JCB

Nek2-Mediated GAS2L1 Phosphorylation and Centrosome-Linker Disassembly Induce Centrosome Disjunction

Kin Chung Franco Au, Kwan Tang Hau, and Robert Qi

Corresponding Author(s): Robert Qi, The Hong Kong University of Science and Technology

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October 17, 2019

Re: JCB manuscript #201909094

Prof. Robert Z Qi
The Hong Kong University of Science and Technology
Division of Life Science The Hong Kong University of Science and Technology Clear Water Bay New Territories
Hong Kong N.A.
Hong Kong

Dear Prof. Qi,

Thank you for submitting your manuscript entitled "Nek2-Mediated GAS2L1 Phosphorylation and Centrosome-Linker Disassembly Induce Centrosome Disjunction". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that the reviewers found the results interesting but requested deeper phenotypic analyses and evidence that the data are robust throughout. We found their comments editorially valid and relevant and feel that addressing the referees' remarks in full will significantly strengthen your study. In particular, we would stress that the following points should be explicitly addressed in a revised manuscript:

- first, while the current work convincingly demonstrates abnormal centrosome behavior and chromosome segregation defects upon improper phosphorylation of GAS2L1, whether these two phenotypes are interlinked remains unknown. The revised manuscript should include a more detailed description of centrosome behavior during the transition from G2 to mitosis as well as a better characterization of the spindle defects. The goal of these experimental additions is to establish whether mitotic defects arise from irregular centrosome positioning at the onset of spindle assembly or whether the two phenotypes are unrelated (points raised by Reviewers #1 and #3).

- second, additional support should be provided for the notion that the 352D mutant does act as a real phospho-mimetic (Reviewer #2, point #2).

- we also feel that all data included in the revised manuscript must be supported with proper statistical analyses. As pointed out by Reviewer #2, simple t-tests may not be reliable for some of the experimental designs reported in the manuscript. Therefore, we ask you to justify the choice of statistical approaches in various experiments. This request does not imply that Student's T-tests should not be used. However, it is important to support and clearly state that the data are appropriate for a particular test, e.g., the distributions are normal and sufficiently large for Student's tests to be applied.

Please do not hesitate to contact us if you anticipate any issue addressing the reviewers' comments. We would be happy to further discuss these points as needed. While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Alexey Khodjakov, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

GAS2L1 is an actin- and microtubule-binding protein. Based on the previous study from the same group (AU et al, Dev. Cell, 2017), GAS2L1 is localized on centrosomes where it mediates the association of centrosomes with microtubules and actin and regulates centrosome disjunction

before mitosis. GALS2L1 and Rootletin exert the opposite effect on centrosome disjunction, with GAS2L1 promoting it and Rootletin suppressing it.
In this manuscript, the authors suggest that phosphorylation of GAS2L1 Ser352 by Nek2 kinase in G2 is required for pre-mitotic centrosome disjunction, proper spindle assembly, and faithful chromosome segregation. They show that GALS2L1 associates with microtubules through GAR region and with actin through its CH region. They demonstrate that the phosphorylation of Ser352 by Nek2A disrupts the interaction between these two domains, relieving autoinhibition between two domains, increasing its association with actin and microtubules. In addition, removal of autoinhibition of GAS2L1 is critical for centrosome disjunction but also necessitates the disassembly of a rootletin-based centrosome linker.
Biochemical part of the manuscript is convincing, and I don't have major concerns. Phospho-profiling and the analysis of binding properties of GAS2L1 domains seems solid. GAS2L1 autoinhibition has also been recently demonstrated (van de Willige et al, EMBO Rep, 2019) . The drawback of the manuscript is that it is hard to judge to what extent the measured chromosome segregation defects in GAS2L1 null cells and Sr352A mutant can be attributed do the defects in centrosome disjunction. The manuscript provides no information about the dynamics of centrosomes before and during mitosis in the absence of GAS2L1 function. It is also lacking an in-depth analysis of mitotic progression, which would be critical to validate this work. So, I don't feel comfortable recommending this work for publication in its current form.

Specific comments:
1.The authors state that GAS2L1-null cells and the clones which express a stable, moderate levels of S352A and S352D do not exhibit any overt defects in cell growth (page 6, second paragraph). Yet, Fig. 3 shows that both, GAS2L1-null cells and S352A clones have mitotic errors in 60% of mitotic population (as determined by the difference in the angle between the axes of the spindle poles and the metaphase plate) These errors are accompanied by DNA segregation errors in ~15% of cells. I am not sure how to reconciliate these observations.

2.It should also be explained why was the angle of 85 degrees taken as a measure and a threshold for spindle abnormalities in Fig. 3? Clearly, only a minority of spindles classified as erroneous using this criterium lead to chromosome segregation errors. So, without an analysis which would allow a direct correlation between the initial centrosome separation in G2, spindle architecture, mitotic duration and the appearance of chromosomal abnormalities, it is difficult to know whether 2 or 4 micrometers of centrosome distance in G2 indeed makes a difference in the quality of ensuing mitosis. Moreover, as centrosome distances from 2 - 6 micrometers are common in control RPE-1 cells but do not lead to chromosome segregation errors.

3.Previous work for the same group shows that GAS2L1 depletion reduces the levels of microtubules and actin associated with centrosomes. Do mitotic spindle poles of GASL1-null cells and S352A cells nucleate comparable levels of microtubules to control cells? It has not been explored whether GAS2L1 depletion perturbs centrosome maturation either. These are all unexplored questions and the authors seem to ignore other possibilities which could, aside from centrosome disjunction issues, contribute to the observed mitotic errors. Thus, a comprehensive analysis of centrosome behavior starting from G2 needs to be performed. DNA morphology needs to be clearly visible. This study was conducted in RPE-1 cells. The benchmark study for centrosome behavior and for spindle assembly in RPE-1 can be find in Magidson et al., Cell, 2011.

4.A method for measurement of centrosome distance is not marked. Were distances measured form 3D recordings and how?

5.Error bars need to be included in figure panels showing enlarged centrosomes.

6.Statistical significance needs to be noted for all histograms.

7.Actual measured values (angles) should be presented in Fig. 3A.

Reviewer #2 (Comments to the Authors (Required)):

In this study Au and colleagues study the mechanism by which the GAS2L1 protein contributes to centrosome separation at mitotic onset. A previous study from the laboratory had shown that the actin and microtubule-binding protein GAS2L1 was required for the initial splitting of the two centrosomes in late G2. Here the same laboratory extends those findings, proposing that the mitotic kinase Nek2 phosphorylates GAS2L1 to prevent an auto-inhibition interaction between the actin- and the microtubule-binding domain, and that this phosphorylation event plays a key role in centrosome splitting. This hypothesis is supported by both cell biological and biochemical experiments.

Overall, this study has the potential for a novel and very exciting publication. The topic and the level of mechanistic insight would certainly be of strong interest for the readership of Journal of Cell Biology, and the technical quality of the experiments is generally high. There are, however, two important concerns that the authors should address for this study to be at an appropriate level for publication. Specifically:

1) Statistics: Generally the statistical analysis is minimal and should be dramatically improved, as this is essential to estimate the reproducibility of the reported results.

a) the experiments shown in Figure 2A, 2C, 2D, 6F, 7A-C have not been evaluated for statistical significance. It is therefore impossible to know if those results are solid or spurious. A statistical analysis is essential. Given that the authors quantify how different treatments lead to different fates, I strongly suggest to use Chi-square tests with the absolute number of cells. This will reveal whether their change in distributions are significant or not (and not rely on a simple t-test, see comment c).

b) For the biochemical experiments shown in Figure 4D, 4E and 5A: does the lack of significance imply that the observed difference are statistically not reproducible, which would dramatically change the interpretation of the results? The authors should indicate whether they tested the significance and with which statistical tests.

C) For the experiments in which the statistical significance is indicated (Fig. 3, 5B-D and 6E) the statistical analysis is based on 3 independent experiments and a t-test. This perfectly fine for experiments 6E and 5D, when one is comparing two conditions, but as soon as one is comparing several conditions, one has to correct for the fact that multiple factors are tested side-by-side (the more factors are tested the higher the chance to observe a statistical significant results with a simple t-test). The authors should therefore perform an ANOVA test to take this aspect in account. This is not just a detail, it can dramatically change the interpretation of a result.

2) One caveat of the experiments that are based on the 325D mutation is that this GAS2L1 mutant might not behave like the real phosphorylation site. The authors could exclude this

possibility, and test their model, by phosphorylating the recombinant GAR domain with active and dead Nek2 and test whether its affinity for the CH domain is diminished after phosphorylation (equivalent to Figure 5A). Such an experiment would provide a strong confirmatory evidence for the fact that the 352D mutation is a real phospho-mimicking mutation.

Patrick Meraldi

Reviewer #3 (Comments to the Authors (Required)):

Gas2l1 was previously shown by the authors to be important for centrosome disjunction at the onset of mitosis. This activity depends on Gas2l1 interaction with microtubules and actin. In this manuscript Au et al. show that Gas2l1 interacts with itself through its GAR and CH domains that interact with microtubule and actin, respectively. The self-interaction decreases the recruitment of actin, and maybe microtubules, at the centrosomes and their disjunction. Phosphorylation by Nek2A of Gas2l1 at Ser352 at mitosis onset releases this auto-inhibitory interaction and promotes disjunction. The authors finally show that Gas2l1 act in conjunction with rootletin (also phosphorylated by Nek2A) to allow centrosome disjunction.
The work described in this paper is quite interesting with a good amount of quality data. However, there are few points that the authors should address before publication:
Major points
1) Most figures lack statistical analysis to support the effects observed, rending difficult the interpretation of the data.

2) The authors, depending on the figures, assess the centriole disjunction (interphase cells), the centrosome disjunction in late G2 or the centrosome movement at the G2/M transition. This is not even clearly stated and readers have to guess so according to the staining used. While mechanisms might be similar for centriole and centrosome disjunction there outcome is likely different. It would be therefore more consistent to concentrate on cells in G2 and thus expressing CENP-F all over the paper.

3) Absence of Gas2l1 impairs centrosome splitting. However, bipolar spindles are still made. Is there a delay in the formation of the spindle? The authors claimed that even if the bipolar spindle is formed spindles are abnormal. That might be so, however, the differences on the figure are not striking. Moreover, as there is no method to explain how the angle between the metaphase plate and the spindle pole was calculated, it is difficult to be convinced by the result.

4) While in vitro data, or data using overexpressed Nek2A are convincing to suggest a phosphorylation of Gas2l1 by Nek2A. Figure 6E showing the phospho status of Gas2l1 in absence of Nek2A is not convincing. The WB with the Phospho antibody is quite blurry preventing to see the decreased phosphorylation. Moreover, the top band revealed by the total Gas2l1 antibody clearly shows the phosphorylated protein and no clear decrease in absence of Nek2A.

5) Gas2l1 KO or siNek2 cells still managed to perform some amount of centrosome disjunction in late G2 (centrosome distance superior to 2um in 20% of G2 cells). Is this disjunction depends on Eg5 even at this stage?
Minor points:
1) In the sedimentation assays there is no control to check that the fragments (CH/ CH-GAR or CH+GAR) do not pellet on their own in absence of polymerised actin or microtubules.

2) The authors address whether the phosphorylation on S352 affect actin binding but not microtubule binding. They also do not assess whether microtubules are decreased at the centrosome as they did for actin.

3) On figure 3C, expression of Gas2l1-S352D in gas2l1-/- cells does not lead to centrosome splitting? Is it because of cell detachment and then how do the authors explain it or is it a poor choice of cell?

4) It is not clear whether the Eg5 inhibitor was added early enough in the different experiments where it is used. Indeed centrosomes are separated (80% at more than 2um in Fig 2C), even in absence of Gas2l1 (60% at more than 2um).

Dear Dr. Khodjakov:

We thank you for your interest in our manuscript entitled "Nek2-mediated GAS2L1 phosphorylation and centrosome-linker disassembly induce centrosome disjunction" (JCB manuscript #201909094). We appreciate the valuable suggestions and comments, all of which have helped us improve the quality of the manuscript.

We have performed all experiments and analyses suggested by the reviewers and have now revised the manuscript to thoroughly address the comments. First, to address the points raised by Reviewers #1 and #3 we have carried out the following analyses and added the obtained data into the manuscript: (1) we analyzed centrosome behaviors, mitotic progression, and chromosome segregation by time-lapse imaging of cells before and during mitosis; and (2) we characterized centrosome maturation at G2/M by examining centrosome-based microtubule nucleation in a microtubule regrowth assay and by analyzing the centrosomal staining of pericentrin and γ-tubulin. We have also improved the characterization of observed spindle defects. Together, our data strongly support that the observed mitotic errors are attributed to defects in centrosome disjunction. Second, we have provided data to further support that GAS2L1(S352D) acts as a phospho-mimetic mutant (Reviewer #2, point #2). We have performed the suggested experiments to compare the effects of wild-type Nek2A and the kinase-dead mutant K37R on interaction between the GAR-Tail (197–681 of GAS2L1) and the CH domain. The results strongly support those of the mutational analyses. Third, we have re-analyzed all quantification data with appropriate statistical tests, and we have included the statistical results and described the statistical methods and the justification of the methods.

We have also revised the manuscript to address all minor points raised by the reviewers. We hope that you will find the revisions satisfactory, and we look forward to your prompt reply.

Sincerely yours,

Robert Z. Qi, Ph.D.
Division of Life Science
The Hong Kong University of Science and Technology

Point-by-point responses to reviewers' comments:

Reviewer #1:

GAS2L1 is an actin- and microtubule-binding protein. Based on the previous study from the same group (AU et al, Dev. Cell, 2017), GAS2L1 is localized on centrosomes where it mediates the association of centrosomes with microtubules and actin and regulates centrosome disjunction before mitosis. GALS2L1 and Rootletin exert the opposite effect on centrosome disjunction, with GAS2L1 promoting it and Rootletin suppressing it.

In this manuscript, the authors suggest that phosphorylation of GAS2L1 Ser352 by Nek2 kinase in G2 is required for pre-mitotic centrosome disjunction, proper spindle assembly, and faithful chromosome segregation. They show that GALS2L1 associates with microtubules through GAR region and with actin through its CH region. They demonstrate that the phosphorylation of Ser352 by Nek2A disrupts the interaction between these two domains, relieving autoinhibition between two domains, increasing its association with actin and microtubules. In addition, removal of autoinhibition of GAS2L1 is critical for centrosome disjunction but also necessitates the disassembly of a rootletin-based centrosome linker.

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Response:

In this revised manuscript, we have included additional experimental results to address the issues raised by this reviewer. First, to strengthen the conclusion that centrosome disjunction defects caused by the loss of GAS2L1 or the blockage of its Ser352 phosphorylation compromise the fidelity of chromosome segregation, we have performed the following experiments suggested in the reviewer's "Specific comments" and added the data into the manuscript: (1) We conducted time-lapse imaging of RPE-1 parental cells, GAS2L1-null cells, and GAS2L1-null cells expressing wild-type, S352A, and S352D GAS2L1 and obtained data to show that chromosome-segregation errors are correlated with centrosome disjunction delays occurred in the GAS2L1-null cells and the S352A-rescuing line (data added as Figures 3 A–C and 4C, and Videos 1–5); and (2) we have carried out assays on the above-mentioned RPE-1 lines to show that centrosome maturation was unaffected by the loss of GAS2L1 or the blockage of its Ser352 phosphorylation. Therefore, chromosome mis-segregation occurred in GAS2L1-null cells and the S352A-rescuing line is not due to any defect in centrosome maturation. Indeed, it has been shown in a number of studies that the fidelity of chromosome segregation is compromised by

defective centrosome separation (Silkworth et al., Mol Biol Cell. 2012, PMID: 22130796; Kaseda et al., Biol Open 2012, PMID: 23213363; Nam and van Deursen, Nat Cell Biol. 2014, PMID: 24776885; Zhang et al., J Clin Invest. 2012, PMID: 23187126).

Second, we have analyzed the dynamics of centrosomes before and during mitosis in the above-mentioned live-cell imaging experiments. The loss of GAS2L1 or its function in centrosome disjunction caused delay of centrosome disjunction before nuclear envelope breakdown (NEBD), but did not affect the poleward movement of centrosomes after NEBD. These data have been added as Figure 3 and Videos 1–5.

Third, we have analyzed mitotic progression in the above-mentioned live-cell imaging experiments. The loss of GAS2L1 or its function in centrosome disjunction resulted in metaphase prolongation, which supports the notion that the timing of centrosome disjunction has impacts on metaphase length (Mardin et al., Dev Cell. 2013, PMID: 23643362). These data have been added as Figure 3 A and D and Videos 1–5.

We will provide more details in our responses below to the "Specific comments" of the reviewer.

Specific comments:
1. The authors state that GAS2L1-null cells and the clones which express a stable, moderate levels of S352A and S352D do not exhibit any overt defects in cell growth (page 6, second paragraph). Yet, Fig. 3 shows that both, GAS2L1-null cells and S352A clones have mitotic errors in 60% of mitotic population (as determined by the difference in the angle between the axes of the spindle poles and the metaphase plate). These errors are accompanied by DNA segregation errors in ~15% of cells. I am not sure how to reconciliate these observations.

Response:

It has been reported that defects in spindle geometry promote the formation of merotelic kinetochore attachments and consequently increase the incidence of chromosome mis-segregation (reviewed in Silkworth and Cimini, Cell Div. 2012, PMID: 22883214; Nam et al., Trends Cell Biol. 2015, PMID: 25455111). Computer simulations also predicted that the geometric factors of mitotic spindles have important impacts on microtubule-kinetochore attachments (Silkworth et al., Mol Biol Cell. 2012, PMID: 22130796 and Paul et al., Proc Natl Acad Sci U S A. 2009, PMID: 19717443). We think that chromosome mis-segregation occurred in GAS2L1-null cells and the S352A-rescuing cells only when spindle geometry was altered to certain extent. We have revised Figure 3A (Figure 4A of the revised manuscript) to show clearly the distributions of spindle angles measured from parental and GAS2L1-null RPE-1 cells as well as GAS2L1-null cells expressing wild-type, S352A, and S352D GAS2L1 in a dot blot.

2. It should also be explained why was the angle of 85 degrees taken as a measure and a threshold for spindle abnormalities in Fig. 3? Clearly, only a minority of spindles classified as erroneous using this criterium lead to chromosome segregation errors. So, without an analysis which would allow a direct correlation between the initial centrosome separation in G2, spindle architecture, mitotic duration and the appearance of chromosomal abnormalities, it is difficult to know whether 2 or 4 micrometers of centrosome distance in G2 indeed makes a difference in the quality of ensuing mitosis. Moreover, as centrosome distances from 2 – 6 micrometers are common in control RPE-1 cells but do not lead to chromosome segregation errors.

Response:

In Figure 3 of last submitted manuscript, we used 85° as the threshold of the angle between the spindle axis and the metaphase plate, and most of the control RPE-1 cells showed the angles of 85–90°. The same criterion was used in a previous report (Nam and van Deursen, 2014, Nat Cell Biol., PMID: 24776885). As stated above, we have revised the figure and presented the angle values in a dot plot to provide a better view of the angle distributions (Figure 4A of the revised manuscript).

We have also added new experimental data in the revised manuscript to support the direct correlation of aberrations in spindle geometry, mitotic progression, and chromosome segregation to the delay of centrosome separation. First, we performed time-lapse imaging of G2 and mitosis in parental and GAS2L1-null RPE-1 cells as well as GAS2L1-null cells expressing wild-type, S352A, and S352D GAS2L1. The analysis of the data obtained from live-cell and immunofluorescence imaging revealed that the timing of centrosome disjunction (inter-centrosomal distance > 2 μm) in G2 have important impacts on spindle geometry, mitotic progression, and chromosome segregation (Figures 3 and 4C–D). Second, we have examined the properties of the pericentriolar material (PCM) as suggested by the reviewer, and we did not find any defect in the size and microtubule-nucleating function of centrosomes in GAS2L1-null cells and the S352A-rescuing cells at G2/M. These results indicate that the knockout of GAS2L1 or the blockage of Ser352 phosphorylation does not interfere with centrosome maturation.

In addition, several studies have documented that centrosome separation delay promotes spindle geometry alteration and chromosomal mis-segregation (Silkworth et al., Mol Biol Cell. 2012, PMID: 22130796; Kaseda et al., Biol Open 2012, PMID: 23213363; Nam and van Deursen, Nat Cell Biol. 2014, PMID: 24776885; Zhang et al., J Clin Invest. 2012, PMID: 23187126). In addition, the timing of centrosome separation has been shown to affect the length of metaphase and mitosis (Mardin et al., Dev Cell. 2013, PMID: 23643362; Kaseda et al., Biol Open 2012, PMID: 23213363). Our current study further supports that defective centrosome separation in late G2 impacts spindle geometry, mitotic progression, and chromosomal segregation.

Under physiological conditions, centrosome separation is initiated in late G2 and the separated centrosomes move further apart after mitotic entry (reviewed in Mardin and Schiebel, J Cell Biol. 2012, PMID: 22472437). We have found that centrosome disjunction (inter-centrosomal distance > 2 μm) occurs only in a small portion (< 5%) of earlier-than-G2 interphase RPE-1 cells (negative in staining of

CENP-F) (Au et al., Dev Cell. 2017, PMID: 28017616). Therefore, most control RPE-1 cells do not undergo premature centrosome disjunction.

3. Previous work for the same group shows that GAS2L1 depletion reduces the levels of microtubules and actin associated with centrosomes. Do mitotic spindle poles of GASL1-null cells and S352A cells nucleate comparable levels of microtubules to control cells? It has not been explored whether GAS2L1 depletion perturbs centrosome maturation either. These are all unexplored questions and the authors seem to ignore other possibilities which could, aside from centrosome disjunction issues, contribute to the observed mitotic errors. Thus, a comprehensive analysis of centrosome behavior starting from G2 needs to be performed. DNA morphology needs to be clearly visible. This study was conducted in RPE-1 cells. The benchmark study for centrosome behavior and for spindle assembly in RPE-1 can be find in Magidson et al., Cell, 2011.

Response:

As suggested, we have performed several assays to analyze centrosome properties before and during mitosis in parental and GAS2L1-null RPE-1 cells as well as GAS2L1-null cells expressing wild-type, S352A, and S352D GAS2L1. First, we performed a microtubule regrowth assay to assess centrosome-based microtubule nucleation in G2/M cells, and we did not find any significant difference among all of the tested RPE-1 lines (data added as Figure S2C). Second, we evaluated the size of the PCM in G2/M cells by staining pericentrin (a PCM matrix protein) and γ-tubulin (a core component of microtubule nucleator γ-tubulin ring complexes), as centrosome maturation is marked by PCM expansion as well as increased microtubule-nucleating activity of the centrosomes (Reviewed in Palazzo et al., Curr Top Dev Biol. 2000, PMID: 11005031). All of these RPE-1 lines showed similar centrosomal staining of both pericentrin and γ-tubulin (data added as Figures S2A and S2B). We have had similar observations from RPE-1 cells after GAS2L1 knockdown (Au et al., Dev Cell. 2017, PMID: 28017616). Together, these results strongly indicate that *gas2l1* knockout and the re-expression of the Ser352 mutants do not impact centrosome maturation.

As stated above, we also conducted time-lapse microscopy on the above-mentioned RPE-1 lines to monitor centrosome dynamics from G2 to mitosis, and nuclear DNA was stained with Hoechst 33342 in the imaging experiments. The dynamic changes of inter-centrosomal distance and the times of centrosome disjunction occurrence have been presented in the manuscript (Figure 3 A–C). Indeed, the analyses allow us to gain more insights into the function of GAS2L1 and its Ser352 phosphorylation in centrosome disjunction and cell-cycle progression.

4. A method for measurement of centrosome distance is not marked. Were distances measured form 3D recordings and how?

Response:

In the revised manuscript, we have described the method for the measurement of centrosome distance in the Materials and methods section. The distance was measured from 2D epifluorescence images of G2 cells or Eg5-inhibited mitotic cells. Under these conditions, the majority of cells contained separated centrosomes well focused in same focal plane and these cells were selected for the distance measurement. It has been shown that in a late stage of prophase, the separated centrosomes move vertically toward the top and bottom sides of the nucleus (Magidson et al., Cell. 2011, PMID: 21854981).

5. Error bars need to be included in figure panels showing enlarged centrosomes.

Response:

We believe that the reviewer should have meant "scale bars" instead of "error bars" for the micrographs showing enlarged centrosomes. Therefore, we have added scale bars into the enlarged images.

6. Statistical significance needs to be noted for all histograms.

Response:

We have re-performed statistical analyses (Please see below our response to Point 1 of Reviewer 2) on the quantification data and presented the statistical results in this revised manuscript.

7. Actual measured values (angles) should be presented in Fig. 3A.

Response:

As stated in our response to Point 1 of the Specific Comments, we have replaced the bar graph in Fig. 3A to present the measured angle values in a dot plot (Fig. 4A of the revised manuscript). This dot plot shows all measured values of the angles between the spindle axis and metaphase plate.

Reviewer #2:

In this study Au and colleagues study the mechanism by which the GAS2L1 protein contributes to centrosome separation at mitotic onset. A previous study from the laboratory had shown that the actin and microtubule-binding protein GAS2L1 was required for the initial splitting of the two centrosomes in late G2. Here the same laboratory extends those findings, proposing that the mitotic kinase Nek2 phosphorylates GAS2L1 to prevent an auto-inhibition interaction between the actin- and the microtubule-binding domain, and that this phosphorylation event plays a key role in centrosome splitting. This hypothesis is supported by both cell biological and biochemical experiments.

Overall, this study has the potential for a novel and very exciting publication. The topic and the level of mechanistic insight would certainly be of strong interest for the readership of Journal of Cell Biology, and the technical quality of the experiments is generally high. There are, however, two important concerns that the authors should address for this study to be at an appropriate level for publication. Specifically:

1) Statistics: Generally the statistical analysis is minimal and should be dramatically improved, as this is essential to estimate the reproducibility of the reported results.

a) the experiments shown in Figure 2A, 2C, 2D, 6F, 7A-C have not been evaluated for statistical significance. It is therefore impossible to know if those results are solid or spurious. A statistical analysis is essential. Given that the authors quantify how different treatments lead to different fates, I strongly suggest to use Chi-square tests with the absolute number of cells. This will reveal whether their change in distributions are significant or not (and not rely on a simple t-test, see comment c).

b) For the biochemical experiments shown in Figure 4D, 4E and 5A: does the lack of significance imply that the observed difference are statistically not reproducible, which would dramatically change the interpretation of the results? The authors should indicate whether they tested the significance and with which statistical tests.

c) For the experiments in which the statistical significance is indicated (Fig. 3, 5B-D and 6E) the statistical analysis is based on 3 independent experiments and a t-test. This perfectly fine for experiments 6E and 5D, when one is comparing two conditions, but as soon as one is comparing several conditions, one has to correct for the fact that multiple factors are tested side-by-side (the more factors are tested the higher the chance to observe a statistical significant results with a simple t-test). The authors should therefore perform an ANOVA test to take this aspect in account. This is not just a detail, it can dramatically change the interpretation of a result.

Response:

We thank the reviewer for the positive comments and helpful suggestions. We have performed statistical tests as suggested to re-analyze our quantification data, and we have included the test results in the revised manuscript. In addition, we have stated the statistical test methods in the figure legends of this revised manuscript.

a) As suggested, we have performed Chi-square tests on the data presented in Figures 2A, 2C, 2D, 7G, and 8A-C (Figures 2A, 2C, 2D, 6F, and 7A-C of last submitted manuscript). The analyses confirmed the significance of the changes in distributions, and the analysis results have been included in the revised figures.

b) We have performed one-way ANOVA tests on the data shown in Figures 5D and 5E (Figures 4D and 4E of last submitted manuscript) and conducted a Student's *t* test on the data in Figure 6A (Figure 5A of last submitted manuscript). These analyses indicate that the differences across the samples are statistically significant, and the analysis results have been included in the revised figures.

c) As suggested, we have performed one-way ANOVA tests on the data presented in Figure 4 (Figure 3 of last submitted manuscript) and Figures 6B and 6C (Figures 5B

and 5C of last submitted manuscript), and the statistical significances have been marked in the figures. In addition, we have performed appropriate statistical tests on the data of newly added experiments in this revised manuscript.

2) One caveat of the experiments that are based on the 325D mutation is that this GAS2L1 mutant might not behave like the real phosphorylation site. The authors could exclude this possibility, and test their model, by phosphorylating the recombinant GAR domain with active and dead Nek2 and test whether its affinity for the CH domain is diminished after phosphorylation (equivalent to Figure 5A). Such an experiment would provide a strong confirmatory evidence for the fact that the 352D mutation is a real phospho-mimicking mutation.

Response:

We have performed the experiment as suggested and have added the results as Figure 7F. In the assay, we compared the effects of wild-type Nek2A and the kinase-dead mutant K37R on the interaction between the GAR-Tail (197–681 of GAS2L1) and CH domain. The expression of wild-type Nek2A significantly increased GAR-Tail phosphorylation at Ser352 and diminished the binding of the GAR-Tail to the CH domain, compared with that of the K37R mutant. These results strongly support our conclusions drawn from the mutational analyses.

Reviewer #3:

Gas2l1 was previously shown by the authors to be important for centrosome disjunction at the onset of mitosis. This activity depends on Gas2l1 interaction with microtubules and actin. In this manuscript Au et al. show that Gas2l1 interacts with itself through its GAR and CH domains that interact with microtubule and actin, respectively. The self-interaction decreases the recruitment of actin, and maybe microtubules, at the centrosomes and their disjunction. Phosphorylation by Nek2A of Gas2l1 at Ser352 at mitosis onset releases this auto-inhibitory interaction and promotes disjunction. The authors finally show that Gas2l1 act in conjunction with rootletin (also phosphorylated by Nek2A) to allow centrosome disjunction.

The work described in this paper is quite interesting with a good amount of quality data. However, there are few points that the authors should address before publication:

Major points
1) Most figures lack statistical analysis to support the effects observed, rending difficult the interpretation of the data.

Response:

As suggested, we have re-analyzed our quantification data with appropriate statistical tests (Please see our responses to Point 1 of Reviewer 2) and included the statistical analyses in this revised manuscript. The statistical results support the conclusions drawn from the quantification data in the manuscript.

2) The authors, depending on the figures, assess the centriole disjunction (interphase cells), the centrosome disjunction in late G2 or the centrosome movement at the G2/M transition. This is not even clearly stated and readers have to guess so according to the staining used. While mechanisms might be similar for centriole and centrosome disjunction there outcome is likely different. It would be therefore more consistent to concentrate on cells in G2 and thus expressing CENP-F all over the paper.

Response:

We used CENP-F–staining-positive cells (i.e., G2 cells) for assays of centrosome separation where appropriate. In the assays of Figures 2A, 7G and 8A of this revised manuscript, we used asynchronous cells to test the activities of overexpressed GAS2L1 proteins (Figures 2A and 7G) and Nek2A (Figure 8A) for inducing premature centrosome separation, and we did not use CENP-F–staining-positive cells in these experiments because a majority of G2 cells show separated centrosomes under physiological conditions. We have revised the figure legends to indicate the cell-cycle status.

3) Absence of Gas2l1 impairs centrosome splitting. However, bipolar spindles are still made. Is there a delay in the formation of the spindle? The authors claimed that even if the bipolar spindle is formed spindles are abnormal. That might be so, however, the differences on the figure are not striking. Moreover, as there is no method to explain how the angle between the metaphase plate and the spindle pole was calculated, it is difficult to be convinced by the result.

Response:

We have newly performed time-lapse imaging experiments to analyze mitotic progression, and we found that *gas2l1* knockout as well as the rescues using the S352A or S352D mutants did not affect the length of prometaphase (Figures 3A and 3D and Videos 1–5) in which bipolar spindles are assembled. Therefore, *gas2l1* knockout or the blockage of Ser352 phosphorylation did not cause any delay of bipolar spindle assembly.

We re-performed a statistical analysis on the spindle angle data collected from parental RPE-1 cells, *gas2l1* knockout cells, and *gas2l1* knockout cells expressing wild-type GAS2L1 and the Ser352 mutants (Figure 4A of this revised manuscript). We have added a description of the method used to measure the angle between the spindle axis to the metaphase plate in the Materials and methods section. The statistical analysis clearly indicates that *gas2l1* knockout or the blockage of Ser352 phosphorylation decreased the angles. Our results support the notion that centrosome-separation delay impacts the geometry bipolar spindles (Silkworth et al., Mol Biol Cell. 2012, PMID: 22130796 and Silkworth and Cimini, Cell Div. 2012, PMID: 22883214).

4) While in vitro data, or data using overexpressed Nek2A are convincing to suggest a phosphorylation of Gas2l1 by Nek2A. Figure 6E showing the phospho status of Gas2l1 in absence of Nek2A is not convincing. The WB with the Phospho antibody is quite blurry preventing to see the decreased phosphorylation. Moreover, the top band revealed by the total Gas2l1 antibody clearly shows the phosphorylated protein and no clear decrease in absence of Nek2A.

Response:

As pointed out by the reviewer, the knockdown of Nek2 did not obviously reduce the amount of phosphorylated GAS2L1 (i.e., upper band in the anti-GAS2L1 immunoblot; Figure 7E of this submitted manuscript or Figure 6E of last submitted manuscript). We have shown in Figure 1D that GAS2L1 undergoes mitotic phosphorylation at multiple sites (12 Ser and Thr residues were identified). Based on a sequence analysis of the identified sites, we think that kinase(s) other than Nek2 should be involved in the mitotic phosphorylation of GAS2L1. In fact, our mass spectrometry analysis showed that some of these identified sites were not phosphorylated *in vitro* by Nek2A. Therefore, we believe that the upper GAS2L1 band in the Nek2-silenced sample contained mainly the protein phosphorylated in Nek2-independent manners.

We have provided evidence to indicate that the anti-pSer352-GAS2L1 antibody specifically recognizes Ser352-phosphorylated GAS2L1 (Figure 7C of this revised manuscript or Figure 6C of last submitted manuscript). Using the antibody, we consistently detected pSer352-GAS2L1 in mitotic extracts, but with some challenges. The relatively weak signals could be due to any of the following reasons: (1) low abundance of pSer352-GAS2L1 in the extracts; and (2) relatively low sensitivity of the anti-pSer352-GAS2L1 antibody. However, we are able to show clearly by the quantitative immunoblotting that the level of pSer352-GAS2L1 was significantly reduced by the knockdown of Nek2.

5) Gas2l1 KO or siNek2 cells still managed to perform some amount of centrosome disjunction in late G2 (centrosome distance superior to 2um in 20% of G2 cells). Is this disjunction depends on Eg5 even at this stage?

Response:

We do not exclude the possibility that Eg5 participates at a certain extent in centrosome separation in late G2, although Eg5 appears at centrosomes (the centrosomal localization is required for Eg5 function) and becomes fully functional after mitotic entry (reviewed in Agircan et al. 2014, Philos Trans R Soc Lond B Biol Sci., PMID: 25047615). In fact, it has been shown in a report that Eg5 also takes part in centrosome separation in late G2 (Smith et al. 2011, EMBO J, PMID: 21522128). It is also likely that centrosome separation under these conditions in late G2 involves other mechanisms, such as those mediated by Kif15 (kinesin-12) and dynein (Tanenbaum et al., Curr Biol. 2009, PMID: 19818618; Raaijmakers et al., EMBO J. 2012, PMID: 23034402).

Minor points:
1) In the sedimentation assays there is no control to check that the fragments (CH/ CH-GAR or CH+GAR) do not pellet on their own in absence of polymerised actin or microtubules.

Response:

Prior to microtubule or F-actin sedimentation assays, the recombinant proteins (i.e., CH, GAR, and CH-GAR) were clarified by centrifugation at 150,000 × *g* (see Materials and methods). We have also performed the suggested control assays and found that in the absence of microtubules and F-actin, the tested recombinant proteins did not precipitate by centrifugation. We have added these control assay results as Figure S3B.

2) The authors address whether the phosphorylation on S352 affect actin binding but not microtubule binding. They also do not assess whether microtubules are decreased at the centrosome as they did for actin.

Response:

GAS2L1 can bind to microtubules through two regions, the GAR domain (i.e., 197–300) and the tail region (Tail; 300–681) (van de Willige et al., EMBO Rep. 2019, PMID: 31486213). The Drosophila GAS2-like protein Pigs (i.e., GAS2L1 orthologue in Drosophila) contains two microtubule-binding regions similar to those of GAS2L1 and furthermore, the deletion of the GAR domain in Pigs did not affect the microtubule-binding activity of the protein (Girdler et al., J Cell Sci. 2016, PMID: 26585311). Indeed, we did not observe any effect of the mutations S352A and S352D on the microtubule-binding activity of GAS2L1 in a microtubule co-sedimentation assay (see the figure below). Therefore, it is unlikely that Ser352 phosphorylation causes any obvious change in the amount of centrosome-associated microtubules.

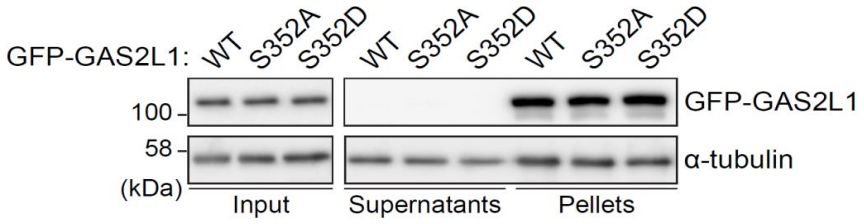

Figure. Extracts of HEK293T cells expressing GFP-GAS2L1 were used in a microtubule sedimentation assay. After sedimentation, the pellets and supernatants were analyzed by anti-GFP and anti-α-tubulin immunoblotting. WT, wild-type.

3) On figure 5C, expression of Gas2l1-S352D in gas2l1-/- cells does not lead to centrosome splitting? Is it because of cell detachment and then how do the authors explain it or is it a poor choice of cell?

Response:

We found that after cell detachment by trypsinization, most (>90%) of G2 RPE-1 cells showed two centrosomes located close to each other (Figures 6C and 6D of this revised manuscript). This is different from the attached G2 cells, approximately half of which showed separated centrosomes. We think that the close positioning of the two centrosomes in the detached G2 cells could be due to cell rounding by detachment. Cell rounding involves the rearrangement of actin filaments and microtubules, which may cause the close positioning of the centrosomes.

4) It is not clear whether the Eg5 inhibitor was added early enough in the different experiments where it is used. Indeed centrosomes are separated (80% at more than 2um in Fig 2C), even in absence of Gas2l1 (60% at more than 2um).

Response:

In the assays of Figures 2D and 8C (Figures 2D and 7C of last submitted manuscript), the Eg5 inhibitor (i.e., S-trityl-L-cysteine, STLC) was added at 6 h post-release of aphidicolin treatment, and at this time point cells were at the S/G2 boundary of the cell cycle. It is known that Eg5 becomes functional at mitotic entry and functions afterwards in the poleward movement of the centrosomes (reviewed in Agircan et al. 2014, Philos Trans R Soc Lond B Biol Sci., PMID: 25047615). Therefore, STLC was added at a much earlier time before Eg5 becomes functional.

It has been shown in several studies that Eg5 inhibition does not affect centrosome disjunction before mitotic entry (Mardin et al., Nat Cell Biol. 2010, PMID: 21076410; Mardin et al., Curr Biol. 2011, PMID: 21723128; Chen et al., J Cell Sci. 2015, PMID: 26220856). Indeed, our data presented in Figure 2D (we believe that it should be Figure 2D instead of Figure 2C) are consistent with those in the previous reports (Mardin et al., Curr Biol. 2011, PMID: 21723128; Chen et al., J Cell Sci. 2015, PMID: 26220856). In addition, we found that a significant population (it should be ~40% instead of 60%, Figure 2D) of STLC-treated *gas2l1*-knockout cells showed centrosome disjunction (inter-centrosome distance > 2 um). As mentioned above, it is likely that in the absence of GAS2L1, centrosome disjunction occurred through other mechanisms, such as those mediated by Kif15 (kinesin-12) and dynein (Tanenbaum et al., Curr Biol. 2009, PMID: 19818618; Raaijmakers et al., EMBO J. 2012, PMID: 23034402).

February 19, 2020

RE: JCB Manuscript #201909094R

Prof. Robert Z Qi
The Hong Kong University of Science and Technology
Division of Life Science The Hong Kong University of Science and Technology Clear Water Bay New Territories
Hong Kong N.A.
Hong Kong

Dear Prof. Qi:

Thank you for submitting your revised manuscript entitled "Nek2-Mediated GAS2L1 Phosphorylation and Centrosome-Linker Disassembly Induce Centrosome Disjunction". The paper has now been assessed by two of the original reviewers (reviewer#1 was not able to re-review the manuscript) and, as you'll see, they both support acceptance of the paper and so we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

**Please be sure to address the remaining concerns voiced by reviewer #3 - you should be able to address these issues with changes to the text and/or to the figure arrangement. Please also attend to the two additional issues:
1. Reviewer 1's comment (in the first round of review) on insufficient description of methodology for measuring angles within the spindle is important. The change in the tilt of metaphase plate reported in the revised manuscript is approximately 3 degrees, which means that for a typical 8-um ling metaphase plate positions of the outwards tips of the drawn line change by a mere 200 nm (sin(3)*8000/2). Reliable detection of such a small movement requires precise criteria that must be described more thoroughly in Materials and Methods.

2. Abnormalities in the chromatin pattern illustrated in the new Fig.3 are very difficult to see due to the color scheme used in the figure. In addition, it appears that the arrow does not point to anything. I had to convert original images into grayscale to see chromatin bridges described in the text. There are multiple ways to improve presentation of these panels. For example, red microtubules can be combined with grayscale chromosomes (this will make presentation of just the DNA channel unnecessary). Alternatively, the DNA channel can be shown in either lighter blue or grayscale beneath the color composite images (as in the current layout). It is imperative here to make the chromatin bridges visible.

Please be sure to provide a point-by-point rebuttal to these remaining issues along with your final revised manuscript.**

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

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2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

5) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

6) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names

of journals according to PubMed.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. At the moment, you are below this limit but please bear it in mind when revising.
Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

9) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

10) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Alexey Khodjakov, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

Reviewer #2 (Comments to the Authors (Required)):

The authors have addressed my comments and the comments of the other reviewers, I therefore fully support publication of this very nice study.

Reviewer #3 (Comments to the Authors (Required)):

The re-submitted manuscript by Au et al. has been substantially improved by the authors. However, I still have concerns on the methods used for some of the quantifications. The methods are insufficiently described at best.
For example, it is not clear how were drawn the lines between the two centrosomes and on the metaphase plate (Figure 4) and whether it is an unbiased drawing. These lines are used to analyse the angles between the axis of the spindle poles and the metaphase plates and are therefore important for the conclusions. Similarly, on the time lapse analysis, the centrosomes appear overexposed and it is difficult to know when they do separate. Could the authors explain the method used to determine the separation and provide less overexposed images?