EMBO reports

PLK1 REGULATES CENTROSOME MIGRATION AND SPINDLE DYNAMICS IN MALE MOUSE MEIOSIS

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1st Editorial Decision 20th Jul 2020

Dear Dr. Gómez Lencero,

Thank you for submitting your manuscript entitled 'PLK1 regulates centrosome migration and spindle dynamics in male mouse meiosis' to EMBO Reports. We have now received three referee reports, which are included below.

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

We concur with the referees that the proposed role of Plk1 in centrosome maturation and the formation of the bipolar spindle during male mouse meiosis is in principle very interesting. However, the referees raise significant concerns that preclude publication in this journal. Referees point out a number of concerns regarding the conclusiveness of the dataset and the methodologies used. In particular, all referees are concerned about the unusually high concentration of BI-2536 used in the study and its possible off-target effects, and do not recommend publication of the manuscript. Given these comments from recognized experts in the field that are also experienced reviewers, and considering the amount of work required to address them, we cannot offer to publish your manuscript.

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

Yours sincerely,

Deniz Senyilmaz Tiebe

Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

This manuscript by Alfaro et al describes the separation of centrosomes during meiosis in murine gametogenesis, and then assesses the functional roles of Plk1 in this process. The models for Plk1 loss of function include BI-2536 at 100 uM of cultured spermatocytes, and Cre-mediated conditional knockout on gonad spermatogenesis of cKO mice. These models concordantly show that inhibition/loss of Plk1 cause monopolar meiotic spindles, which is consistent with the well-established effect of Plk1 inhibition in mitosis. More surprisingly the knockout cells lack pericentrin in 15% of the cells.
Many of the findings here are new, importantly demonstrate key functional roles of Plk1 in meiosis, and delineate the process of centrosome separation during the phases of meiosis. The finding of monopolar spindles is not surprising since that is seen also in mitosis-though it is new. The finding of PCNT loss in 15% of cells in the conditional knockout system is surprising, but needs to be better validated to rule out artifacts-at a minimum centriole proteins should be identified when trying to demonstrate that PCNT is missing so the proper location where it should be found is identified. However, there are some other major issues with the models (see #2 below), and lacking

quantitation (3). Although I recognize the efforts, these issues lead me to have very serious reservations about this manuscript.

Major:
(1) The concentration of BI-2536 is 1000x too high-100 uM instead of the typical 100 nM. I'm not opposed to this in principal but it risks off-target effects. The bar isn't that high to convince me that monopolar spindles are from inhibition of Plk1-that is the expected mitotic effect, and it is seen with knockout. However, any other phenotypes with BI-2536 could be off-target.
a. The main claim based on 100 uM BI-2536 is monopolar spindles, this is fine. This is not suprising. Yet, it might be considered showing the knockout data first or presenting the inhibitor/ knockout data together rather than trying to convince the reader that 1000x concentration won't cause off-target effects.
b. The effect on AurTph is much harder to accept as 1000x levels could have inhibited any mitotic kinase. Further, you can see residual AurTph in the knockout condition, as expected if this were off-target-although apparently reduced in the one cell shown, it is not quantified and we cannot make conclusions on that single cell. AurA is known to autophosphorylate, so a much simpler/better explanation is that 1000X BI-2536 inhibits AurKa to a degree.
(2) The validation with MLF1P is problematic at multiple levels:
a. Although validation of Plk1 inhibition is great, this doesn't address the issue of off-target effects which is the major risk of 1000x known inhibitor.
b. Naming-the standard gene name is CENPU and this one should be used. PBIP1 is the alternate name used commonly in the cited literature. MLF1P is not an alternate name-it should be MLF1IP - MLF1 interacting protein. However, I see little value in using this unusual name when the existing literature use the other two names.
c. Most important, in cite [52], inhibition or knockdown increases PBIP1 KT recruitment to the KT. Here, inhibition/knockout decreases PBIP1 KT recruitment. So this validation seems to have inadvertently called all the models into question. I'm at a loss to explain this result, but it is seriously concerning.
(3) Some data is not presented or lacking:
a. Line 228: "excision of the target exon...was confirmed by PCR." No data is shown. Additionally, it would be important to know what fraction of cells had knockout at the timepoint selected. This could be inferred indirectly from WB or southern blot, or perhaps Plk1 IF (with careful validation b/c levels of Plk1 can change markedly in mitosis-and presumably meiosis-compared with interphase).
b. There is no apparent quantitation of data displayed in Figures 1-4, and Figure 8, or information about replicates. No controls are displayed for Figure 6. This makes it challenging to make generalized conclusions.
(4) The PLK1 knockout causes 15% of cells to lose PCNT and is said to respresent "unequivocal absence of pericentrin signals." This seems to strong based on limited data, and should be bolstered with controls and experiments that provide more detail.
a. In mitosis in Hela cells, BI-2536 does not cause PCNT loss in mitotic cells (Figure 2E of cite 13--Lee and Rhee JCB 195:1093, 2011)-I think this is miscited ; In fact the follow up paper by this group (Lee and Rhee Nat Communications 6:10076, 2015-not cited her) shows that Plk1 phosphorylation of PCNT licenses its removal after mitotic exit. If this latter mechanism were to be relevant to meiosis, then inhibition of Plk1 should increase, not decrease PCNT. It is of course possible that a different mechanism is at play here, but additional validation would be required.
i. Show the retention of other centrosome proteins so that PCNT loss is verified at a known centrosome structure.
ii. Evaluation of cell-by cell Plk1 loss to determine if it is heterogenous and if the 15% of cells without PCNT.
iii. Deeper analysis of mechanisms underlying Plk1 regulation of PCNT-are the same

phosphorylation events described in the Lee and Rhee papers?
b. Discussion lines 340-343 say "In these cells, the inhibition of PLK1 with BI2536 causes the reduction of Pericentrin levels, although the effect is attenuated if the cells were already in mitosis [13]." This doesn't reflect the contents of cite 13 well-that paper showed that BI2536 affected phospho-pericentrin, but not total pericentrin, and did so only with monastrol pretreatment (Figure 2E). So there was no apparent measure of attenuation if cells were already in mitosis. Unless I'm mistaken, the results here for meiosis are very different than what is reported previously for mitosis and the discussion should clear about these differences, assuming they are validated as in (a) above.
Minor:
1. As a minor point, it is great that the manuscript is very up front about using high BI-2536 concentrations but the actual amount 100 uM, 1000x higher than typically used in human cell culture, is buried in methods. It should be in the text or legend or both.
2. Selection of the pS137 antibody to detect Plk1 is unusual, and may not be the best choice. This is not known to be a critically important phosphorylation, and may not be phosphorylated in all Plk1. A total Plk1 antibody would have been a better choice.
3. The figures were had to follow-they are not numbered so I had to count and Figures 2 and 3 were similar in appearance and adjacent making counting harder.
4. Line 230-says minimal/no apoptosis (Fig EV3C)-the better example seems to be EV3B.

Referee #2:

By staining proteins of the PCM, the authors describe in detail the centrosome cycle during male meiosis. They furthermore describe that high concentrations of the Plk1 inhibitor BI2536 result in disruption of centrosome separation in prophase I (although possibly not centrosome splitting, i.e. severing of the intercentrosomal linker). Centrosome maturation does not seem to be affected. This in turn results in failure to establish a bipolar spindle. Similar results are observed upon abrogation of Plk1 expression in genetically engineered mice. In this case a stronger effect on centrosome maturation seems to be observed.

The results and conclusions, although not surprising in view of what is known about Plk1 function in different cell types and organisms, are strong and result from well-done experiments. Overall the manuscript contributes to the better understanding of the different roles of the polo kinase in specialized cell types.

I would suggest completing the study with some new immunolabelings, with the aim of better documenting centriole duplication during meiosis and clarifying whether Plk1 activity is necessary for centrosome maturation in spermatocytes.

Figure 1& 2
- Staining for pericentrin, the authors show how the centrosomes (or more accurately, the PCM) behave during the different phases of meiosis. This is supported with figure EV1, detecting γ-tubulin. Centrosomes seem to be mature but unseparated in early prophase I, being detected as a single pericentrin/γ-tubulin spot -that one assumes contains 4 centrioles. This is in contrast to meiosis II or to what happens in other cell types that split centrosomes in G2.
The data would be stronger if it included immunolabeling of centrin or another centriolar marker. I would suggest doing this additional experiment. This would also document when, in between meiosis I and II, centriole duplication occurs, as discussed in the manuscript.

- The two centrosomes seem to be independently visible around diplotene. Is this always the case or there is some variance regarding the timing of centrosome splitting? are centrosomes detached from the nucleus at this phase as the figure seems to imply? Do they separate significatively before prometaphase in at least some cells?
- The authors could speculate on how the duplicated centrosomes are maintained together without c-Nap1. Are other components of the intercentrosomal linker such as rootletin present in spermatocytes?
- The PCM or at least pericentrin seems to fragment in late meiosis I. Is this always so, maybe suggesting functional importance of the process? does it always correlate with telophase?

Figure 3
- Why is total Plk1 localization not reported? Also explain why a specific phosphorylated form of the kinase (Plk1 S137P) is labeled and to what this form corresponds (active kinase, I presume). If the authors wanted to detect active kinase, why didn't they stain with the more commonly used anti-Plk1 Ser210P antibody?
- "After confirming the localisation of PLK1 in mouse spermatocytes" is not correct, should be "confirming the localization of active Plk1".

Figure 4 & 5
This is in my opinion the weakest part of the manuscript, although the use of genetically engineered mice later on supports its main conclusions (while at the same time rendering this part a bit redundant).
- The IC50 of BI2536 is ~1nM. Elaborate a bit more on how to explain the need to use much higher concentrations of the drug (100 uM). Would the effects be specific? even at 10 uM BI is able to inhibit a significant number of kinases (see for example http://www.kinase-screen.mrc.ac.uk/screening-compounds/341035)
- Speculate on why BI2536/Plk1 inhibition does not result in centrosome maturation failure in meiosis I (in contrast to mitosis).
- And on why it does result in separation failure after nuclear envelope breakdown. At this point, different independent pathways would normally collaborate to separate the centrosomes but one expects that these would fail as a result of an abnormal maturation of the centrosome and thus its inability to nucleate enough MTs.
- Does BI2536 affect centrosome maturation and separation in early meiosis II?
- The section title "PLK1 inhibition impairs the formation of bipolar spindles I and II" should be changed to "BI2536 impairs the formation of bipolar spindles I and II".

Figure 6&7
- How do the results relate to these of the previous figures? especially regarding the observation that lack of Plk1 has a severe effect on PCM structure/centrosome maturation.
- 7C represents both meiosis I and II, why the first and second meiotic divisions had not been quantified separately?
- Based on their data with BI, the authors state that "the absence of PLK1 kinase activity does not affect γ-Tubulin location during prophase I in male mouse meiosis.". Is this confirmed in the conditional KO model? Plk1 is central to centrosome maturation in a variety of cell types. Data should be provided in this regard (γ-tubulin staining and quantification, pericentrin quantification - and, ideally, MT (α or β-tubulin) quantification).

Figure 8
- Describe with more detail the antibody used. I can't seem to find its description in the text.
- Why has the pattern of total Aurora A not been studied? note that changes in the pattern of the

phosphorylated kinase could be the result of changes of the localization of the total Aurora pool, of its phosphorylation state, or both.
- As the antibody used an anti- pan-aurora-P do not state that the results correspond to one type of aurora kinase (i.e. "PLK1 inhibition alters Aurora A phosphorylation in male mouse meiosis" should be changed to something along the lines of "(...) alters the pattern of Aurora kinase phosphorylation in male mouse meiosis").

Minor points
- The part of the introduction dealing with centrosomes and their cycle could use more work.
- I would suggest changing "S137ph" and "AuroraTph" to the more common "S137P" and "AuroraTP" (or to simplify, "Aurora P").

Referee #3:

This manuscript addresses PLK1 kinase functions in mouse spermatocytes. PLK1 is interesting to pursue in male meiosis, since it is understudied compared to its established roles in mitosis and female meiosis. The authors combined genetic manipulation and pharmaceutical perturbation of PLK1 to show that PLK1 is critical for the integrity of pericentriolar material (PCM), an integral centrosome component linking centrioles to microtubule nucleators. PLK1 inhibition also had deleterious effects on bipolar spindle formation in both meiosis I and II. The manuscript provides limited insights, however, beyond these initial observations, and this reviewer is also concerned about the different results obtained from PLK1 chemical inhibition and conditional knockout mice. The data shows that PLK1 chemical inhibition has bigger impacts on apoptotic spermatocyte rates, monopolar spindle rates, and phospho-Aurora signals compared to PLK1 conditional knockout. They discuss that "in vitro analyses are usually more aggressive than the genetic models" (line 426). However, in contrast, PLK1 knockout showed stronger effects on PCM integrity compared to chemical inhibition: lack of Pericentrin in knockout vs slight deformation of Pericentrin signals in chemical inhibition. Therefore, it cannot be simply explained by the aggressiveness of the perturbation. This raises a question regarding the specificity of BI 2536 in the condition used in this study (100 uM in 10% DMSO for 8h). Off-targeting of BI 2536 to other PLK (e.g., PLK4) or Aurora kinases, which regulate similar pathways, may explain the different results between BI 2536 treatment and knockout experiments. This could also explain why g-tubulin stayed intact in BI 2536 treatment, while it was absent in the PLK1 conditional knockout mice used in the back-to-back paper. To resolve these contradictions, the authors should validate the specificity of the BI 2536 treatment in their system or focus on the genetic model to avoid confusions.
The authors claim in the abstract that they studied both PLK1 and Aurora A, but the data for Aurora A is very limited in this manuscript. The only data relevant to Aurora A is the immunofluorescence analysis of phospho-Aurora kinases (staining all Aurora kinases: A, B, and C) upon PLK1 inhibition in figure 8. Further, it is difficult to interpret this data, because there are no quantification of signal intensities and no grey scale images just showing the phospho-Aurora signals. I suggest to stain just for Aurora A in PLK1 knockout spermatocytes, which would tell if PLK1 regulates phosphorylation of Aurora A and/or its localization at centrosomes. Also, directly perturbing Aurora A will provide further insights into the contribution of Aurora A on centrosome maturation and bipolar spindle formation in spermatocytes.
Some additional mechanistic insight into the roles of PLK1 in centrosome maturation and migration would make the paper stronger. For example, is Eg5 kinesin downstream effector of PLK1 to establish the bipolar spindle? This was proposed as a future direction in the Discussion section, but I think it would be nice to be included in the current study.

Additional comments:
-Plant cells also don't have centrioles (line 32).
-Correct embryogenesis to oogenesis (line 69).
-Explain what is known about PLK1-S137ph (line 131).
-Terms kinetochoric MTs and metaphasic plate are uncommon in the field. Change them to kinetochore MTs and metaphase plate.

Dear Editor, Dr. Senyilmaz

Thank you very much for the work put into the manuscript by the three reviewers and yourself, it is helpful for us.

Although I assume from your reply that our publication is no longer considered for publication, for our peace of mind, I would like to address the main concern raised by the reviewers: concentration of BI.

The methodology that we used for culturing spermatocytes is different to the one used for other cell lines/tissues. By using a slightly modified protocol of Sato et al 2011 (Ref#100 of the manuscript), fragments of seminiferous tubules are placed over an agarose gel (half-soaked) in MEMα culture medium (in controls) of medium with BI (in treated samples). After extracting the testis, the fragments of the seminiferous tubules need to be cut transversally to keep the tissue as similar to the original status as possible. In addition, the fragments are not immersed in the media, but deposited over the agarose gel absorbing the media from the bottom. This means that the material used is not a suspension of cells, but an entire fragment of the original tissue. The media does not surround the cells, but instead, the seminiferous tubules absorb the media via an organotypic culture. In our experience, the spermatocytes need the intact tissue (seminiferous epithelium with Sertoli cells) to remain in good conditions. This protocol was also used in Gomez et al 2016 (EMBO Reports). Over the years, we have done several trials with suspension of spermatocytes (extracted from disaggregated seminiferous tubules), but the morphology of the cultured cells under this approach show significant aberrations after only 2 hours of control cultures. As you can deduct out of our previous publications, we are very cautions with the chromosome structure so we adequate our experiments in order to keep the three-dimensional structures of the cells in its original appearance. This is why we approach our studies by the protocol stablished by Sato et al., assuming that spermatocytes need the entire tissue with Sertoli and seminiferous epithelia to resemble the original appearance. This implies that concentration of inhibitors need increase, however, the final concentration that reaches the cells is reduced by the effect over the entire tissue and the metabolism of the Sertoli Cells, and the absorption by the agarose gel (that is 99% water). When approaching the treatment for this specific manuscript, we were also aware of the high concentration used for BI, however, lower concentrations gave no effects at all with this methodology, obtaining samples identical to controls (IF images can be provided if requires). With the high concentration of the drug, the percentage of monopolar spindles observed after the BI treatment in cultures spermatocytes is similar to the percentage obtained in the genetic model used in this work. In addition,

levels of apoptosis are not high after the 8h BI treatment, indicating that the high doses are not lethal for spermatocytes under this culturing conditions.

As a scientist, I felt that this explanation was needed, as our group is responsible for the methodologies used and we would like to avoid any misunderstandings or interpretations of a misuse of proper procedures. We are very sorry if this explanation was not properly transmitted in the text, and we assume our failure in this.

I also want to express our concerns about the future of this work. As you could read in this publication, we agreed with Dr. Phil Jordan for a co-submission of our manuscripts, as we recently realized (after attending to a conference last February 2020) that we were planning of submitting complementary results. I know from Dr. Jordan that his publication received favorable comments yesterday. I am honestly happy for him, and wish him the best for the publication of his work. However, it deeply worries me as, once his work is published, ours has a significantly reduced impact. In this sense, would it be possible that the journal might reconsider our manuscript after the explanation of the methodologies used? We will be happy addressing any other suggestion that the referees have. We will understand any decision.

Best regards and looking forwards to hearing from you soon.

Rocio Gomez

2nd Editorial Decision 23rd Jul 2020

Dear Dr. Gómez,

Thank you for contacting us regarding the recent decision taken on your manuscript and the clarification that the experimental set-up used necessitates usage of the drug in different concentrations. I have now read your points carefully and discussed the study and the reports with our Head of Publications Dr. Bernd Pulverer.

Should you be willing to address all concerns of the referees, we would be open to re-consider a revised manuscript. We would expect the revised manuscript to be submitted in 4 months, and this timeframe should be considered when deciding whether it is realistic to resubmit. However, at this time we cannot promise that we can publish the study, given the fact that the revisions would have to include substantive experimentation to be compelling, and we can only move forward if the referees are convinced by the revision. In line with this, please note that the next decision will be the final one. We are of course aware of the co-submitted manuscript, therefore we'd clearly understand if you wanted to publish without delay elsewhere. In fact, this was the reason for our previous decision.

Kind regards,

Deniz Senyilmaz Tiebe

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Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Point-by-point response for EMBOR-2020-51030V2

- Please, be aware that the numbers referred in the details below correspond to the pages and lines of the new version submitted. To address the referee´s suggestions, we have changed the order of the results, and included new Figures. For a better understanding of the changes, a list of Figures of the revised version and the correspondent original figures is listed below.
- Please, be also aware that the references of the new version are updated with the EMBO Report style (recommendation effective since 1 July 2020, after first submission).

Revised version Figures:

Figure 1: Distribution of PCNT and SYCP3 during the first meiotic division. *This figure is equal in content to the original submission but has smaller number of columns to save space.*

Figure 2: Distribution of PCNT and SYCP3 during interkinesis and the second meiotic division. *This figure is equal to the original submission.*

Figure 3: Distribution of CETN3 and SYCP3 during spermatogenesis. *New figure upon referee´s request.*

Figure 4: PLK1S137P distribution during male mouse meiosis. *This figure is equal to Fig. 3 in the original submission.*

Figures 5: Triple immunolabelling of PCM, CETN3 and SYCP3 during prophase I in control, *Plk1*(Δ/Δ) and BI2536-treated spermatocytes. This is a *revised version of previous Fig. 6, including graphs from previous Fig. 7. New data of Centrin-3 is included.*

Figure 6: Immunolabelling of γ-Tubulin and SYCP3 during prophase I in control, *Plk1*(Δ/Δ) and BI2536-treated spermatocytes; Immunolabelling of γ-Tubulin CETN3 in control and *Plk1*(Δ/Δ) spermatocytes. *This is a revised version of previous Fig. 4 that includes γ-Tubulin immunolabelling and quantification, upon referee´s request.*

Figure 7: Meiotic spindles I and II in control, *Plk1*(Δ/Δ), BI2536-treated and MLN8237-treated spermatocytes. *This is a revised merged version of previous Fig. 5 and graphs from previous Fig. 7, with the addition of new data from* MLN8237 experiments*.*

Figure 8: AURK distribution in control, *PLK1(Δ/Δ),* BI2536-treated and MLN8237-treated spermatocytes. *This is a revised version of previous Fig. 8 including new quantification analysis and new data from* MLN8237*.*

Figure 9: Distribution of CEP250, CETN3 and SYCP3 in control and *Plk1*(Δ/Δ) spermatocytes; Distribution of KIF11 and SYCP3 in control, *Plk1*(Δ/Δ) and BI2536-treated spermatocytes. *New figure upon referee´s request.*

***EV1*: Distribution of γ-Tubulin and SYCP3 during male mouse meiosis.** *This figure is equal to the original submission, plus the addition of an image of a metaphase II.*

EV2: Distribution of PLK1S137P and PLK1T210P. *This a revised version of the previous EV2 with the addition of PLK1T210P data, upon referee´s request.*

EV3: Gene strategy and PCR analysis and for *Plk1*(Δ/Δ) mouse model. *New figure upon referee´s request***.** *Histological section of Plk1(Δ/Δ) testis from previous Fig EV3C are now shown in this figure. In addition, liver histological section and testis cryosection have been included. Quantification of PLK1 at centrosomes in control and Plk1(Δ/Δ) metaphases is also included.*

EV4: Optimization of *in vitro* studies for control, BI2536 and MLN8237 organotypic cultures of seminiferous tubules. *This a revised merged version of the previous EV3 and EV4 with the addition of MLN8237 and all quantitative data requested. This figure presents the experimental design of the analysis of the use of BI2638-treated and MLN8237-treated cultured seminiferous tubules. Analysis of the optimal treatment duration, concentration, and level of apoptosis in each experimental condition are displayed.*

EV5: Distribution of CENP-UP in control metaphases I and monopolar spindles of *Plk1*(Δ/Δ), BI2536-treated and MLN8237-treated spermatocytes. *This a revised version of the previous EV5, including data from Plk1*(Δ/Δ) and MLN8237-treated spermatocytes.

EV6: Table of primary and secondary antibodies used in this study. *This figure is equal to EV6 in the original submission.*

Suppl. Video 1*:* Three-dimensional reconstruction of WT bipolar spindle I.
Suppl. Video 2: Three-dimensional reconstruction of *Plk1(Δ/Δ)* monopolar spindle I.

Response to the reviewers

- Please notice that all referee´s comments are copied in blue. Author´s responses are in black.

Response to the Referee #1

This manuscript by Alfaro et al describes the separation of centrosomes during meiosis in murine gametogenesis, and then assesses the functional roles of Plk1 in this process. The models for Plk1 loss of function include BI-2536 at 100 uM of cultured spermatocytes, and Cre-mediated conditional knockout on gonad spermatogenesis of cKO mice. These models concordantly show that inhibition/loss of Plk1 cause monopolar meiotic spindles, which is consistent with the well-established effect of Plk1 inhibition in mitosis. More surprisingly the knockout cells lack pericentrin in 15% of the cells.

Many of the findings here are new, importantly demonstrate key functional roles of Plk1 in meiosis, and delineate the process of centrosome separation during the phases of meiosis. The finding of monopolar spindles is not surprising since that is seen also in mitosis-though it is new. The finding of PCNT loss in 15% of cells in the conditional knockout system is surprising, but needs to be better validated to rule out artifacts-at a minimum centriole proteins should be identified when trying to demonstrate that PCNT is missing so the proper location where it should be found is identified. However, there are some other major issues with the models (see #2 below), and lacking quantitation (3). Although I recognize the efforts, these issues lead me to have very serious reservations about this manuscript.

Author response: We are very grateful to referee #1 for sharing his/her expertise and offering positive comments on our work. We respond to all concerns below.

Major:

The concentration of BI-2536 is 1000x too high-100 uM instead of the typical 100 nM. I'm not opposed to this in principal but it risks off-target effects. The bar isn't that high to convince me that monopolar spindles are from inhibition of Plk1-that is the expected mitotic effect, and it is seen with knockout. However, any other phenotypes with BI-2536 could be off-target.

We previously explained to the editor why we used this concentration but are happy to offer a detail explanation in this response.

When approaching our study, we were also aware of the atypical high concentration used for BI. The methodology that we use for culturing spermatocytes is different to the one generally used for other

cell lines. By using a slightly modified protocol of Sato et al 2011 (Sato et al., 2011, *Nat Commun* 2: 1-7), fragments of seminiferous tubules are placed over an agarose gel (half-soaked) in MEMα culture medium (in controls) or medium with BI (in treated samples). After extracting the testis, the fragments of the seminiferous tubules need to be cut transversally to keep the tissue as similar to the original status as possible. In addition, the fragments are not immersed in the media, but deposited over the agarose gel absorbing the media from the bottom. This means that the material used is not a suspension of cells, but an entire fragment of the original tissue. The media does not surround the cells, but instead, the seminiferous tubules absorb the media via an organotypic culture. In our experience, the spermatocytes need the intact tissue (seminiferous epithelium with Sertoli cells) to remain in good conditions. This protocol was also used in Gómez *et al.,* 2016 (EMBO Rep. 2016 May; 17(5): 695–707). Over the years, we have done several trials with suspension of spermatocytes (extracted from disaggregated seminiferous tubules), but the morphology of the cultured cells under this approach show significant aberrations after only 2 hours of control cultures. As you can deduct out of our previous publications, we are very cautious with the chromosome structure so we adequate our experiments in order to keep the three-dimensional structures of the cells in its original appearance. Therefore, we approach our studies by the protocol stablished by Sato *et al*., 2011 (Nat Commun. 2011 Sep 13;2:472) assuming that spermatocytes need the entire tissue with Sertoli and seminiferous epithelia to resemble the original appearance. This implies that concentration of inhibitors need to increase, however, the final concentration that reaches the cells is reduced by the effect over the entire tissue and the metabolism of the Sertoli Cells, and the absorption by the agarose gel (that is 99% water). Nevertheless, discussion about this methodological approach in now included (page 15-16 lines 454-466) and potential 1000X BI-2536 off-targets is now included in the discussion (page 19 lines 583-584). Explanation of the methodology is also included in page 21 of material and methods.

Our data shows that using this high concentration the percentage of monopolar spindles observed after the BI treatment in cultures spermatocytes is similar to the percentage obtained in the genetic model used in this work. In addition, levels of apoptosis are not high after the 8h BI treatment, indicating that the high doses are not lethal for spermatocytes under this culturing conditions. Nevertheless, we now offer data that support our results. The revised version includes EV4, which offers all data of experimental design and optimization of the protocol for time and concentration, level of apoptosis and quantification of monopolar spindles in 1 µM, 10 µM and 100µM. We opted for analyzing the highest concentration of BI2536 since it offers a stronger phenotype: higher concentration shows higher incidence of monopolar spindles (without reaching lethal concentrations/high incidences of Caspase-3 positive apoptotic cells). This figure also includes the optimization of the experiments using MLN8237.

In our opinion, the main concern of whether BI2536 has off targets cannot be fully explained, since PLK1 and AURKA kinases share the same cascade of events in the regulation of centrosome, leading to the alteration of phosphorylated AURKA both in depletion -*Plk1*(Δ/Δ)- or inhibition -BI2536- of PLK1, and in MNL8237-treated spermatocytes. Quantification of these data is also now included in Figure 8, see below response point b). However, differences results are obtained with BI2536 and MN8237.

a. The main claim based on 100 uM BI-2536 is monopolar spindles, this is fine. This is not surprising. Yet, it might be considered showing the knockout data first or presenting the inhibitor/ knockout data together rather than trying to convince the reader that 1000x concentration won't cause off-target effects.

Following the referee suggestion, we have reorganized the order of the figures and results. The current revised version of the manuscript presents the study of the centrosome dynamics in wildtype male mouse (Fig 1-4), then the study of the genetic model *Plk1*(Δ/Δ) and finally the analysis of the chemical inhibition of PLK1 in cultured spermatocytes with BI2536 (Fig. 5-9). As mentioned, new version also includes the analysis of MLN8237- treated cultures.

b. The effect on AurTph is much harder to accept as 1000x levels could have inhibited any mitotic kinase. Further, you can see residual AurTph in the knockout condition, as expected if this were off-target-although

apparently reduced in the one cell shown, it is not quantified and we cannot make conclusions on that single cell. AurA is known to autophosphorylate, so a much simpler/better explanation is that 1000X BI-2536 inhibits AurKa to a degree.

Please note that in all figures AurorTph is now called AURKP, as suggested by referee#2.
Quantification of AURKP levels at centrosomes in all experimental conditions (control, *Plk1*(Δ/Δ), BI2536 and MLN8237) is now displayed in Fig 8.
To present clearer images, we have changed the image of the *Plk1*(Δ/Δ) monopolar spindle I, because we agree with the reviewer that this particular image could be confusing (as one of the bivalents was placed very near to the centrosome in the Z projects and this might be misinterpreted). We apologize for presenting an unclear image. The new image shows the significative reduction of AURKP at centrosomes in *Plk1*(Δ/Δ) and BI2536-treated spermatocytes. Nevertheless, discussion about the potential 1000X BI-2536 inhibiting AURKA to a degree is now included in the discussion (page 19 lines 583-584).

(2) The validation with MLF1P is problematic at multiple levels:

a. Although validation of Plk1 inhibition is great, this doesn't address the issue of off-target effects which is the major risk of 1000x known inhibitor.

The objective of testing phosphorylated CENP-U (CENP-UP/MLF1P) was the validation of PLK1 inhibition at centrosomes. Complementary data about this protein is now offered also under AURKA inhibition in Figure EV4 (previous Figure EV4). Our data shows that centrosomal signal of CENP-UP is lost in BI2536 and *Plk1*(Δ/Δ), although it persists in AURKA inhibition with MLN8237. This is only one validation, however, we are with the reviewer that this particular approach does not fully address the issue of off-target effects with BI2536. Nevertheless, the phenotype showed in the genetic model *Plk1*(Δ/Δ) is consistent with BI2536 results: induction of monopolar spindles is equal in BI2536 and *Plk1*(Δ/Δ), and the explanation of the different results of centrosome maturation in early prophase I are due to the different timings of both experiments. This suggest that *Plk1*(Δ/Δ) and BI2536 results could be correlated.

b. Naming-the standard gene name is CENPU and this one should be used. PBIP1 is the alternate name used commonly in the cited literature. MLF1P is not an alternate name-it should be MLF1IP - MLF1 interacting protein. However, I see little value in using this unusual name when the existing literature use the other two names.

The manuscript now referrers to CENP-U in all the text and figures. We thank the reviewer for this suggestion and apologize for using an incorrect alternative name.

c. Most important, in cite [52], inhibition or knockdown increases PBIP1 KT recruitment to the KT. Here, inhibition/knockout decreases PBIP1 KT recruitment. So this validation seems to have inadvertently called all the models into question. I'm at a loss to explain this result, but it is seriously concerning.

The antibody used for the detection of CENP-UP shows a very light signal at kinetochores in the control spermatocytes whereas the signal at centrosomes is strongly detected (see EV4A). We have focused on this centrosomal signal to assess the validation of *Plk1* inhibition. In addition, data about the presence of CENP-UP in MLN8237 is presented in EV4D.
We would like to inform that we are currently working on the role of PLK1 at centromeres, so data referring to KT will be addressed in another manuscript. For this reason, discussion about centromeres has been avoided in this manuscript. We have preliminary results which indicate that PLK1 plays an important role at centrosomes. This might explain why CENP-UP at centrosomes is altered in *Plk1*(Δ/Δ) and BI2536, but persists in MLN8237.

(3) Some data is not presented or lacking:

a. Line 228: "excision of the target exon...was confirmed by PCR." No data is shown. Additionally, it would be important to know what fraction of cells had knockout at the timepoint selected. This could be inferred indirectly from WB or southern blot, or perhaps Plk1 IF (with careful validation b/c levels of Plk1 can change markedly in mitosis-and presumably meiosis-compared with interphase).

We had no intention in missing important data in our manuscript. The *Plk1*-deficient mice was generated previously at Dr. Malumbre´s lab [53,54], therefore we didn't include the data about the excision of the target exon because it has been already published. It is already reported that these mice reduce 86% *Plk1* levels in testis [Wachovicz *et al.* 2016].

However, as requested, gene strategy from Wachovicz *et al.* 2016 is now displayed in Fig EV3 and cited in the text. PCR results of *Plk1*(Δ/Δ) are also included in EV3.

In addition, quantification of PLK1 at centrosomes has been done for control and *Plk1*(Δ/Δ). Immunofluorescence signals for PLK1 at centrosomes in *Plk1*(Δ/Δ) metaphases presented levels below the detection threshold, offering significance in quantification analysis in comparison to control metaphases (Fig EV3C).

We believe that unfortunately, the fraction of knockout cells in *Plk1*(Δ/Δ) cannot be inferred indirectly from WB in testis extracts, since It must be noted that testis comprises a mixture of heterogeneous and asynchronous cell populations, both at the seminiferous tubules (i.e. Sertoli cells, spermatocytes and spermatids) and at the testis interstitium (i.e. Leydig cells and fibroblasts), which could present different turnover rates of PLK1 protein levels, especially considering it is a conditional model.

However, trying to further ascertain this request, we also developed a triple immunolabelling of PLK1, CDK5RAP2 and γ-Tubulin on testis cryosections, but unfortunately immunolabelling were not clear enough to depict one to one labelling of PLK1. These images could be sent to the editor is requested.

b. There is no apparent quantitation of data displayed in Figures 1-4, and Figure 8, or information about replicates. No controls are displayed for Figure 6. This makes it challenging to make generalized conclusions.

Fig 1-4 describe the dynamics of centrosome maturation and migration. Images are representative of all spermatocytes in each stage. We have complemented this data with new Fig 3, which describes Centrin-3 signal and infers the timing of duplication of centrioles in meiosis.

Quantification data of previous Fig 7 is now disclosed in the revised manuscript in Figs 5, 6 y 7.

The number of replicates for each experimental condition (WT, *Plk1*(Δ/Δ), BI2536, and MLN8237) are included in the material and methods section (page 23 line 700-704) and supplementary excel with the complete raw quantification data.

(4) The PLK1 knockout causes 15% of cells to lose PCNT and is said to represent "unequivocal absence of pericentrin signals." This seems to strong based on limited data, and should be bolstered with controls and experiments that provide more detail.

Control images are now displayed in Fig 5. The quantification of number of cells that lack PCNT comparing control to *Plk1*(Δ/Δ) is now included. We consider that the absolute absence of zygotenes/ pachytenes/diplotenes lacking PCNT signal in control individuals allows us to suggest that the data obtained in *Plk1*(Δ/Δ) are clear enough to conclude that this is a phenotype result of the knockdown of *Plk1* in this genetic model. Most importantly, we have now studied Centrin-3: new results indicate the absence of Pericentrin in the PCM, as centrioles are positively marked with Centrin-3 in that area.

a. In mitosis in Hela cells, BI-2536 does not cause PCNT loss in mitotic cells (Figure 2E of cite 13--Lee and Rhee JCB 195:1093, 2011)-I think this is miss-cited ; In fact the follow up paper by this group (Lee and Rhee Nat Communications 6:10076, 2015-not cited here) shows that Plk1 phosphorylation of PCNT

licenses its removal after mitotic exit. If this latter mechanism were to be relevant to meiosis, then inhibition of Plk1 should increase, not decrease PCNT. It is of course possible that a different mechanism is at play here, but additional validation would be required.

Kim, Lee and Rhee, Nat Communications 6:10076 (2015) is now cited. We apologize for this absence and the mistaken citation of Lee and Rhee JCB 195:1093 (2011). Extreme length of prophase I in compassion with mitotic prophase are important to consider. Moreover, mammalian male meiosis is a finalist process, as cell enter the first meiotic division, continue through the second and then specialized as mature gametes during spermiogenesis. This points out the differences between mitosis and meiosis. Our BI2536 data does not show a decrease of PCNT at poles in monopolar spindles. This is due to the short time of treatment in relation to the duration of meiosis. Previous work of Griswold (Griswolsd 2016, Physiol Rev 96: 1-17) and Page´s lab (Enguita-Marruedo *et al*, PLoS Genet; e1007439 2019) support this timing of male mouse prophase I. An scheme of meiotic duration could also be visited in Fig EV2 of Viera et al., 2020 (EMBO Rep (2020)21:e49273). We believe that cell observed in metaphase I after the 8h treatment had a preloaded PCM, meaning that PCNT was already at centrosomes before BI2536 treatment. In contrast, *Plk1*(Δ/Δ) show absence of PCNT, but only in early prophase I, after 7 TX days treatment. This indicates that our data of *Plk1*(Δ/Δ) about lack of PCNT at early prophase are due to the entry into meiosis already under the effect of TX. This is not observed in BI2536 as the timing of the treatment is less than 24h.

This line of discussion is now included in page 16 lines 454-466.

i. Show the retention of other centrosome proteins so that PCNT loss is verified at a known centrosome structure.

Immunolabelling of Centrin-3 is now presented in Figures 3 and 5. Centrin-3 positive signal in PCNT negative zygotene and pachytene verify the localization of the centrosome in figure 5 for *Plk1*(Δ/Δ).

ii. Evaluation of cell-by cell Plk1 loss to determine if it is heterogenous and if the 15% of cells without PCNT.

The quantification of number of cells that lack PCNT comparing control to *Plk1*(Δ/Δ) is now included in Fig 5. The total amount of quantified cells is included in que quantification raw data, images shown are representative of the cells in each stage. Trying to further evaluate a cell-by cell PLK1 loss, we also developed a triple immunolabelling of PLK1, CDK5RAP2 and γ-Tubulin on testis cryosections, but unfortunately immunolabelling were not clear enough to depict one to one labelling of PLK1. However, a PLK1 antibody was used for quantification at centrosomes in Fig EV3C, albeit this particular antibody only offers signals at centrosomes at metaphase, but no apparent signal at centrosomes during prophase I (please read minor point 2).

iii. Deeper analysis of mechanisms underlying Plk1 regulation of PCNT-are the same phosphorylation events described in the Lee and Rhee papers?

b. Discussion lines 340-343 say "In these cells, the inhibition of PLK1 with BI2536 causes the reduction of Pericentrin levels, although the effect is attenuated if the cells were already in mitosis [13]." This doesn't reflect the contents of cite 13 well-that paper showed that BI2536 affected phospho-pericentrin, but not total pericentrin, and did so only with monastrol pretreatment (Figure 2E). So there was no apparent measure of attenuation if cells were already in mitosis. Unless I'm mistaken, the results here for meiosis are very different than what is reported previously for mitosis and the discussion should clear about these differences, assuming they are validated as in (a) above.

We agree with the reviewer that Fig 2E of Lee and Rhee presented data for phosphorylated PCNT, and apologize if these data were mis-cited. Following the response in 4a (above), and after including Kim et al., 2015 (Nat Communications 6:10076, 2015), and the careful reading of Lee and Rhee, 2011 (JCB

195:1093) we understand that PCNT (not phosphorylated PCNT) persisted at poles after BI2536 treatment in those mitotic studies. In agreement, we observe PCNT at poles after BI2536 treatment. Both publications are cited in the correspondent parts of the discussion (pag16 lines 462 and page 17 line 523).

Minor:

1. As a minor point, it is great that the manuscript is very up front about using high BI-2536 concentrations but the actual amount 100 uM, 1000x higher than typically used in human cell culture, is buried in methods. It should be in the text or legend or both.

   It is now included in results (page 9 line 228), material and methods (page 21 line 636) and in all the correspondent legend of figures.

2. Selection of the pS137 antibody to detect Plk1 is unusual, and may not be the best choice. This is not known to be a critically important phosphorylation, and may not be phosphorylated in all Plk1. A total Plk1 antibody would have been a better choice.

   We have tried several antibodies against total PLK1 in mouse spermatocytes (Santa Cruz #sc5585 Lot. K1407 and #sc-17783 Lot. A2510, Abcam #Ab1057 Lot. 916830 and #Ab14209 Lot. 153348 Lot. A2510) but unfortunately none of them gave positive immunolabelling during the entire meiotic divisions. The PLK1 antibody used for quantification at centrosomes in Fig EV3C only offers signals at centrosomes at metaphase, but no apparent signal at centrosomes during prophase I. This could be explained after antigen recognition. This antibody (SC Ref# SC-5585) has been kindly given by a colleague, but it is currently discontinued by Santa Cruz. We are sorry that we can't address a distribution of PLK1 during the entire meiosis with none of the other antibodies that we tried. However, as requested by referee·3, immunolabelling of PLK1T210ph is now displayed in Figure EV2 and mentioned in page 7 lines 183-185 of the results. We would like to confirm that we used Plk1S137P antibody because it detects centrosomes in mouse spermatocytes throughout the entire meiotic divisions and because It has been previously described in mouse oocytes (Du et al 2015; Cell Cycle 14: 3566-79.].

3. The figures were had to follow-they are not numbered so I had to count and Figures 2 and 3 were similar in appearance and adjacent making counting harder.

   All figures are numbered in the uploaded version to the EMBO Reports submission online system. We apologize if the previous compiled Pdf of Text+JPEG-figures was difficult to follow for the reviewer.

4. Line 230-says minimal/no apoptosis (Fig EV3C)-the better example seems to be EV3B.

   We thank the reviewer for noticing this erratum. It has been corrected in the new figure configuration.

Response to the Referee #2

By staining proteins of the PCM, the authors describe in detail the centrosome cycle during male meiosis. They furthermore describe that high concentrations of the Plk1 inhibitor BI2536 result in disruption of centrosome separation in prophase I (although possibly not centrosome splitting, i.e. severing of the intercentrosomal linker). Centrosome maturation does not seem to be affected. This in turn results in failure to establish a bipolar spindle. Similar results are observed upon abrogation of Plk1 expression in genetically engineered mice. In this case a stronger effect on centrosome maturation seems to be observed.

The results and conclusions, although not surprising in view of what is known about Plk1 function in different cell types and organisms, are strong and result from well-done experiments. Overall the manuscript contributes to the better understanding of the different roles of the polo kinase in specialized cell types.

I would suggest completing the study with some new immunolabelings, with the aim of better documenting centriole duplication during meiosis and clarifying whether Plk1 activity is necessary for centrosome maturation in spermatocytes.

Author response: We are very grateful to referee #2 positive comments on our work, and appreciate the suggestions, as they have helped to improve our work. We respond to all concerns below.

Figure 1&2

- Staining for pericentrin, the authors show how the centrosomes (or more accurately, the PCM) behave during the different phases of meiosis. This is supported with figure EV1, detecting γ-tubulin. Centrosomes seem to be mature but unseparated in early prophase I, being detected as a single pericentrin/γ-tubulin spot that one assumes contains 4 centrioles. This is in contrast to meiosis II or to what happens in other cell types that split centrosomes in G2.

The data would be stronger if it included immunolabeling of centrin or another centriolar marker. I would suggest doing this additional experiment. This would also document when, in between meiosis I and II, centriole duplication occurs, as discussed in the manuscript.

We have now included data of the distribution of Centrin-3 in mouse spermatocytes in new Fig 3, together with additional data of Centrin-3 in Fig 5 and 6. We would like to explain that these data were not included before as none of the previously tested antibodies against Centrin-1 and 2 (Abcam anti Centrin-1 #ab-11257 and #156858; and Abcam anti Centrin-2 #196554) worked in mouse spermatocytes. To address the referee's requirements, we have now successfully used the Anti Centrin-3 (Abnova H00001070-M01).

Our data suggest that centrosomes duplicate twice during male mouse meiosis: first in the transition from leptotene to zygotene, and second in late interkinesis.

- The two centrosomes seem to be independently visible around diplotene. Is this always the case or there is some variance regarding the timing of centrosome splitting? are centrosomes detached from the nucleus at this phase as the figure seems to imply? Do they separate significatively before prometaphase in at least some cells?

Our data suggest that centrosome duplication occurs in the transition from leptotene to zygotene, and migration starts in diplotene. Late diplotenes can be identified by the chromocentres signals with DAPI and the immunolabelling with SYCP3. At this stage, centrosomes seem to be detached from the nucleus, and continue migrating once that nuclear envelope break down in diakinesis. Visible separation is seen before prometaphase I (at diakinesis), and before prometaphase II (in late prophase II). Representative images are presented in new Figure 3. The disorganization of NE components in this stage is presumably coincident with centrosome separation towards the opposite poles, as the formation of the bipolar spindle takes place in the transition from diakinesis to metaphase I.

- The authors could speculate on how the duplicated centrosomes are maintained together without c-Nap1.

Data of C-NAP1 (protein CEP250) is now included in new Fig 9. This has been achieved after optimization of the fixation protocol (explained in material and methods section, page 22 line 660).

Our results indicate that duplication of centrioles occur in the transition between leptotene to zygotene (new Fig 3). This means that duplication of centrioles does not occur prior to meiosis, differing to mitotic cells where duplication occurs in S phase (Conduit et al,2015, Nat Rev Mol Cell Biol 16: 611-24). CEP250 was retained till diplotene stage in control spermatocytes, and no CEP250 signals were observed at centrosomes from diakinesis onwards. However, CEP250 link is still connecting adjacent non-migrated centrosomes on monopolar spindles under *Plk1* depletion -*Plk1*(Δ/Δ)-, accordingly to mitotic cells without Plk1 activity (Mardin et al., 2011, Curr. Biol. 21, 1145–1151. (10.1016/j.cub.2011.05.047)). This suggests PLK1 has a role in severing CEP250 link between parental centrioles before the onset of centrosome separation in diplotene.

Are other components of the intercentrosomal linker such as rootletin present in spermatocytes?

As previously mentioned, new CEP250 data is now included. We have not tried rootletin in our studies. We are very sorry, but we could not develop this experiment on time during this period of revision,

mainly considering the limitation in budget and laboratory occupation in Covid restrictions. We are planning this for further studies of meiotic centrosomes.

- The PCM or at least pericentrin seems to fragment in late meiosis I. Is this always so, maybe suggesting functional importance of the process? does it always correlate with telophase?

We believe that the unique characteristics of meiosis, where two consecutive rounds of cell divisions without DNA replication in between, offers a completely different scenario to mitosis (see response to referee#1 4a). This discussion is now included in pages 14-15 lines 407-424.

Figure 3

- Why is total Plk1 localization not reported? Also explain why a specific phosphorylated form of the kinase (Plk1 S137P) is labeled and to what this form corresponds (active kinase, I presume). If the authors wanted to detect active kinase, why didn't they stain with the more commonly used anti-Plk1 Ser210P antibody? - "After confirming the localisation of PLK1 in mouse spermatocytes" is not correct, should be "confirming the localization of active Plk1".

As mentioned above, we have not been able to find an antibody against PLK1 that works in squashed in all meiotic stages (see response to referee#1 minor concern 2). We managed to have a kind donation of a discontinued antibody against PLK1 (SC Ref# SC-5585), but unfortunately this antibody doe not offer the possibility of studying centrosomes in the entire meiotic division. It only offers positive reaction in dividing spermatocytes. Centrosomes are labeled with this antibody in control metaphases but it is absent in monopolar spindles of *Plk1*(Δ/Δ) (Fig EV3C).

Addressing referee#2 question, we have used Plk1S137P antibody because it detects centrosomes in mouse spermatocytes in each stage thought the both meiotic divisions and was previously described in mouse oocytes (Du et al 2015; Cell Cycle 14: 3566-79.]. In contrast, anti-Plk1T210P does not detect centrosomes in spermatocytes. As requested, immunolabelling of Plk1T210P is now included in Fig EV2 and mentioned in page 8 lines 182-185 of the results.

Both PLK1 modifications (PLK1S137P and PLK1T210P) are now introduced in page 7 lines 75-76, lines 170 and 184 of results, and lines 436-437 of the discussion.

Figure 4 & 5

This is in my opinion the weakest part of the manuscript, although the use of genetically engineered mice later on supports its main conclusions (while at the same time rendering this part a bit redundant). - The IC50 of BI2536 is ~1nM. Elaborate a bit more on how to explain the need to use much higher concentrations of the drug (100 uM). Would the effects be specific? even at 10 uM BI is able to inhibit a significant number of kinases (see for example http://www.kinase-screen.mrc.ac.uk/screeningcompounds/341035)

The new version of the manuscript first presents the study of the centrosome dynamics in wildtype male mouse, then is focuses in the study of the genetic model *Plk1*(Δ/Δ) and finally analyses the chemical inhibition of PLK1 in cultured spermatocytes with BI2536.

As mentioned above in response to the referee#1 (please see pages 2 and 3 of this document), we have used this high concentration due to the special requirement of the organotypic cultures of seminiferous tubules. This is now better explained in discussion (page 16 lines 454-466). Explanation of the methodology is also included in page 21 of material and methods. In addition, complete data of the optimization experimental design are displayed in new Fig EV4.

- Speculate on why BI2536/Plk1 inhibition does not result in centrosome maturation failure in meiosis I (in contrast to mitosis).

This concern has also been addressed above in respond to referee#1 (please see response 4a and b in pages 5 and 6). We believe that the differences between mitosis and meiosis are responsible for these diverse roles of PLK1. In addition, the different timings of mitosis and meiosis inevitable change the

potential results obtained with short treatments of BI2536. This is included now in discussion page 16 lines 454-479.

- And on why it does result in separation failure after nuclear envelope breakdown. At this point, different independent pathways would normally collaborate to separate the centrosomes but one expects that these would fail as a result of an abnormal maturation of the centrosome and thus its inability to nucleate enough MTs.

We thank the reviewer for addressing this interesting approach. We suggest that separation failure in *Plk1*(Δ/Δ) or BI2536 occurs in late diplotene, when NE has not yet been breakdown (see new Figure 5), discussed in page 14 lines 397-406. We agree that different independent pathways would normally collaborate to separate the centrosomes. One of them is the role of C-NAP1 (CEP250 protein). New data regarding CEP250 is now presented. Data regarding CEP250 persistence between non-migrated centrosomes in monopolar spindle partly explains the mechanistic effect of PLK1 functions. In addition, new data also present KIF11/kinesin EG5 as a downstream effector of PLK1 to form bipolar spindles. These new data are presented in Fig 9.

Our overall data suggest that PLK1 is needed at least thrice during meiosis: it is needed prior to pachytene to ensure centrosome maturation, at diplotene to disentangle CEP250 and trigger centrosome separation, and its later needed again in diakinesis and prophase II to migrate centrosomes via KIF11/EG5 ensuring the formation of the meiotic spindles I and II, respectively.

- Does BI2536 affect centrosome maturation and separation in early meiosis II?

Yes. As seen in Fig 7, BI2536 induces the formation of monopolar spindles II. This is corroborated in *Plk1*(Δ/Δ). MLN8237 treatment also impairs the formation of bipolar meiotic spindles II. All data are presented in Fig 7, which includes quantification data for metaphases I and II.

- The section title "PLK1 inhibition impairs the formation of bipolar spindles I and II" should be changed to "BI2536 impairs the formation of bipolar spindles I and II".

This sentence has been eliminated as headline. As requested by referee#1, data from *Plk1*(Δ/Δ) is presented first in this work. This led to the reorganization of the figures. Spindle analysis are now presented altogether for control, *Plk1*(Δ/Δ), BI2536 and MLN8237 in the new Figure 7.

Nevertheless, the sentence “BI2536 impairs the formation of bipolar spindles I and II” suggested by the reviewer is now included in page 11 lines 296-297.

In concordance with the reviewer’s suggestion, title´s results section cites “BI2536 impairs centrosome migration in prophase I” in page 9 line 223.

Figure 6&7

- How do the results relate to these of the previous figures? especially regarding the observation that lack of Plk1 has a severe effect on PCM structure/centrosome maturation.

New Figure 6 presents now the comparison between control, *Plk1*(Δ/Δ) and BI2536. The explanation of why in BI2536 no lack of PCM in early prophase I is observed, whereas in contrast it is observed in *Plk1*(Δ/Δ) prophases, is based on the timing of male mouse meiosis and the short time of treatment. As mentioned above, the entire meiosis lasts around 2 weeks in male mouse (Griswolsd 2016, Physiol Rev 96: 1-17). An scheme of meiotic duration could also be visited in Fig EV2 of Viera et al., 2020 (EMBO Rep (2020)21:e49273). Only prophase I takes 9 days. This indicates that the 7 days TX treatment in *Plk1*(Δ/Δ) is long enough to induce a phenotype in early prophase. Our results suggest that the lack of PCNT in early prophase is due to the entry into meiosis of these cells in the absence of PLK1 kinase activity, arresting a proper maturation of the pericentriolar matrix. In contrast, BI2536 8h treatment is not long enough to show a phenotype in prophase, only for the transition between diakinesis and metaphase I (appearance of monopolar spindles). Although the organotypic culture of seminiferous

tubules allow us to design interesting experiments, it has some disadvantages. The experimental design presented in new EV4 demonstrates that culturing seminiferous tubules for longer that 8h is not trustable, as apoptosis levels significantly increase. This might be due to the methodology itself and the need of the seminiferous tubules for hormone supply by Sertoli cells and nutrients and O_2 by the blood flow. This is now discussed in page 16 lines 468-479 of discussion.

- 7C represents both meiosis I and II, why the first and second meiotic divisions had not been quantified separately?

For a better understanding of the results, data are presented now separately for metaphase I and II in Fig 7. We thank the reviewer for this suggestion.

- Based on their data with BI, the authors state that "the absence of PLK1 kinase activity does not affect γTubulin location during prophase I in male mouse meiosis.". Is this confirmed in the conditional KO model? Plk1 is central to centrosome maturation in a variety of cell types. Data should be provided in this regard (γtubulin staining and quantification, pericentrin quantification - and, ideally, MT (α or β-tubulin) quantification).

This data is now provided in Figure 6 (Page 10 lines 255-274). This experiment indicates that the absence of PCM (PCNT) in *Plk1*(Δ/Δ) impairs γ-Tubulin localization, as it has been described in mitosis. These results are not appreciated in the BI2536 treatment as 8h is not long enough to induce alteration in PCM assembly.

A triple immunolabelling for PCNT, Centrin-3 and SYP3 was conducted in new Figure 6. Quantification of the number of cells showing Pericentrin is also provided in that figure. As expected, all *Plk1*(Δ/Δ) cells that lack PCNT also showed absence of γ-Tubulin, suggesting that a PLK1-regulated presence of PCNT at meiotic centrosomes is essential for the location of γ-Tubulin.

Figure 8

- Describe with more detail the antibody used. I can't seem to find its description in the text. - Why has the pattern of total Aurora A not been studied? note that changes in the pattern of the phosphorylated kinase could be the result of changes of the localization of the total Aurora pool, of its phosphorylation state, or both.

Please note that in all figures AurorTph is now called AURKP, as suggested in minor points.

The details of the antibody used to detect AURKP is included in the Table of Antibodies (EV6). We are sorry that, by mistake, this was not included before. Unfortunately, we have tried several Anti-AURKA antibodies bur we could not obtain positive immunolabelling in WT spermatocytes. This is the reason why we opted for AURKP. The different options suggested by the reviewer about the localization of the total AURKAP pool/its phosphorylation state/both are now explained in page 12 lines 323-326. In addition, we consider that AURKP is a good option as it labels AURKB/C at centromeres, that serves as an internal positive control when AURKAP is affected at centrosomes. MLN8237 experiments, that specifically inhibit AURKA, are in accordance with this hypothesis.

- As the antibody used an anti- pan-aurora-P do not state that the results correspond to one type of aurora kinase (i.e. "PLK1 inhibition alters Aurora A phosphorylation in male mouse meiosis" should be changed to something along the lines of "(...) alters the pattern of Aurora kinase phosphorylation in male mouse meiosis").

This sentence has been changed in the title of the results (page 11 line 317). We thank the reviewer for this adequate suggestion.

Minor points

- The part of the introduction dealing with centrosomes and their cycle could use more work.

  We have improved the introduction and now it presents more information about centrosomes (lines 39-57). We thank the reviewer for this suggestion.

- I would suggest changing "S137ph" and "AuroraTph" to the more common "S137P" and "AuroraTP" (or to simplify, "Aurora P").

  Following the reviewer´s suggestions, these changes have been made. After consulting Uniprot database, and in accordance with the term PLK1, we opted for the term AURK when referring to Aurora Kinases, and AURKP when referring to the antibody that detects the three phosphorylated Aurora Kinases. The term AURKAP is referred to the centrosomic signal offered by the antibody AURKP.

Response to the Referee #3

This manuscript addresses PLK1 kinase functions in mouse spermatocytes. PLK1 is interesting to pursue in male meiosis, since it is understudied compared to its established roles in mitosis and female meiosis. The authors combined genetic manipulation and pharmaceutical perturbation of PLK1 to show that PLK1 is critical for the integrity of pericentriolar material (PCM), an integral centrosome component linking centrioles to microtubule nucleators. PLK1 inhibition also had deleterious effects on bipolar spindle formation in both meiosis I and II. The manuscript provides limited insights, however, beyond these initial observations, and this reviewer is also concerned about the different results obtained from PLK1 chemical inhibition and conditional knockout mice.

The data shows that PLK1 chemical inhibition has bigger impacts on apoptotic spermatocyte rates, monopolar spindle rates, and phospho-Aurora signals compared to PLK1 conditional knockout. They discuss that "in vitro analyses are usually more aggressive than the genetic models" (line 426). However, in contrast, PLK1 knockout showed stronger effects on PCM integrity compared to chemical inhibition: lack of Pericentrin in knockout vs slight deformation of Pericentrin signals in chemical inhibition. Therefore, it cannot be simply explained by the aggressiveness of the perturbation. This raises a question regarding the specificity of BI 2536 in the condition used in this study (100 uM in 10% DMSO for 8h). Off-targeting of BI 2536 to other PLK (e.g., PLK4) or Aurora kinases, which regulate similar pathways, may explain the different results between BI 2536 treatment and knockout experiments. This could also explain why gtubulin stayed intact in BI 2536 treatment, while it was absent in the PLK1 conditional knockout mice used in the back-to-back paper. To resolve these contradictions, the authors should validate the specificity of the BI 2536 treatment in their system or focus on the genetic model to avoid confusions.

As mentioned above in response to the referee#1 (please see pages 2 and 3 of this document), we have used this high concentration due to the special requirement of the organotypic cultures of seminiferous tubules. This is now better explained in discussion (page 16 lines 454-466). Explanation of the methodology is also included in page 21 of material and methods. In addition, data of optimization of the experimental design is displayed in new EV4.

We believe that explanation of the different results between BI2536 treatment and conditional knockout experiments is not based on the off targets of the drug but rather on the procedures itself. As mentioned above, the timing meiosis is much longer than the mitotic cell cycle. This also explains why in BI2536 no lack of PCM in early prophase I is observed, whereas in contrast it is observed in leptotene and zygotenes of (please see response to referee#2 above). Nevertheless, we are aware that this high concentration could be perturbing other pathways, and so is cited in the discussion in page 19 lines 583-584. However, the phenotype showed in the genetic model *Plk1*(Δ/Δ) is consistent with BI2536 results: induction of monopolar spindles is equal in BI2536 and *Plk1*(Δ/Δ), and the explanation of the different results of centrosome maturation in early prophase I are due to the different timings of both experiments.

The authors claim in the abstract that they studied both PLK1 and Aurora A, but the data for Aurora A is very limited in this manuscript. The only data relevant to Aurora A is the immunofluorescence analysis of phospho-Aurora kinases (staining all Aurora kinases: A, B, and C) upon PLK1 inhibition in figure 8. Further, it is difficult to interpret this data, because there are no quantification of signal intensities and no grey scale images just showing the phospho-Aurora signals. I suggest to stain just for Aurora A in PLK1 knockout spermatocytes, which would tell if PLK1 regulates phosphorylation of Aurora A and/or its localization at centrosomes. Also, directly perturbing Aurora A will provide further insights into the contribution of Aurora A on centrosome maturation and bipolar spindle formation in spermatocytes. Some additional mechanistic insight into the roles of PLK1 in centrosome maturation and migration would make the paper stronger. For example, is Eg5 kinesin downstream effector of PLK1 to establish the bipolar spindle? This was proposed as a future direction in the Discussion section, but I think it would be nice to be included in the current study.
We thank the reviewer for this comments as it has helped us to improve our work.

Please note that upon suggestion of referee#2, Aurora Kinases are now called AURK and the antibody AurorTph is now called AURKP in the text and all figures.

The revised version includes now experiments of directly perturbing AURKA (use of MLN8237/Alisertib), a well-known specific inhibitor of AURKA. This new data is displayed in Fig 8. In addition, as suggested by the reviewer, the quantification of the signal intensities and grey scale images for AURKP are presented.

We believe that these new experiments reinforce our discussion of the crosstalk between PLK1 and AURKA kinases within the centrosome regulation context. Moreover, it now justifies the mention of AURKA in the abstract and, under our opinion, improves the scope of the manuscript. We also understand that this new data complements the genetic model presented in the back-to-back manuscript by Wellard *et al.*

In addition, as suggested, we have studied kinesin KIF11 (EG5). New data regarding KIF11 is now displayed in Fig 9. We believe that KIF11 is altered after PLK1 disruption, and this impedes centrosome migration in monopolar spindles. This new data is included in the results and discussion.

In addition, CEP250 (C-NAP1) protein has also been studied. No apparent signals of CEP250 are observed at centrosomes after diplotene in control spermatocytes. However, CEP250 link is still connecting adjacent non-migrated centrosomes on monopolar spindles under *Plk1* depletion -*Plk1*(Δ/Δ)-, accordingly to mitotic cells without PLK1 activity (Mardin et al., 2011, Curr. Biol. 21, 1145–1151. (10.1016/j.cub.2011.05.047)).

As mentioned above, Our overall data suggest that PLK1 is needed at least thrice during meiosis: it is needed prior to pachytene to ensure centrosome maturation, at diplotene to disentangle CEP250 and trigger centrosome separation, and its later needed again in diakinesis and prophase II to migrate centrosomes via KIF11/EG5 ensuring the formation of the meiotic spindles I and II, respectively.

Additional comments:

-Plant cells also don't have centrioles (line 32).

These suggestions have been corrected (page 3 lines 36-37).

We thank the reviewer for these adequate comments.

-Correct embryogenesis to oogenesis (line 69). This has been corrected.

-Explain what is known about PLK1-S137ph (line 131).

Both PLK1 modifications (PLK1S137P and PLK1T210P) are now introduced in page 7 lines 75-76, lines 170 and 184 of results, and lines 436-437 of the discussion.

-Terms kinetochoric MTs and metaphasic plate are uncommon in the field. Change them to kinetochore MTs and metaphase plate.

Following the reviewer´s suggestions, these changes have been made.

1st Revision - Editorial Decision 4th Jan 2021

Dear Dr. Gómez,

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

As you can see, the referees find that the study is significantly improved during revision and recommend publication. Before I can accept the manuscript, I need you to address some minor points below:

• There are currently 7 keywords, and we can accommodate maximum 5 keywords for technical reasons. Please remove two of the keywords.
• As per our guidelines, please add a 'Data Availability Section', where you state that no data were deposited in a public database.
• As of January 2016, new EMBO Press policy asks for all corresponding authors to link to their ORCID iDs. You can read about the change under "Authorship Guidelines" in the Guide to Authors here: http://emboj.embopress.org/authorguide

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We regret that we cannot do this linking on your behalf for security reasons.
• Please include the funding information in the Acknowledgements section.
• We note the following regarding the figure callouts:
o Fig 3J-L callouts are missing the '3'.
o Fig 7 panels are not alphabetically ordered.
o Fig EV1 panels are not called out.
• Data Set 1 need to be renamed to Dataset EV1 and it needs a legend.
• The movies need to be ZIPped with their legends and their nomenclature needs to be corrected.
• The figure legends need to be moved to the end of the Article file.
• Figure EV6 is the primary & secondary antibody tables. It should be corrected to Table EV1 and in excel or word format.
• The EV figure file names need to be corrected to Figure EV#.
• Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size

cannot exceed 550x400 pixels.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated. I am aware that the comments were made on an earlier version of the manuscript, please use the attached document as a reference and perform the changes on the latest version of the text.

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

Kind regards,

Deniz Senyilmaz Tiebe

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Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The reviewers have made substantial responses to all critiques in my initial review, have politely pointed out where I misunderstood (and revised to make the manuscript more clear anyway), and have attempted challenging experiments which sometimes technically failed or were unable to completely address some critiques. I appreciate the effort and believe that the manuscript is substantially improved and should be published in EMBO Reports.

Referee #2:

The authors have either experimentally addressed or adequately argued this reviewers' comments.

My main concern, that some of the results central to the work were obtained using a remarkable high concentration of the Plk1 inhibitor BI2536 -thus raising doubts about specificity-, has been somehow addressed. The authors now focus on the study of the Plk1(Δ/Δ) animals presenting the resulting observations first, and discuss the rational for using such high concentrations with the support of additional data. The discrepancies observed between results using Plk1(Δ/Δ) animals and those using the Plk1 inhibitor are also better discussed.

The new version of the manuscript is in my opinion improved, much more complete, and adequately support the authors' claims. It thus can be accepted for publication.

Referee #3:
The authors have addressed to all of my concerns, and I think the manuscript is now acceptable for publication.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 21st Jan 2021

Dear Dr. Gomez,

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

Congratulations on a nice work!

Before we can transfer your manuscript to our production team, there are is one minor thing to sort out. I have taken the liberty of making some changes in the bullet points to increase clarity/accessibility. Please take a look and confirm, or feel free to make further changes:

Bullet points
• Centrosomes duplicate twice during male mouse meiosis: in the leptotene/zygotene transition and during interkinesis.
• Meiotic centrosomes separate during diplotene and during interkinesis/prophase II to facilitate the assembly of bipolar meiotic spindles.
• Genetic ablation or chemical inhibition or PLK1 result in monopolar spindles in both meiotic divisions in mouse spermatocytes.
• In vitro inhibition of PLK1 and AURKA suggest impairs formation of meiotic bipolar spindles.
• Inhibition of PLK1 and AURKA impairs KIF11 localization to monopolar spindles.

Kind regards,

Deniz Senyilmaz Tiebe
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample size was chosen to ensure there was no bias due to cell positioning on the slices or due to antibodies malfunctioning. 100 cells per slice were considered randomely and individually analized.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	3 individuals were used per genotype and treatment as similar works have previously done. This number is considered the minimun to appreciate if any of them show some kind of abnormality.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Animals randomization was induced by the fact animals were receive from the animal facility and were not picked by the researcher. For genetically modified mice all available animals were used.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done as was not neccesary for the experiments shown in this work
5. For every figure, are statistical tests justified as appropriate?	statistical tests used were the same used by similar works before to ensure results to be clearly comparable
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Variance homoskedasticity was assess by Levene test. Normality tests were also used (kolmogorov-smirnov). Not all data meet all assumptions for parametric tests, however t-test was still used as sample differences were small and simetrical and using t-test ensures comparability of results with previous similar works. Non parametric analyses were also perform and the same results were obtained.
Is there an estimate of variation within each group of data?	Yes

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

C- Reagents

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

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Adult C57BL/6 wild-type (WT) male mice and Plk1 conditional depletion male mice. All details are found in the manuscript "materials and methods"
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animals were kept at the Animal Facility of the Spanish National Cancer Research Centre (CNIO) or the Animal Facility of Faculty of Medicine of Universidad Autónoma de Madrid under specific pathogen-free conditions in accordance with the recommendation of the Federation of European Laboratory Animal Science Associations (FELASA). All animal procedures were approved by local and regional ethics committees (Institutional Animal Care and Use Committee and Ethics Committee for Research and Animal Welfare, Instituto de Salud Carlos III) and performed according to the European Union guidelines.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
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22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA