relate their findings to centriole number dynamics in cancer cells. However, as alluded to in the discussion, the proliferative disadvantage of cells harboring supernumerary centrosomes may be dependent on p53, which is often mutated in several types of cancer. In addition, the authors explore a single non-cancer-derived cell line. We consider that this section should be rewritten as the results here presented may not be generalizable to a cancer setting.

Materials and methods

Mathematical model: although the description of the model is clear, we recommend that the authors write the equations and parameter values.

Reviewer #2 (Remarks to the Author):

Centrosome numbers are exquisitely regulated throughout cell cycle. This is important to prevent loss or amplification of centrosomes, which would impair proper chromosome segregation and compromise genetic stability. In normal cells, both loss and amplification of centrosomes can trigger p53-induced cell cycle arrest to prevent proliferation of cells with abnormal centrosome number. In normal tissue culture cells, amplification of centrosome numbers has been shown to have a negative effect on cell proliferation, leading to loss of the extra centrosome over time. While the exact mechanism for loss of extra centrosomes has not been formally investigated, previous work suggested that differences in cell cycle (likely via stabilisation of p53), slower mitosis progression, could lead to the slow decline of these cells over time in tissue culture. Here, the authors investigate the mechanisms by which normal cells maintain centrosome numbers upon overexpression of PLK4 to induce centriole overduplication. They found that in fact, increase time in cell cycle (interphase and mitosis) could explain the "disappearance" of cells with extra centrosomes from the population. They also found that in some cases, asymmetric segregation of extra centrosomes could result in the appearance of daughter cells with normal centrosome numbers and that although this might not a major mechanism, it can contribute to the loss of cells with extra centrosomes over time.

Overall, this manuscript strongly suggests that decrease in cell proliferation leads to loss of cells with extra centrosomes, which has been previously proposed but not formally demonstrated. Thus, while it somehow lacks novelty it adds value to the field. The experiments are well designed and the data clear. There are however some concerns that should be address by the authors prior to publication.

Major concerns

#1. Lack of novelty. The authors provide a clear characterization of centriole number homeostasis in
RPE-1 cells after inducing centrosome amplification by PLK4 overexpression. They show that centriole number homeostasis can be explained by the proliferative disadvantage in cells with extra centrioles compared to normal cells. While this work is of importance to the field, the results are a confirmation of previous published work. 1) It has been shown that, in tissue culture, cells lose extra centrosomes upon induction of centrosome amplification. 2) It is also known that centrosome amplification leads to growth disadvantage via p53 activation in vitro (Holland et al., 2012 and Fava et al., 2017, papers are mentioned in the manuscript). 3) Delay in mitosis caused by extra centrosomes has been extensively characterised previously, although these papers are not referenced by the authors (see below), the authors could counterbalance this by using other cell lines and make this work more general. Since all the work has been done in RPE-1 cells there are limitations regarding the generalisations of the findings. At least, some important experiments could be assessed in other normal cell lines.

#2. Testing the model. The authors use a mathematical model where they fit their observations to demonstrate that decrease proliferation induced by extra centrosomes could lead to the slow decrease of cells with extra centrosomes overtime. While it is interesting to see the model fitting the experimental data, it would be stronger to have the model tested. For example, does inducing a similar cell cycle delay results in similar loss of cells from the population in the same time frame? Does loss of p53 in RPE cells with extra centrosomes prevent centrosome loss or delays it?

#3. Crucial references missing. I was surprised by the amount of references that were missing. I am assuming the authors forgot to add these important contributions:
- Delay in mitosis due to centrosome amplification has been already demonstrated in 3 independent articles: Basto et al, Cell 2008; Kwon et al, Genes&Dev 2008 and Yang et al., Nat Cell Biol 2008. These should be added to the manuscript when the delay in mitosis is discussed.
- On page 3, line 40, the multipolar intermediates have been suggested initially by Ring et al., J Cell Biol 1982 and demonstrated by Kwon et al., Gen&Dev 2008. These should be added as well.
- The authors correctly refer to the work of Sabino et al., Curr Biol 2015 when discussing the possibility that extra centrosomes could be inactivated during mitosis, as demonstrated in flies. However, Rhys et al 2018 demonstrated that using similar PLK4 overexpression system, inactivation of centrosomes in mitosis in human cells does not seem occur, which is what the authors also found here. Thus, this work should also be cited here.

#4. Misleading statement about the role of asymmetric segregation of extra centrioles in contributing to loss of extra centrioles. The authors make the following statement about asymmetric segregation of extra centrioles in their abstract: 'We find that return of normal centriole number in the population of induced cells cannot be explained by [...] grossly asymmetric segregation of extra centrioles in mitosis.' While the expectation is that if all cells undergo asymmetric distribution of centrosomes the rate at which cells with extra centrosomes would disappear from the population would be much faster, it is not possible to neglect its role in centrosome homeostasis. First, the cell line used has ~20% of cells with normal centrosome number upon PLK4 overexpression (that is just the nature of the inducible systems). Thus, the cells with normal centrosome number will naturally outcompete cells with extra centrosomes. However, the authors observed that in ~30% of asymmetric centrosome segregation. Thus, and according to the authors model, the expectation is that if 100% of cells had extra centrosomes, asymmetric segregation would likely be the main contributor for the loss of centrosome amplification. Though it would likely take longer time. Therefore, due to the limitations of the system the authors should tone down this statement. This could also be tested by single cell sorting or isolation of one-cell derived colonies to assess if colonies that derived from 1 cell with extra centrosomes lose centrosomes over time. This certainly seems to be the case in FACS sorted tetraploid cells (Ganem
et al., Nature 2009).

Minor concerns

1. On Figure 1C: The PLK4 expression levels could be confirmed.
2. On Figure 2A: It is not clear at which timepoint after doxycycline washout this experiment is performed.
3. On Figure 2A and 3A: quantification of data is missing.
4. On Figure 2C: The graph could be better explained in the figure.
5. On the reference list: Nigg, Holland 2018 paper appears two times in reference list as "a" and "b" but are the same.
Response to reviewers of MBoC E19-04-0195

We thank the reviewers and the editor for their comments on the manuscript. We have revised the original version of the manuscript, as indicated in the responses to specific reviewer comments, and hope that it now is acceptable for publication.

Reviewer #1:

Figure 1: the authors investigate if extra centrioles undergo precise duplication, using a procentriole marker, and perform live cell imaging to see if the number of centrioles in the daughter cells corresponds to that of the mother, before mitosis (to further confirm if there are defects in duplication or if extra centrioles are eliminated), after inducing Plk4 overexpression with doxycycline. The authors conclude neither seem to be playing a role in the return to number equilibrium. We have two major remarks regarding this data.

1) first, if it is true that extra centrioles are able to duplicate efficiently, it is an important finding. However, we do not think this conclusion can be drawn from the experimental results. It could be that excess Plk4 still resides at the centrosome after doxycycline washout for a couple of cell cycles. This could be addressed by doing a western blot in the days following drug washout, or alternatively, a qPCR. We checked PLK4 expression by qPCR after doxycycline treatment and performed Western blots to assay the level of PLK4 present after washout of doxycycline (Figure 1D-D’) and include the data in the revised figure as indicated. Importantly, we found that PLK4 protein returned to the pre-induction level after 72 h of doxycycline washout. This demonstrates that at the timepoints assayed for duplication - five days after washout of doxycycline - PLK4 protein would not be present in excess. We also note that all observed duplication events were of paired centrioles, and not rosettes of centrioles as might be expected were PLK4 still higher than normal.

A mutually illuminating approach would be to collect cells near the end of the experiment (for example, at 8 or 9 days), repeat the SAS-6 staining, and score duplication events associated to extra centrioles.

This is an interesting suggestion; however, at later days during the time course such as those suggested only a small number of cells with extra centrioles are present in the population, as shown in Fig. 1C, and it would be challenging to have a sufficient number of cells for analysis. We hope that the reviewer agrees that the results concerning PLK4 level described above are sufficient to make this point.

2) second (fig. 1C), the authors state that they image cells live for three days and score centrioles at the beginning and at the end of the experiment. Then, they compare centriole numbers between mother and daughter cells. However, given the doubling time of RPE-1, it is possible that cells divide more than once during that period. Can the authors present the results in a different way, indicating division events along time and centriole duplication/segregation events.

We believe the reviewer is referring to figure 2C, in which we quantified the number of centrioles in cells after a period of live imaging that allowed us to identify daughter cells from divisions. At issue is the explanation for the cell pairs that have more total centrioles than the mother cell from which they were derived. It is true that the cells divided either once or twice in this experiment, information that we have for each cell pair and have now included in the main text. Specifically, 83% of the analyzed cells underwent 1 mitotic
division during the course of the live imaging session; the remaining 17% includes cells that divided a second time. However, the cells were imaged over three days at low magnification to observe divisions, and at high magnification to count centrioles only at the beginning and end, as described in the original version of the Materials and Methods:

“hTERT-RPE-1 GFP-Centrin-2 tetON-PLK4 cells were diluted 1:10 with WT hTERT-RPE-1 cells to facilitate long-term tracking of the fluorescent cells as they migrated and divided. The cells were seeded onto glass-bottom dishes (World Precision Instruments) one day prior to imaging; 30 min prior to imaging, the medium was changed to phenol-free DMEM-F12 (Life Technologies) supplemented with 10% CCS. Images were acquired every 5 min on a Keyence digital optical microscope (Keyence Corporation) with a Nikon S Plan Fluor ELWD 20X/0.45 NA objective. Still images were taken using a Nikon Plan Apo 60X/1.40 NA objective. Cells were maintained in a humidified chamber at 37 °C under 5% CO2 during image acquisition.”

The low magnification adopted during live imaging did not allow us to observe centriole duplication events directly. We therefore cannot include this piece of information in our data.

3) third, the sample size for the live-cell imaging setup is substantially low. However, although it does not seem centrioles are being lost along cell division, it does seem there are daughter cells with higher numbers than the mother (that can be ascribed by the fact that they account for centriole duplication in the daughter cells), suggesting ongoing centrosome amplification. We acknowledge it may be time-consuming to repeat the experiment so we suggest the address this issue using statistics. For example:

H0: the number of daughter cells, after centriole duplication, containing different number of centrioles compared to their mother (before mitosis) is equal to zero (p=0) - i.e. extra centrioles duplicate normally and there is no centriole elimination

H1: the number of daughter cells, after centriole duplication, containing different number of centrioles compared to their mother (before mitosis) is greater than zero (p>0) - i.e. extra centrioles duplicate have impaired duplication and/or there is centriole elimination

And assuming a binomial distribution under the null, the authors can then determine the p-value and statistical power of their test.

We appreciate the suggestion, but we feel that the description above adds sufficient information to understand and interpret the experiment. Also, we note that if there were continued centriole amplification, which, overlaid on centriole elimination, would yield data resembling those shown in Fig. 2C, that amplification should be apparent as either free centrioles (from de novo formation), or centriole rosettes. However, we saw neither of these in the data shown in Fig. 2A, in which we imaged Sass6, a component of the cartwheel and present only in new centrioles in mammalian cells. Rather we saw only doublets (with centrin or CP110 as a marker), showing that whatever duplication takes place is standard centriole duplication. These data were collected 5d after washout of doxycycline. From the experiment suggested in Major Remark 1, we now know that PLK4 protein levels at a population level have returned to normal by this time (Figure 1D-D’).

Sample sizes and replicates: although we could find sample sizes and number of replicates for all experiments, the authors do not report them consistently. For example, in the legend corresponding to figure 3C, it reads "The numbers with each bar indicate n numbers". After reading the text, it becomes clear that this corresponds to 32 cells in total, collected from 4 experiments. We suggest that the authors go through all figures and ensure that they report
samples sizes per experiment and number of experiments such that it is immediately readable.

Thank you for pointing this out. We have fixed this issue.

Figure 3C: the authors compare centriole numbers in the two daughter cells after cell division and observe that most of the daughter cells (70%) differ by at most one centriole. The authors conclude centriole segregation is symmetric. However, it should be noted that this result likely depends on absolute centriole numbers. In other words, if there is only one extra centriole, it is expected that the cell would fall under this category. We recommend that the authors show the distribution of centriole numbers per cell and, within each category, make reference to the percentage of cells in the 0-1 category, 2-3, etc. Nevertheless, as it is, it can be assumed that centriole segregation is not grossly asymmetric.

Yes, we agree with this conclusion. We presented this data in this format as the number of extra centrioles varies consistently between cells, depending on where they are during the cell cycle, and how much PLK4 overexpression they experienced. We have added a histogram (Figure 3D) to show the frequency of symmetric and asymmetric segregation events binned by total centriole number.

Figure 4F: the authors define a simple mathematical model, using experimentally measured parameters (division rates, probability of cell cycle arrest or cell death, etc.) as inputs, and compare it to experimental data. Although the authors claim that the model "accurately recapitulates the population dynamics of the experimental cultures", we consider this is impossible to assess without any quantitative measure (such as the coefficient of determination). In fact, visually, the predicted curves differ substantially from the experimental results. On the other hand, their approach may not be ideal. Instead of plugging-in experimentally measured parameter values, we recommend that the authors fit the model (for example using a maximum-likelihood-based approach) and, posteriorly, compared the estimated parameter values with their measurements. We would also like to stress that while the models does capture the trend in the experiments, in a strict modelling perspective, it is not possible to ascribe why this model outperforms other models based on any other of the competing hypotheses.

The model was only intended to show that the small starting population of cells with normal centriole number can theoretically overtake the population given the empirical values measured by our live imaging data (frequency of death, arrest, division, et cetera). Since you are absolutely correct that this is not strictly a model, we have changed the language and no longer refer to this as a model.

Minor remarks
Abstract: "However, extra centrioles in these cells cause an increase in the duration of the cell cycle, resulting in a proliferative disadvantage relative to normal cells in the population. The extent of this disadvantage is sufficient to account for the observed rate of return to normal centriole number. (l. 14-17)"

It is not clear if "increase in the duration of the cell cycle" also includes cell cycle arrest, which by our interpretation seems to play a significant role in determining the population (cf. figure 4C - approximately, 40% of cells do not divide in the time window of observation”). If that is the case, we suggest the authors should rewrite these sentences.
Thank you, we have rephrased this statement to make this clearer.

Introduction
Comparison to Wong et al. 2015: the authors seek to understand the mechanisms underlying the return to centriole number equilibrium, which was first described by this paper: https://www.ncbi.nlm.nih.gov/pubmed/25931445. In that study, the authors treat the cells with a drug (centrinone) that abrogates centriole duplication, in order to deplete centrioles from the population. They find that, in the absence of p53, cells continue cycling and assemble the centrioles de novo, which results in transient centrosome amplification followed by return to wild-type numbers.

Here, the authors induce Plk4 overexpression directly, which is known to induce canonical and de novo amplification. Therefore, the source of centrosome amplification in the two systems is not exactly identical. Furthermore, centriole loss constitutes a potential selective step (by inducing cell cycle arrest) that is absent in this work.

In our perspective, the authors should stress the differences between the two systems, and we consider that the observation that centriole numbers return to their original distribution after transient Plk4 overexpression is novel. In other words, the observation is the same, but the system is different.

Our questions were first inspired by this work, but we indeed acknowledge that the systems are different. We have added text to the Introduction addressing this issue.

Results and discussion
Figure 1C: for completeness purposes, the authors should have provided negative controls for every day of the experiment (i.e. percent of DMSO-treated cells with and without centrosome amplification). This is but a minor concern, as we expect the results would not differ significantly from the control the authors present.

That is indeed the case. For the purpose of keeping the figure clear, we didn’t think that it was necessary to add counts for DMSO at each time point. We added the DMSO washout data in Fig. S1A.

Figure 1B: the authors label the cells with EdU to score cycling cells and observe a relative decrease in the proportion of EdU-positive cells with centrosome amplification with respect to wild-type numbers. Intriguingly, the effect only becomes apparent after 48h. Can this be attributed to variation within the experiments or is it possible that the proliferative disadvantage imparted by extra centrosomes is delayed?

From our live imaging analysis, cells with extra centrioles show a delay in cell cycle progression. The EdU data could be ascribed to the same reason, meaning that because these cells proliferate slower than normal ones, the effect is seen after more than 24h.

Segregation bias (l. 183-4): clarification is required in what the authors mean by "segregation bias".

Thank you for this comment. We have changed the text to read: “The remaining 30% show asymmetric segregation, with decreasing frequency with more uneven segregation that is best described as a Poisson distribution centered on λ=1.3 (R²=0.93).”
Proposed mechanism: we consider that the authors are overly assertive regarding the contribution of cell cycle delay to their observations, and in general. Their results indicate that approximately 40% of the cells with centrosome amplification undergo cell cycle arrest, suggesting a considerable effect in the population dynamics. Furthermore, as previously mentioned, we are not entirely convinced that the other hypothetical mechanisms (impaired duplication, centriole elimination and asymmetric segregation) are so negligible. Finally, the authors should refer to Baudoin et al. (https://www.biorxiv.org/content/biorxiv/early/2019/01/21/526731.full.pdf), where they investigate the return to wild-type centriole numbers after tetraploidization, and propose a different mechanism from the one in this study, despite sharing a form of negative selection against centrosome amplification.

We have moderated the discussion and interpretation of our results. We have now cited Baudoin paper, which was recently published.

Generalizability to cancer: the authors relate their findings to centriole number dynamics in cancer cells. However, as alluded to in the discussion, the proliferative disadvantage of cells harboring supernumerary centrosomes may be dependent on p53, which is often mutated in several types of cancer. In addition, the authors explore a single non-cancer-derived cell line. We consider that this section should be rewritten as the results here presented may not be generalizable to a cancer setting.

We have now added a section to the Results and in Fig. 5A,B and Fig. S4 in which we assess data for gene expression of PLK4 and other centriole replication proteins and p53 status in different cancers from publicly-available information in TCGA (The Cancer Genome Atlas). This reveals that PLK4 is more highly expressed in some cancers than others and that in some of these cancers this is not accompanied by TP53 mutations or other p53-inhibiting alterations. Although we do not have data for centriole numbers in these TCGA cancers, we do cite instances in which centriole amplification is known to be a phenotype of a given cancer type (e.g. testicular germ cell cancer has high PLK4 and centriole amplification). The lack of a correlation between PLK4 mRNA level and p53 status suggests that at least some cancers have elevated PLK4 and normal p53 activity, and that these would be subject to the same considerations we demonstrate here.

Materials and methods
Mathematical model: although the description of the model is clear, we recommend that the authors write the equations and parameter values.

We agree with this recommendation, and have rewritten the description of the methods and show the equations in Figure 5C.

Reviewer #2:

Centrosome numbers are exquisitely regulated throughout cell cycle. This is important to prevent loss or amplification of centrosomes, which would impair proper chromosome segregation and compromise genetic stability. In normal cells, both loss and amplification of centrosomes can trigger p53-induced cell cycle arrest to prevent proliferation of cells with abnormal centrosome number. In normal tissue culture cells, amplification of centrosome numbers has been shown to have a negative effect on cell proliferation, leading to loss of the extra centrosome over time. While the exact mechanism for loss of extra centrosomes has not
been formally investigated, previous work suggested that differences in cell cycle (likely via stabilization of p53), slower mitosis progression, could lead to the slow decline of these cells over time in tissue culture. Here, the authors investigate the mechanisms by which normal cells maintain centrosome numbers upon overexpression of PLK4 to induce centriole overduplication. They found that in fact, increase time in cell cycle (interphase and mitosis) could explain the "disappearance" of cells with extra centrosomes from the population. They also found that in some cases, asymmetric segregation of extra centrosomes could result in the appearance of daughter cells with normal centrosome numbers and that although this might not a major mechanism, it can contribute to the loss of cells with extra centrosomes over time.

Overall, this manuscript strongly suggests that decrease in cell proliferation leads to loss of cells with extra centrosomes, which has been previously proposed but not formally demonstrated. Thus, while it somehow lacks novelty it adds value to the field. The experiments are well designed and the data clear. There are however some concerns that should be addressed by the authors prior to publication.

We thank reviewer #2 for these comments and will address the following suggestions individually.

Major concerns

#1. Lack of novelty. The authors provide a clear characterization of centriole number homeostasis in RPE-1 cells after inducing centrosome amplification by PLK4 overexpression. They show that centriole number homeostasis can be explained by the proliferative disadvantage in cells with extra centrioles compared to normal cells. While this work is of importance to the field, the results are a confirmation of previous published work. 1) It has been shown that, in tissue culture, cells lose extra centrosomes upon induction of centrosome amplification. 2) It is also known that centrosome amplification leads to growth disadvantage via p53 activation in vitro (Holland et al., 2012 and Fava et al., 2017, papers are mentioned in the manuscript). 3) Delay in mitosis caused by extra centrosomes has been extensively characterised previously, although these papers are not referenced by the authors (see below). The authors could counterbalance this by using other cell lines and make this work more general. Since all the work has been done in RPE-1 cells there are limitations regarding the generalizations of the findings. At least, some important experiments could be assessed in other normal cell lines.

With respect to novelty, we would stress that this work is the first time that the full range of possible mechanisms for return-to-normal have been examined; furthermore, we have characterized the effects of CA without the introduction of tetraploidy, which occurred in previous work from other groups that used cytokinesis failure to induce CA. Also, our work differs somewhat with respect to the involvement of p53 and in the homogeneity of the response (addressed in our work by single-cell analysis). The failure to cite the papers the reviewer lists in point 3 below was an oversight on our part with respect to final referencing in the manuscript, and we have fixed the issue by referencing these works, which clearly are relevant to our findings.

#2. Testing the model. The authors use a mathematical model where they fit their observations to demonstrate that decrease proliferation induced by extra centrosomes could lead to the slow decrease of cells with extra centrosomes over time. While it is interesting to see the model fitting the experimental data, it would be stronger to have the model tested. For example, does inducing a similar cell cycle delay results in similar loss of cells from the
population in the same time frame? Does loss of p53 in RPE cells with extra centrosomes prevent centrosome loss or delays it?

Although we agree that would be useful in theory, we do not have a good way to do the experiment in practice. With respect to TP53 status, this is an experiment that we would like to do in the RPE1-hTERT cells that we use here, but we have not yet been able to, and, given the current constraints in doing lab work, we feel that this would be best left to future work. Also, the work from the Cimini lab published in eLife that we now reference, used TP53-/- RPE1-hTERT cells and obtained similar results, as we now describe (see above), albeit in tetraploid cells.

#3. Crucial references missing. I was surprised by the amount of references that were missing. I am assuming the authors forgot to add these important contributions:
- Delay in mitosis due to centrosome amplification has been already demonstrated in 3 independent articles: Basto et al, Cell 2008; Kwon et al, Genes&Dev 2008 and Yang et al., Nat Cell Biol 2008. These should be added to the manuscript when the delay in mitosis is discussed.
- On page 3, line 40, the multipolar intermediates have been suggested initially by Ring et al., J Cell Biol 1982 and demonstrated by Kwon et al., Gen&Dev 2008. These should be added as well.
- The authors correctly refer to the work of Sabino et al., Curr Biol 2015 when discussing the possibility that extra centrosomes could be inactivated during mitosis, as demonstrated in flies. However, Rhys et al 2018 demonstrated that using similar PLK4 overexpression system, inactivation of centrosomes in mitosis in human cells does not seem occur, which is what the authors also found here. Thus, this work should also be cited here.

We thank the reviewer for pointing out these oversights; these references are now in the manuscript.

#4. Misleading statement about the role of asymmetric segregation of extra centrioles in contributing to loss of extra centrioles. The authors make the following statement about asymmetric segregation of extra centrioles in their abstract: 'We find that return of normal centriole number in the population of induced cells cannot be explained by [...] grossly asymmetric segregation of extra centrioles in mitosis.' While the expectation is that if all cells undergo asymmetric distribution of centrosomes the rate at which cells with extra centrosomes would disappear from the population would be much faster, it is not possible to neglect its role in centrosome homeostasis. First, the cell line used has ~20% of cells with normal centrosome number upon PLK4 overexpression (that is just the nature of the inducible systems). Thus, the cells with normal centrosome number will naturally outcompete cells with extra centrosomes. However, the authors observed that in ~30% of asymmetric centrosome segregation. Thus, and according to the authors model, the expectation is that if 100% of cells had extra centrosomes, asymmetric segregation would likely be the main contributor for the loss of centrosome amplification. Though it would likely take longer time. Therefore, due to the limitations of the system the authors should tone down this statement. This could also be tested by single cell sorting or isolation of one-cell derived colonies to assess if colonies that derived from 1 cell with extra centrosomes lose centrosomes over time. This certainly seems to be the case in FACS sorted tetraploid cells (Ganem et al., Nature 2009).

This is an excellent point that we tried to address by multiple methods. Unfortunately, we have not been able to specifically quantify centriole number by GFP fluorescence intensity using FACS. We tested several other methods to obtain single clones starting from a single cell with extra centrioles, such as dilution of cells and seeding onto micropatterns to achieve 1 cell/micropattern. We were able to achieve this first step but encountered several technical problems while maintaining the cells on micropatterns for several days. This
precluded us from performing a clonal analysis of centrioles fate starting from a single cell. We are continuing to try variations on this experiment and hope to be able to report the results in the future.

Minor concerns

1. On Figure 1C: The PLK4 expression levels could be confirmed.
   To address this, we assayed PLK4 expression by qPCR after dox treatment and performed Western blots to assay the level of PLK4 present after washout of dox (Figure 1D-D’).

2. On Figure 2A: It is not clear at which timepoint after doxycycline washout this experiment is performed.
   This is an important point, and we thank the reviewer for noticing. We have made the figure legend more clear.

3. On Figure 2A and 3A: quantification of data is missing.
   The quantification is reported in text, but we rewrote it now in the figure legend so that it is immediately accessible.

4. On Figure 2C: The graph could be better explained in the figure.
   We have expanded the text associated with this figure.

5. On the reference list: Nigg, Holland 2018 paper appears two times in reference list as "a" and "b" but are the same.
   This is now fixed.
RE: Manuscript #E19-04-0195R  
TITLE: "Growth disadvantage associated with centrosome amplification drives population-level centriole number homeostasis"

Dear Dr Stearns,
It seems to me that you considered carefully all the comments raised by your two reviewers and addressed them properly. I am happy to accept directly your publication without sending it back to the reviewers and congratulate you for this interesting study.

Sincerely,
Manuel Thery
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Stearns:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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