EMBO reports

Spindle assembly checkpoint insensitivity allows meiosis-II despite chromosomal defects in aged eggs

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DOI: 10.15252/embr.202357227

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Review Timeline:

Submission Date:	22nd Mar 23
Editorial Decision:	12th Apr 23
Revision Received:	22nd Jun 23
Editorial Decision:	18th Aug 23
Revision Received:	7th Sep 23
Accepted:	19th Sep 23

Editor: Ioannis Papaioannou / Achim Breiling

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

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As you will see, the referees acknowledge that the findings are potentially interesting and relevant, and most of the data are robust. However, they also point out that the conclusions are not fully supported by the available data and that other possible interpretations have not been considered. They also identify other limitations in the study, and they provide useful suggestions for the improvement of the study and the manuscript.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript. If you have any questions or comments, we can also discuss the revisions in a video chat, if you like.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we usually recommend a revision within 3 months (July 11th). Please discuss with me the revision progress ahead of this time if you require more time to complete the revisions.

Please note that you can format your manuscript either as a Report or as an Article. For Reports, the manuscript should not exceed 27,000 characters (including spaces but excluding Materials & Methods and References) and 5 main plus 5 Expanded View figures. The Results and Discussion sections must be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For an Article there are no length limitations, but it should have more than 5 main figures and the Results and Discussion sections must be separate. In both cases, the entire Materials and Methods must be included in the main manuscript file.

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- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter plots showing the individual data points.

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

Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

The spindle assembly checkpoint (SAC) constitutes an important mechanism to monitor the success of chromosome segregation during both mitotic and meiotic cell divisions. In cases where chromosomes are not or are incorrectly attached to microtubules via the kinetochores, at these kinetochores the spindle assembly checkpoint initiates the production of the mitotic checkpoint complex which inhibits the anaphase promoting complex, preventing anaphase onset. During somatic cell divisions, the spindle assembly checkpoint is critical for accurate chromosome segregation, and therefore most somatic cells have a very robust spindle assembly checkpoint. It has been known for some time, though, that meiotic division are highly prone to chromosome mis-segregations, suggesting that the SAC is not very effective during those circumstances. This phenomenon has been studied fairly extensively in meiosis I oocytes, but so far, the role of the SAC in the second meiotic division, meiosis II, has not been analysed, mainly for the technical reasons that it is difficult to manipulate meiosis II without disrupting meiosis I. As the implications of chromosome mis-segregation in meiosis are far-reaching, including the development of trisomies in the embryo or infertility altogether, there is an intense interest in understanding the role of the SAC in meiosis. This is particularly pertinent for the question of how advancing maternal age increases the risk of aneuploidy in germ cells, leading to reproductive failure.

Mihajlović and colleagues now report the analysis of the role of the SAC specifically in mouse meiosis II oocytes. The authors hypothesise that aneuploidy is due to failure of the spindle assembly checkpoint (SAC) in meiosis II to prevent mis-segregation of the already misaligned chromatid pairs. To test this hypothesis, they generated mice with an oocyte-specific knock-out of the KIF18A motor protein, which is known to promote chromosome segregation in both mitosis and meiosis. Their use of KIF18A-KO was prompted by the observation that GFP-KIF18A is only observed in meiosis II oocytes, not meiosis I oocytes, indicating a meiosis II-specific role, and hence providing an opportunity to induce meiosis II defects without affecting meiosis I. Using this tool, Mihajlović et al. demonstrate that the loss of KIF18A during meiosis causes chromosome mis-segregation specifically in meioisis II in the form of highly unaligned chromosomes, often displaying syntelically attached sister chromatids. They show that these are not detected by the spindle assembly checkpoint, as evidenced by the lack of GFP-MAD2 kinetochore localisation. A similar lack of spindle checkpoint engagement was observed when chromosome mis-segregation was induced by a combination of monastrol and nocodazole treatment, indicating that their observations were not specific for KIF18A knock-out.
The study is nicely conducted with very high-quality images, and the observations made are important and highly relevant for the field. However, a major drawback of the study is that the analysis is mainly observational, and there is no insight into why the spindle assembly checkpoint is not activated during meiosis II. It would be extremely valuable if the authors could provide some level of insight into why this is.

Major issues:

• The authors observe that sister chromatid pairs are not aligned on the Meiosis metaphase II plate in KIF18A-KO mice. The kinetochores of these mis-aligned chromatids are not decorated by GFP-MAD2 and are hence assumed to be spindle checkpoint silent. In somatic cell divisions, the spindle assembly checkpoint works together with Aurora B-mediated error correction. Active Aurora B is also critically important for MPS1 recruitment and hence spindle checkpoint signalling in mitotic divisions. The authors' results seem to suggest that error correction is not happening in meiosis II, and syntelic attachments,

once formed, are not turned over. Is Aurora B active at all? Is a lack of Aurora B activity the underlying reason for the absence of SAC activation?
To check Aurora B activity, the authors could check known Aurora B targets (e.g. CENP-A) to see whether they are phosphorylated.
• The authors base their conclusion that the spindle assembly checkpoint is not active on the absence of GFP-MAD2 kinetochore signal but it is potentially possible that GFP-MAD2 behaviour is not representative of the spindle checkpoint per se. Have the authors carefully checked that the timing of mitosis is not altered in KIF18A-cKO oocytes in comparison to WT oocytes? Is cyclin B degradation altered in any way?
• None of the graphs in the main figures have error bars or any statistical tests. Were the experiments conducted more than once?
• A recent publication (Kouznetsova et al., 2019) suggested that incorrect microtubule-kinetochore attachments in meiosis II are largely corrected during anaphase, resulting in a lower percentage of aneuploid gametes than analysis of meiosis II metaphase plates would suggest. Have the authors checked the numbers of aneuploid gametes in their different experimental situations? How do their data relate to the Kouznetsova study?
Minor issues:
Figure 2:
• The title of this figure/results section is inaccurate. Although SAC activity is inferred from the data, no direct demonstration of the SAC (in)activity is made in this figure (i.e. recruitment of checkpoint proteins). Title would be more appropriate for figure 3.
• It would be helpful to include a legend in Figure 2C.
• It is not clear at what point the measurement of chromosome oscillation is begun. It would be helpful to describe the interval measured on the x-axis of the graphs in Figure 2B. Also, is 10 mins of measurement sufficient?
Figure 3:
• At what point during meiosis are oocytes treated with Monastrol/Nocodazole? Methods state treatment is 4h (2h each monastrol and nocodazole). Does this overlap with meiosis I and if so what is the effect of Mon/Noc treatment on meiosis I?

Referee #2:

In their manuscript entitled "Spindle assembly checkpoint failure in meiosis-II underpins age-related developmental aneuploidy," Mihajlović, FitzHarris, and colleagues investigate the basis of chromosome missegregation events in meiosis-II using mouse oocytes. Previous studies on mammalian meiosis-I and various model organisms have shown that the spindle assembly checkpoint (SAC) is not very effective in promoting accurate chromosome segregation in female meiosis. The authors aim to determine if this observation applies to meiosis-II in mouse oocytes. They employ three different mechanisms to observe chromosome misalignment and missegregation in meiosis-II: deletion of the mitotic kinesin KIF18A, treatment with a combination of spindle-disrupting drugs, and observation of oocytes from aged mice. In all three cases, minimal checkpoint activity was observed for clearly misaligned chromosomes, and chromosome missegregation was a frequent outcome. The imaging is overall well done, and the data appear robust. However, there are no measurements of the main output of the SAC - a delay in anaphase - for meiosis-II that would directly test the ability of the SAC to allow for extra time for the biorientation of chromosomes. Additionally, many of the conclusions about the activation of the SAC and its effect on chromosome segregation are confusing.

Major concerns:

1. The function of the SAC is to delay anaphase give the chromosomes additional time to properly attach to spindle microtubules. However, given enough time, the SAC will frequently "slip" even when all of the chromosomes are not properly attached. Furthermore, the duration of the delay is proportional to the amount of SAC signaling and the number of unattached chromosomes (Collin et al. 2014). Therefore, to determine the degree of SAC signaling, the authors will need to determine if there is an anaphase delay resulting from each of the perturbations that disrupt chromosome alignment. Curiously, the authors show this for meiosis I (Figure 1C), but not meiosis II. The authors do measure the rate of Cyclin B1 degradation following treatment with high levels of nocodazole, which shows a robust anaphase delay and suggests that the oocytes do indeed have a functional checkpoint in Metaphase-II. It is unclear why the authors continue to state repeatedly that there is a "SAC failure" in the title and throughout the manuscript despite this result that clearly demonstrates an active SAC.

2. The SAC responds to a lack of microtubule attachments at the kinetochore, not to misaligned chromosomes directly. It is unclear if the lack of Mad2 signal that the authors observe at misaligned kinetochores is because they lack unattached kinetochores or if the unattached kinetochores fail to activate the SAC. For misaligned chromosomes such as the ones created by the conditions the authors use, unattached kinetochores are a downstream effect of other factors that respond to misalignment or lack of tension (Aurora A, Aurora B, MCAK, etc.). These factors then create unattached kinetochores that can be recognized by the SAC. It's worth noting that low-dose nocodazole likely acts by suppressing MT dynamics instead of depolymerizing MTs, and therefore does not activate the SAC directly. I think a more likely conclusion is therefore that the meiosis-II spindles have difficulty with "error correction" mechanisms instead of, or in addition to, decreased SAC activity.

3. The authors argue that the aneuploidy created in aged mice results from a combination of disrupted cohesion between sisters and a lack of SAC activity. However, no amount of SAC activity will allow for the proper segregation of sister chromatids that are no longer attached to each other. The title of the manuscript therefore makes no sense to me. Are the authors arguing that strong enough SAC activity would arrest these oocytes indefinitely and prevent them from developing? This is unclear, as their model does not show what would happen if robust SAC activity was active in the oocytes. Furthermore, I am not convinced that such a mechanism would increase fertility.

Minor points:

4. Possibly the most interesting aspect of this study is the difference between Meiosis-I and Meiosis-II for the requirement of KIF18A. I feel that further exploration or discussion if this result could greatly benefit the manuscript.

Overall, the experiments presented appear to be well executed; however I do not feel that they support the main conclusions of the paper.

Referee #3:

Mihajlović generate a KIF18A conditional knockout and GFP tagged mouse to examine the effects in female meiosis. They present evidence indicating that KIF18A is not important in meiosis I but critical in meiosis II for chromosome alignment. They then use this model, where only a small number of chromosomes misalign in meiosis II to address the sensitivity of the checkpoint in meiosis II. This is an interesting area and a good approach to test an important question. However, the conclusions are not always supported by the data as other interpretations are possible. In particular, the limitations of the system preclude concluding that KIF18A is dispensable for meiosis I. In addition, the authors argument that the spindle assembly checkpoint (SAC) is ineffective in meiosis II is too strong. While it is true that the authors present data indicating that the SAC is weak, better analysis is required to show whether there is any delay at all and therefore to demonstrate how weak the checkpoint is.

Major concerns

1. More in-depth analysis of the mouse model should be performed. Are the mice with KiF18A absent from the female germline fertile?
2. Although the authors using imaging to examine the depletion of KIF18A in the KIF18A-cKO it is possible that residual protein remains that cannot be detected by this method. Persistence of KIF18A could explain why the authors find no phenotype in meiosis I. The authors should perform futher analyses (ideally western blotting) to confirm that Kif18A protein has been depleted already in meiosis I. Establishing that the gene has been deleted is not sufficient.
3. Statistical analyses should be carried out throughout the manuscript. For example, line 98/Figure 2A, although the authors claim "the frequency of misaligned chromosomes was significantly increased in KIF18A-cKO Met-II eggs.", the authors cannot make this conclusion without proper statistical tests on their data.
4. The authors conclude that KIF18A is important specifically for maintaining the alignment of chromosomes in metaphase II, but not metaphase I (line 102/103). However, the authors have not tested chromosome alignment in metaphase I. It is possible that chromosomes fail to maintain alignment in metaphase I but that this has no adverse affect on chromosome segregation, potentially due to meiosis I-specific backup mechanisms. Can the authors measure chromosome alignment in metaphase I from their movies?
5. Line 105-122. This paragraph claims that there is no functional checkpoint as evidenced by the onset of anaphase in KIF18A-cKO eggs with misaligned chromosomes. However, this experiment only shows that anaphase is possible in this situation, but does not reveal whether there is a checkpoint that is eventually overridden. To determine whether there is a checkpoint at all, the authors would need to measure the timing of anaphase onset after activation, ideally by measuring the time of securin/cyclin B degradation as a direct read out of SAC satisfaction. In the example shown there does indeed appear to be a delay in anaphase onset in the KIF18A-cKO egg (6 min vs 3 min in the control). I agree with the authors that their data show that the checkpoint cannot maintain an indefinite arrest but I do not think that this is evidence that there is no checkpoint at all.
6. Similarly, in Figure 3, the authors claim that cyclin B is degraded at a normal rate in the MonNoc eggs, but they have not shown the kinetics. Additionally, a concern with this experiment is that the eggs have enough time to align chromosomes before anaphase so they are not actually measuring the effect of mis-aligned chromosomes. How can the authors rule out this possibility?

Referee #1:

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Major issues:

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attachments, once formed, are not turned over. Is Aurora B active at all? Is a lack of Aurora B activity the underlying reason for the absence of SAC activation? To check Aurora B activity, the authors could check known Aurora B targets (e.g. CENP-A) to see whether they are phosphorylated.

We thank the reviewer for this excellent suggestion. To address whether Aurora B is normally active in eggs, we examined Aurora kinase activity by immunofluorescence of the phosphorylated form of INCENP (a well-known Aurora B target; see *Bishop & Schumacher, 2002; J. Biol. Chem.*). We found that pINCENP is very clearly present on all chromosomes at metaphase-II, and is even slightly elevated level on misaligned versus aligned chromosomes. Thus, the lack of Aurora B activity is unlikely to explain the absence of SAC activation at misaligned chromosomes. These results have now been added to Figure EV4C, and are described at lines 152-158 and 359-361.

• The authors base their conclusion that the spindle assembly checkpoint is not active on the absence of GFP-MAD2 kinetochore signal but it is potentially possible that GFP-MAD2 behaviour is not representative of the spindle checkpoint per se. Have the authors carefully checked that the timing of mitosis is not altered in KIF18A-cKO oocytes in comparison to WT oocytes? Is cyclin B degradation altered in any way?

This excellent point is in fact made by all three reviewers. To address this, we re-analyzed our time-lapse movies and found that there is no difference in the timing of anaphase onset between WT and KIF18A-cKO eggs. Importantly, we also found that presence of misaligned chromosome did not cause a delay in anaphase onset either in KIF18A-cKO or MonNoc eggs, further supporting the idea that SAC is not activated by misaligned chromosomes. We also analysed the dynamics of Cyclin B1-GFP degradation in MonNoc eggs, and found no change in Cyclin B1-GFP destruction in the presence of misaligned chromosomes. Thus, the idea that misaligned chromosomes fail to activate SAC in meiosis-II oocytes is now supported not only by the observation that anaphase happens, but also the timing is unchanged, and the rate of Cyclin B1 destruction is unaffected. Many thanks (to all three reviewers!) for raising this point, as we agree that this analysis strengthens the paper. This data is now included in Figures 2D, 3A and EV3C and described at lines 119-120, 138-144, 267 and 274-277.

• None of the graphs in the main figures have error bars or any statistical tests. Were the experiments conducted more than once?

Yes, each experiment in this study was performed on at least two separate days (most on 3 or more), and n-numbers are that of the total number of eggs. Error bars have not been presented in the graphs where the results have been expressed as proportions. Statistical tests have been annotated in the figure legends and have now been added at the places where missing, as well as some clarifying comments regarding n-numbers/replicates. Please see for example lines 238, 242, 254, 290 and 340.

• A recent publication (Kouznetsova et al., 2019) suggested that incorrect microtubule-kinetochore attachments in meiosis II are largely corrected during anaphase, resulting in a lower percentage of aneuploid gametes than analysis of meiosis II metaphase plates would suggest. Have the authors

checked the numbers of aneuploid gametes in their different experimental situations? How do their data relate to the Kouznetsova study?

Whilst we have not presented karyotyping in post-activation eggs (this is far from trivial as the pronuclei form rapidly), our data show clearly that misaligned chromosomes consist of sister chromatid pairs and always travel intact towards the nearest spindle pole during anaphase-II - as opposed to splitting sisters apart towards the opposing spindle poles. Thus, given that both sister chromatids end up in the same cell this, this unquestionably results in aneuploidy.

The comparison of our work with Kouznetsova et al. 2019 is an interesting point, and its true we had not explained the distinction between our work and theirs. In their paper they first show in control-young eggs that immediately after Pb1 formation, while the Met-II spindle is first forming, there are misaligned sister pairs (that do indeed look like the ones in our paper), but these are rapidly aligned to establish the steady state spindle and are thus not present at anaphase. Later in their paper they also discuss correction of MT attachments on aligned chromosomes to avoid lagging (analogous to classic work in somatic cells, see *Cimini et al. 2003; J Cell Sci*). Our observation is very different to all of this. Rather we are showing that when there is a misalignment that persists to anaphase onset (not addressed in the Kouznetsova paper), that these fail to activate SAC and are therefore missegregated.

As such the reviewers point illustrates that we should have explained this, and so we are very grateful for the comment. To do so we have now added a sentence that explains the difference between chromosome misalignment that we study and that reported by Kouznetsova et al. 2019 (see lines 105-106).

Minor issues:
Figure 2:
• The title of this figure/results section is inaccurate. Although SAC activity is inferred from the data, no direct demonstration of the SAC (in)activity is made in this figure (i.e. recruitment of checkpoint proteins). Title would be more appropriate for figure 3.

We thank the reviewer for this insightful comment. We have now changed the title of Figure 2 to 'Misaligned chromosomes fail to delay anaphase onset in KIF18A-cKO eggs'.

• It would be helpful to include a legend in Figure 2C.

Many thanks, we have now included the legend in the Figure 2C as suggested.

• It is not clear at what point the measurement of chromosome oscillation is begun. It would be helpful to describe the interval measured on the x-axis of the graphs in Figure 2B. Also, is 10 mins of measurement sufficient?

Chromosome oscillations were measured in the metaphase of meiosis-II (in the period 12-18 h post-IBMX release). This information has now been added in the material and methods (see lines 533-534). In somatic cells, chromosome oscillatory movements that are usually attributed to KIF18A are very rapid

and occur at the frequency 0.5-1/min (see *Iemura et al., 2021; Cancers*), thus if chromosomes exhibited rapid oscillatory movements in eggs, 10 mins of live imaging would have been ample time to detect them. To make this clearer we have now strengthened the description of this at line 96.

Figure 3:

• At what point during meiosis are oocytes treated with Monastrol/Nocodazole? Methods state treatment is 4h (2h each monastrol and nocodazole). Does this overlap with meiosis I and if so what is the effect of Mon/Noc treatment on meiosis I?

Monastrol/Nocodazole treatment was performed on ovulated Met-II oocytes (14-16 h following hCG stimulation). So, at no point did the treatment overlap with meiosis-I. To clarify this, we have now added this information into the material and methods section, see line 454.

Referee #2:

In their manuscript entitled "Spindle assembly checkpoint failure in meiosis-II underpins age-related developmental aneuploidy," Mihajlović, FitzHarris, and colleagues investigate the basis of chromosome missegregation events in meiosis-II using mouse oocytes. Previous studies on mammalian meiosis-I and various model organisms have shown that the spindle assembly checkpoint (SAC) is not very effective in promoting accurate chromosome segregation in female meiosis. The authors aim to determine if this observation applies to meiosis-II in mouse oocytes. They employ three different mechanisms to observe chromosome misalignment and missegregation in meiosis-II: deletion of the mitotic kinesin KIF18A, treatment with a combination of spindle-disrupting drugs, and observation of oocytes from aged mice. In all three cases, minimal checkpoint activity was observed for clearly misaligned chromosomes, and chromosome missegregation was a frequent outcome. The imaging is overall well done, and the data appear robust. However, there are no measurements of the main output of the SAC - a delay in anaphase - for meiosis-II that would directly test the ability of the SAC to allow for extra time for the biorientation of chromosomes. Additionally, many of the conclusions about the activation of the SAC and its effect on chromosome segregation are confusing.

Major concerns:

1. The function of the SAC is to delay anaphase give the chromosomes additional time to properly attach to spindle microtubules. However, given enough time, the SAC will frequently "slip" even when all of the chromosomes are not properly attached. Furthermore, the duration of the delay is proportional to the amount of SAC signaling and the number of unattached chromosomes (Collin et al. 2014). Therefore, to determine the degree of SAC signaling, the authors will need to determine if there is an anaphase delay resulting from each of the perturbations that disrupt chromosome alignment. Curiously, the authors show this for meiosis I (Figure 1C), but not meiosis II. The authors do measure the rate of Cyclin B1 degradation following treatment with high levels of nocodazole, which shows a robust anaphase delay and suggests that the oocytes do indeed have a functional checkpoint in Metaphase-II. It is unclear why the authors continue to state repeatedly that there is a "SAC failure" in the title and throughout the manuscript despite this result that clearly demonstrates an active SAC.

Thank you for this helpful comment. As noted above, the same remarks in regard to anaphase timing and Cyclin B1 destruction rates are also made by the other two reviewers. As described above, we have now carefully analysed our data and found no delay in anaphase onset in the presence of misaligned chromosomes in either of our two models, or any change in Cyclin B destruction dynamics in the presence of a misaligned chromosome. Please see this new data which is presented in Figures 2D, 3A and EV3C.

In addition, this comment led us to realise that we should qualify some of the language, avoiding comments such as 'lack of SAC', and instead spelling out more carefully that SAC failed to prevent anaphase in cases where there were misaligned chromosomes. This led to a several changes, including the title of the paper, which is now 'Spindle assembly checkpoint insensitivity permits completion of meiosis-II despite chromosomal defects in aged oocytes'. For examples of other changes please see lines 14, 17, 111, 132, 146, 154, 157-158, 164, 172-173, 187 and 285.

2. The SAC responds to a lack of microtubule attachments at the kinetochore, not to misaligned chromosomes directly. It is unclear if the lack of Mad2 signal that the authors observe at misaligned kinetochores is because they lack unattached kinetochores or if the unattached kinetochores fail to activate the SAC. For misaligned chromosomes such as the ones created by the conditions the authors use, unattached kinetochores are a downstream effect of other factors that respond to misalignment or lack of tension (Aurora A, Aurora B, MCAK, etc.). These factors then create unattached kinetochores that can be recognized by the SAC. It's worth noting that low-dose nocodazole likely acts by suppressing MT dynamics instead of depolymerizing MTs, and therefore does not activate the SAC directly. I think a more likely conclusion is therefore that the meiosis-II spindles have difficulty with "error correction" mechanisms instead of, or in addition to, decreased SAC activity.

This is an interesting point. To address this we have now performed an experiment where we exposed eggs with misaligned chromosomes to cold-media to remove unstable microtubules and thereby examine the stable attachment status of misaligned chromosomes ('cold-shock analysis'). Our results revealed that misaligned chromosomes are predominantly unattached (82.8%; see Figure EV4C). Thus, our results suggest that unattached kinetochores of misaligned chromosomes fail to robustly activate SAC. This new experiment is presented as Figure EV4C and described at lines 158-159, 361-363 and 505-506.

3. The authors argue that the aneuploidy created in aged mice results from a combination of disrupted cohesion between sisters and a lack of SAC activity. However, no amount of SAC activity will allow for the proper segregation of sister chromatids that are no longer attached to each other. The title of the manuscript therefore makes no sense to me. Are the authors arguing that strong enough SAC activity would arrest these oocytes indefinitely and prevent them from developing? This is unclear, as their model does not show what would happen if robust SAC activity was active in the oocytes. Furthermore, I am not convinced that such a mechanism would increase fertility.

This interesting comment has triggered much discussion here! Overall, it made us realise that we had not expressed well enough the relevance/importance to embryo aneuploidy of our observation that

misaligned chromosomes do not activate SAC and thus get missegregated. And this also led us to perform an experiment that we present for the reviewer's curiosity here.

Firstly, we agree with the reviewer that no amount of SAC activity would allow for proper segregation of sister chromatids. It was not our intention to imply this. Rather the question this study answers is – how can Met-II eggs with misaligned chromosomes (the result of the aging cohesion-loss effect) give rise to classic aneuploid embryos with gains or losses of individual chromatids? The answer to this is not obvious, since one might imagine that misalignments would trigger a SAC (and thus indefinite arrest? Slippage? death? – see more on this below) and therefore not generate aneuploid embryos. And this had not been addressed because no one had generated a model that gave Met-II specific problems with which to test the notion. Our data reveal a very weak SAC at Met-II, and this explains how it is possible to generate classic aneuploid embryos (as frequently seen in the clinic) from eggs with misaligned chromosomes.

Secondly, ...

Figure for referee with unpublished data and its description has been removed upon request by the authors.

Minor points:

4. Possibly the most interesting aspect of this study is the difference between Meiosis-I and Meiosis-II for the requirement of KIF18A. I feel that further exploration or discussion if this result could greatly benefit the manuscript.

Ultimately in this study we used the KIF18A mouse as a tool to examine errors in Met-II, and the biological explanation as to why KIF18A is non-essential in MI, is not really the focus of the paper. That said, we have also been trying to address more broadly why Met-II knockout of KIF18A causes misalignment. One thing we have done that we can also include is a test of MT turnover levels (using the PAGFP-tubulin assay) to determine whether KIF18A might cause misalignment by affecting MT turnover. This assay effectively tests the potential for spindles to perform MT-kinetochore 'error correction', and we wondered whether a change in this ability to correct errors was a possible contributor to misalignment in KIF18A-cKO oocytes. This turns out not to be the case, however, as MT turnover is unaffected in the KIF18A-cKO eggs. Further examination of the role of KIF18A-cKO in oocytes and embryos is certainly of interest, but we have included all we can here, and ultimately of course this is not the question we are addressing in this paper.

Please see the new data which is presented as Figure EV3D, and described at lines 93, 342-348 and 508-519.

Overall, the experiments presented appear to be well executed; however I do not feel that they support the main conclusions of the paper.

From the points raised above we understand that this final comment relates to the previous lack of timing and Cyclin B1 destruction rate data, and this has been rectified. Many thanks overall for your very helpful comments which we are certain have improved the paper.

Referee #3:

Mihajlović generate a KIF18A conditional knockout and GFP tagged mouse to examine the effects in female meiosis. They present evidence indicating that KIF18A is not important in meiosis I but critical in meiosis II for chromosome alignment. They then use this model, where only a small number of chromosomes misalign in meiosis II to address the sensitivity of the checkpoint in meiosis II. This is an interesting area and a good approach to test an important question. However, the conclusions are not always supported by the data as other interpretations are possible. In particular, the limitations of the system preclude concluding that KIF18A is dispensable for meiosis I. In addition, the authors argument that the spindle assembly checkpoint (SAC) is ineffective in meiosis II is too strong. While it is true that the authors present data indicating that the SAC is weak, better analysis is required to show whether there is any delay at all and therefore to demonstrate how weak the checkpoint is.

Major concerns

1. More in-depth analysis of the mouse model should be performed. Are the mice with KiF18A absent from the female germline fertile?

Yes, indeed, female mice lacking KIF18A in the germ line are fertile. We initiated a small fertility trial a few months ago in which females of 3 different genotypes: *Zp3-Cre+ Kif18a*$^{wt/wt}$ (WT oocytes), *Zp3-Cre+ Kif18a*$^{wt/GFP\text{-}flox}$ (HET oocytes) and *Zp3-Cre+ Kif18a*$^{GFP\text{-}flox/GFP\text{-}flox}$ (KIF18A-KO oocytes) were crossed with non-transgenic B6D2F1/J (WT) stud males. As you can see from the graph below, we were able to obtain live progeny from *Zp3-Cre+ Kif18a*$^{GFP\text{-}flox/GFP\text{-}flox}$ females that lack KIF18A in germline thus suggesting that KIF18A-KO eggs are viable and could produce embryos. Note of course that this is as expected, given that the majority of oocytes in KIF18A-cKO mice have correctly aligned chromosomes. The fertility trial data is now included as Figure EV2E, and is discussed at lines 324-328 and 430-434 of the revised manuscript.

2. Although the authors using imaging to examine the depletion of KIF18A in the KIF18A-cKO it is possible that residual protein remains that cannot be detected by this method. Persistence of KIF18A could explain why the authors find no phenotype in meiosis I. The authors should perform futher

analyses (ideally western blotting) to confirm that Kif18A protein has been depleted already in meiosis I. Establishing that the gene has been deleted is not sufficient.

Many thanks for this comment. Western blotting is of course extremely useful in some situations – for example when a protein is homogeneous/cytoplasmic and knowing the extent to which it has been knocked out is crucial. However, for proteins with a very specific subcellular localisation related to their function, as for KIF18A, Western blots are less useful, as any small residual amounts of protein could theoretically still localise to the spindle and provide function. So, in this situation immunofluorescence to show on an egg-by-egg basis that there is nothing detectable on the spindle - as we have done - is far more meaningful. To breed many KIF18A-cKO mice to attempt a Western that might require many hundreds of oocytes without giving much more information feels hard to justify (hundreds of mice given the breeding scheme). Also, the point of the paper is that the KIF18A-cKO gives a phenotype we can analyse, rather than really being about the nuanced role of KIF18A.

All this having been said, we now realise that our presentation of the knock-out would be more convincing if we presented quantitative analysis. Please see the new Figure EV2F (lines 328-330), where we have used carefully controlled quantitative fluorescence analysis to demonstrate that KIF18A really is gone from the spindle.

3. Statistical analyses should be carried out throughout the manuscript. For example, line 98/Figure 2A, although the authors claim "the frequency of misaligned chromosomes was significantly increased in KIF18A-cKO Met-II eggs.", the authors cannot make this conclusion without proper statistical tests on their data.

Many thanks. The proper statistical analysis had indeed been performed prior to submission, but had been accidentally deleted in one of the latest versions of the manuscript. We have now added the missing information back (please see lines 238, 242, 254 and 290).

4. The authors conclude that KIF18A is important specifically for maintaining the alignment of chromosomes in metaphase II, but not metaphase I (line 102/103). However, the authors have not tested chromosome alignment in metaphase I. It is possible that chromosomes fail to maintain alignment in metaphase I but that this has no adverse affect on chromosome segregation, potentially due to meiosis I-specific backup mechanisms. Can the authors measure chromosome alignment in metaphase I from their movies?

The extent of chromosome alignment in meiosis-I is in Figure 1B. We found no difference in the extent of chromosome misalignment between KIF18A-cKO and controls oocytes, and also no aneuploidy at Met-II by direct chromosome counting, thus leading us to conclude that KIF18A is dispensable for chromosome alignment in meiosis-I. We have now reinforced the section of the results at lines 84-85 to ensure this is clear to the reader.

5. Line 105-122. This paragraph claims that there is no functional checkpoint as evidenced by the onset of anaphase in KIF18A-cKO eggs with misaligned chromosomes. However, this experiment only shows

that anaphase is possible in this situation, but does not reveal whether there is a checkpoint that is eventually overridden. To determine whether there is a checkpoint at all, the authors would need to measure the timing of anaphase onset after activation, ideally by measuring the time of securin/cyclin B degradation as a direct read out of SAC satisfaction. In the example shown there does indeed appear to be a delay in anaphase onset in the KIF18A-cKO egg (6 min vs 3 min in the control). I agree with the authors that their data show that the checkpoint cannot maintain an indefinite arrest but I do not think that this is evidence that there is no checkpoint at all.

As you will see above, the question regarding the timing of the anaphase onset was raised by the other two reviewers also. We performed these analyses and find that the timing from parthenogenetic egg activation to anaphase onset does not change in the presence of misaligned chromosomes in any of the examined conditions, thus supporting our claims that the spindle assembly checkpoint had not been activated by the presence of misaligned chromosome. Please see the new data that is presented in Figure 2D, 3A and EV3C.

As for the delay the reviewer is referring to from our example, time-points shown are relative to anaphase onset (which was determined as the first time-point when chromosomes are optically separate and marked as 0 min.) and as such does not represent a delay in anaphase onset. To make this clearer we have now added a sentence to state that misaligned chromosome had no impact on anaphase onset (see lines 119-120).

6. Similarly, in Figure 3, the authors claim that cyclin B is degraded at a normal rate in the MonNoc eggs, but they have not shown the kinetics. Additionally, a concern with this experiment is that the eggs have enough time to align chromosomes before anaphase so they are not actually measuring the effect of mis-aligned chromosomes. How can the authors rule out this possibility?

Thank you for this comment. We now included the graph that shows Cyclin B1-GFP degradation rate in MonNoc eggs. As you will see, we find no difference in Cyclin B1-GFP degradation rate between the eggs with aligned and misaligned chromosomes, thus supporting the idea that misaligned chromosome do not alter the kinetics of Cyclin B1 destruction in mouse eggs. Additionally, we performed the analysis of misaligned chromosome behaviour prior to anaphase-II in MonNoc eggs (identical to that in Figure 2C done for KIF18A-cKO eggs) that nicely shows that misaligned chromosomes remain misaligned when anaphase-II commences. Please see the newly added data in Fig. 3A described at lines 138-144.

Dear Dr. FitzHarris,

Thank you for submitting your revised manuscript to EMBO reports and for your patience during its peer review. I apologize for the delayed response, which was due to the unavailability of the referees during the summer vacation period, and a backlog in our editorial offices. We have now received the full set of reports from the three referees that were asked to re-evaluate your study. Their comments are included below.

As you will see, all three referees are satisfied with the revision, they explain that most of their concerns have been successfully addressed with the addition of new data and textual clarifications, and they now recommend publication. There are only two minor remaining requests from referees #1 and #2, which we would like you to address in a revised version of your manuscript and in a point-by-point response describing in detail all new changes you might make in the manuscript.

From the editorial side, there are also a few things that we need from you before we can proceed with acceptance of your manuscript:

- Please check that the title and the abstract of the revised manuscript are brief, yet explicit, even to non-specialists. The length of the title should not exceed 100 characters (including spaces), and the abstract should be a single paragraph not exceeding 175 words.

- Please provide up to 5 keywords in your revised manuscript (after the Abstract).

- We noticed that callouts for Fig. EV2E&F and EV4B are missing; please make sure that all Figure panels are called out (in alphabetical order) in your revised manuscript.

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Yours sincerely,

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

The authors have addressed all of my concerns, and I now support publication. This is a very interesting manuscript! My only additional comment would be, could the authors just speculate a little bit more in the discussion why the SAC is not established in meiosis II, even if mis-aligned chromosomes are clearly Aurora B-positive and unattached? Is the SAC potentially blocked at a specific step?

Referee #2:

In their revised manuscript entitled "Spindle assembly checkpoint insensitivity permits completion of meiosis-II despite chromosomal defects in aged oocytes," Mihajlović, FitzHarris, and colleagues have made a number of important improvements to their study investigating the basis of chromosome missegregation events in meiosis-II using mouse oocytes. The authors addressed many of my concerns by modifying the conclusions of the paper, some of the phrasing, and by adding additional experiments. Although the manuscript is substantially improved, I am still not convinced that the checkpoint itself is compromised in meiosis II instead of other upstream regulatory mechanisms.

Concern:

One of my main initial concerns was about the potential for the lack of arrest being the result of decreased activity of other regulatory mechanisms that are necessary to trigger the checkpoint by creating unattached kinetochores. To address this issue, the authors added two new experiments. The first looks at Aurora B activity at the kinetochore through immunofluorescence of phosphorylated INCENP at aligned and unaligned kinetochores. The authors observe staining of the kinetochores and a slight increase at misaligned kinetochores. However, there is no point of comparison for what the levels would be for a cell with robust CPC-based error correction. There is also no statistical analysis of the difference between the levels at aligned vs unaligned chromosomes. It is therefore difficult to conclude much beyond the presence of some active CPC at a relevant location. It does not demonstrate a robust degree of error correction.

The second assay that the authors conducted to address this issue is to look at the number of cold-stabilized microtubules. For this figure, I am not really sure what is being shown and what to compare it to. All of the images shown are cropped to the point of losing context within the cell. In addition, there is no comparison to aligned chromosomes to see if there is a difference in attachment. There is also no indication of how attachment was determined, as most of the examples appear to have proximal tubulin that could be interpreted as bound to the kinetochores. Based on the example images shown, I would have categorized all of the kinetochores as "attached", as there appear to be microtubules in their direct vicinity.

I would suggest that the authors remove these two experiments and simply conclude that they cannot yet distinguish between an inability to activate the SAC and an inability to detach microtubules from misaligned chromosomes. I don't think this is an important distinction to make for this paper, and the authors can therefore address it by suggesting either mechanism could be contributing to the inability to arrest the cells.

Referee #3:

The authors have addressed the concerns of the reviewers with additional experiments and clarifications in the text. This is an interesting study that will be well-received by the field.

We would like to thank all three reviewers for their thoughtful comments that certainly helped improve our manuscript. Please find our responses to the remaining specific points below:

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We have now incorporated a few sentences of discussion wherein we speculate about why spindle assembly checkpoint remains inactivated in meiosis-II despite the activation of Aurora B kinase (*see lines 245-252 of the revised manuscript*).

Referee #2:
In their revised manuscript entitled "Spindle assembly checkpoint insensitivity permits completion of meiosis-II despite chromosomal defects in aged oocytes," Mihajlović, FitzHarris, and colleagues have made a number of important improvements to their study investigating the basis of chromosome missegregation events in meiosis-II using mouse oocytes. The authors addressed many of my concerns by modifying the conclusions of the paper, some of the phrasing, and by adding additional experiments. Although the manuscript is substantially improved, I am still not convinced that the checkpoint itself is compromised in meiosis II instead of other upstream regulatory mechanisms.

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authors can therefore address it by suggesting either mechanism could be contributing to the inability to arrest the cells.

We have now removed two panels originally presented in Figure EV3C. We agree the paper works fine without them.

Referee #3:
The authors have addressed the concerns of the reviewers with additional experiments and clarifications in the text. This is an interesting study that will be well-received by the field.

Dr. Greg FitzHarris
University of Montreal Hospital Research Center (CRCHUM)
900 Rue St Denis
Montreal, Quebec H2X 0A9
Canada

Dear Dr. FitzHarris,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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

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Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgement

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figures
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Material and Methods

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figures
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figures

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Material and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	