Loss of centromeric RNA activates the spindle assembly checkpoint in mammalian female meiosis I

Tianyu Wu, Simon Lane, Stephanie Morgan, Feng Tang, and Keith Jones

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January 15, 2021

Re: JCB manuscript #202011153

Dr. Tianyu Wu
Shanghai Medical College of Fudan University
NO. 131 Dong’an Road
Shanghai 200032
China

Dear Dr. Wu,

Thank you for submitting your manuscript entitled "Loss of centromeric RNA activates the spindle assembly checkpoint in mammalian female meiosis I". The manuscript has now been evaluated by three reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, my editorial decision is against publication in JCB.

You will see that reviewers recognize the potential importance of your study, suggesting a role of centromeric RNA in maintaining the centromere integrity during meiosis, but they all express major concerns about the specificity of Cen-ASO, and point out the necessity of new diagonal approaches to address this concern. Since a substantial amount of new experimental data would be needed to satisfactorily address the concerns of the reviewers, I expect that this would require considerable time and effort.

Given interest in the topic, however, I would be open to resubmission of a significantly revised and extended manuscript that fully addresses the reviewers' concerns and is subject to further peer-review, endeavoring to use the same reviewers. To avoid investing in a revision that may not be sufficient for re-review, however, if you are interested in resubmitting to JCB, please first contact the journal office to initiate discussion about a potential revision plan. Assuming that the novelty of the findings has not been compromised in the interim, it is likely that a suitably revised manuscript could be re-reviewed and further considered for publication in our journal.

Although your manuscript is intriguing, I feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. If you wish to expedite publication of the current data, it may be best to pursue publication at another journal. Our journal office can transfer your reviewer comments to another journal upon request.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss the reviewer comments further once you've had a chance to consider the points raised in this letter. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Hironori Funabiki, Ph.D.
Reviewer #1 (Comments to the Authors (Required)):

This manuscript describes the phenotype of mouse oocytes following introduction of an anti-sense oligonucleotide against the minor satellite of centromeres (Cen-ASO). First, they showed that Cen-ASO depleted minor satellite transcripts. Second, they observed highly frequent meiotic arrest in Cen-ASO-microinjected oocytes. This arrest was associated with failure of securin degradation and depended on the spindle checkpoint activity. Live imaging and quantitative chromosome dynamics analysis showed severe chromosome alignment defects in Cen-ASO-microinjected oocytes. Notably, they frequently observed breaks at major satellite regions, which depended on bipolar forces exerted via spindle microtubules. Such centromere damages have not been described in previous studies using somatic cells, and therefore the observation described here suggests oocyte-specific requirement of minor satellite transcripts for protecting major satellite DNAs from damages. Overall this is an interesting manuscript reporting unexpected phenotypes. However, their conclusion about the role for minor satellite transcripts should require additional experiments to be robustly supported. Especially, major concerns would be on (1) the questionable specificity of Cen-ASO and (2) lack of explanation to how minor satellite transcripts might remotely act to prevent damages at major satellite DNAs.

Major points
1) Most importantly, all the dataset came from experiments using only one oligonucleotide targeting the minor satellite transcript (Cen-ASO). Could the authors design another oligonucleotide and show that same phenotypes are observed? Alternatively, any other experimental tools that may specifically deplete minor satellite transcripts?
2) The specificity of Cen-ASO is questionable, especially based on the images of the chromosomes of 100uM-Cen-ASO-microinjected oocytes (Figure 2A). They appear to have less-condensed chromosome arms, which may suggest off-targets. In the experiments shown in Figure 3 and 4, they used 40uM Cen-ASO conditions, in which chromosome arms appear to be morphologically normal. But can the authors exclude the possibility that invisible levels of side effects along chromosome arms induced spindle-checkpoint-dependent arrest?
3) Figure 1D and E - these experiments should be done in 40 uM Cen-ASO conditions, instead of 100 uM. It would be informative to add Reversine before meiotic resumption, and compare the timing of anaphase onset between H2O+Reversine and Cen-ASO+Reversine, which would be a good test for spindle-checkpoint-independent effects of Cen-ASO.
4) There could be some side effects even at 40 uM conditions. How about testing lower concentrations, such as 20 uM and 10 uM? In such conditions, could RNA depletion levels be correlated with the phenotypes observed?
5) Figure 3 - please test endogenous CENP-C levels by immunostaining. Quantitative data of CENP-C signals (antibodies and CenpC-mCherry) would be helpful.
6) The authors suggest that microtubule attachments to kinetochores are defective in Cen-ASO oocytes. This could be experimentally addressed by immunostaining.
7) Please quantify Mad2 and/or other active spindle checkpoint markers on kinetochores in Cen-ASO oocytes. The results of 6) and 7) would help support the activation of the spindle checkpoint in these oocytes.

8) How minor satellite transcripts play a role in the integrity of major satellite DNAs? The minor satellite transcripts translocate to major satellite DNA regions to protect them? It would be ideal if the authors could experimentally address these questions, or at least discussions should be provided.

9) page9: "These transcripts are abundant" - This statement should require a measurement of centromeric RNA transcripts.

Minor points:
(1) Fig 1B - 100 uM and 40 uM labels are swapped.
(2) The concentration of Reversine used is not described.
(3) How much is the concentration of 5MM-ASO?
(4) Fig 3B - arrows do not indicate chromosomes shown in the insets.
(5) Fig 4E - any explanations to RNase H in the text or legend?
(6) typos: resent (p3 line15), ubiquitinisation (p5 line12), time-lase (p8 line11)

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

In the manuscript by Wu et al., the authors describe a fascinating feature of mouse oocyte meiosis that could be implicated in contributing to the high rates of aneuploidy that occur in the female germline. Following findings from cancer cell biology, the authors examine mouse oocytes for the presence of non-coding RNAs that are made from centromeres. They find by using anti-sense oligo knockdown of these RNAs, that mouse oocytes arrest in meiosis I and frequently have "damaged" centromeres that split apart from the bivalents. Partial rescue of the MI arrest with Reversine treatment suggests that the arrest is SAC-mediated; and rescue of the splitting phenotype by reducing kinetochore-microtubule tension via monastrol treatment suggests that the RNAs provide a protective mechanism in a high tension microtubule setting. Overall, this reviewer is excited by the model presented, but my enthusiasm is reduced because of over-interpretations, small sample sizes, and lack of experimental details. These are described below:

1. The rescue with reversine is an important experiment. Currently only 19 oocytes were assessed making this underpowered to conclude that the arrest is SAC induced. Furthermore, if this experiment was performed with any replicates (it is not indicated), then the number of oocytes per experiment is extremely limited to draw a meaningful conclusion. More replicates are required. The authors show that 12/19 extrude a polar body upon reversine treatment. Why do the authors think the other 7 fail to extrude a polar body? Do they think the SAC activated in an MPS1- independent manner? Does MAD2 still localize to these kinetochores? Does MAD2 get more enriched at the broken centromeres? Could a combination of Reversine + Monastrol treatment support 100% polar body extrusion? Ultimately the connection between the cell cycle defects and centromere/kinetochore defects are interesting but unfortunately not well substantiated.

2. Fully grown oocytes are transcriptionally silent. When do the authors speculate that these centromeres are being transcribed? It would be helpful to explain this point to the reader for cell type context.

3. In Figure 1D it looks like securin-yfp starts to re-accumulate in the reversine treated ASO group.
Is this a representative panel of images? If so, why do the authors think securin starts to accumulate again? If not representative, then the authors should consider another oocyte to show.

4. The threads of satellite DNA shown in Figure 3B remind me of findings from Zielinska AP and Schuh M in Current Biology in 2019. Can the authors speculate and connect findings here to kinetochore splitting and chromosome fibers found in the 2019 paper?

5. Page 3: Balboula et al did not specifically show that "non-coding RNAs are essential for meiosis progression". Their experiments injecting RNaseA into oocytes cannot distinguish coding vs non-coding RNAs.

6. Figure 3B: I'm confused by the zoom panels. The legend indicates that the arrows correspond to the zoom, but they do not seem to correlate. Perhaps a box would be more accurate?

7. Figure 3E- what is the gray line of tubulin measuring? From pole to pole? Are these lines in 3E an average or just one representative scan?

8. The authors need to detail how their images were analyzed. What do they mean by "angle" in the 3D style plots? Is this a necessary measurement? It is not discussed in the results. If it is not necessary, I recommend removing these 3D style plots and presenting the data is a standard 2D mode.

9. Error bars are missing from many figures such as 1C,

10. The 100 and 40 uM labels in Figure 1B are flipped.

11. P value in text for Fig 3B, C looks like a place holder?

12. Insert appropriate primary literature citations for: monastrol function (page 8), SAC function (page 2), major satellite definition (page 2)

Text issues:

Centromere text is written specific for describing mouse centromeres (highly ordered repeats) but is conveyed that this is a ubiquitous property of centromeres. Many organisms don't have sequence defined centromeres. This introduction section needs to be written with more precision.

"reversine to overcome a SAC arrest" is not precise. Reversine inhibits MPS1, the kinase that triggers recruitment of SAC MCC components. Therefore, reversine treatment prevents SAC signaling.

GV stage oocytes; GV is not a stage- it is more appropriate to say GV intact. Prophase I is a cell cycle stage.

The manuscript should be carefully edited by the native English speaking co-authors. There are many typos, sections of awkward phrasing and grammatical errors to tend to.

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

This paper presents a functional analysis of centromere-derived RNA transcripts during mouse meiosis. Using antisense oligonucleotides targeting the mouse minor satellite repeats, the authors find that injection of these oligos results in meiotic chromosome segregation defects due to extensive DNA damage. Based on these findings, the authors provide a model in which centromere transcription prevents DNA damage. These studies are focused on an interesting and still poorly understood topic of centromere transcription and centromere-derived transcripts. However, all of the data presented in this paper relies on their antisense targeting strategy, and I am strongly concerned about this data and their interpretations. In the absence of an orthologous approach to selectively target minor satellite-derived transcripts and the other experiments listed below, this
1. There have been a variety of models put forward describing roles for the transcription of centromere regions or roles of centromere-derived transcripts, including recent work suggesting that these transcripts don't play a functional role. However, protecting centromere regions from DNA damage has not been a phenotype observed to my knowledge in any prior work, creating caution for the findings here. This could be related to the organism or the fact that this present study is focused on oocytes, but due to the fundamental nature of centromeres, it seems surprising that this would vary that much between other systems. Thus, instead this phenotype could be an artifact of the technical strategy used to target the centromere RNAs.

2. The simplest explanation for this data is that the antisense oligo is acting to induce the DNA damage, either by targeting to centromere regions and complexing with DNA there, or indirectly through RNA interactions. The authors provide a negative control for a similar oligo that lacks the ability to associate with minor satellite repeats due to sequence changes. However, they do not provide a positive control for a sequence that targets a different genomic repeat and a distinct long non-coding RNA. As I am concerned that the ASO oligo and this targeting strategy itself is responsible for the observed phenotypes and does not reflect a role for centromere-derived RNAs, the authors should conduct similar experiments with a sequence that recognizes a different prevalent repeat region (for example, LINEs).

3. Similar to the point above, it would be important to show a similar phenotype to that observed for their ASO strategy using an alternative targeting scheme. One possibility would be for them to use their Transcription Activator-like Effector (TALE) construct, which they show recognizes the minor satellite repeats and centromere regions. They could create a fusion between the TALE and an RNAse to locally degrade RNA in the vicinity of centromeres.

4. In contrast to somatic cells where transcriptional inhibition will rapidly result in a variety of phenotypes including cell death, oocytes have a pool of maternal RNAs such that they don't require ongoing transcription for viability. A recent study found that completely inhibiting transcription in oocytes (albeit from a different organism) did not result in meiotic chromosome segregation defects (PMID: 31422918). The authors should conduct similar studies to completely inhibit RNA Polymerase II, such as using Triptolide or alpha-Amanitin.

5. DNA damage is assessed visually based on chromosome morphology. It would be helpful to have an additional marker for DNA damage, such as gamma-H2AX.

6. As a minor point, the authors state that "Centromeric RNA was detected in oocytes at all 3 timepoints, with peak expression during MI." However, they are not measuring expression, but instead are measuring levels, which will reflect both transcription and degradation.
We thank all the reviewers for their helpful suggestions and comments. You can see that the manuscript has undergone extensive revision with the inclusion of further experiments and accurate description. Here we give a detailed response to the points raised.

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

This manuscript describes the phenotype of mouse oocytes following introduction of an anti-sense oligonucleotide against the minor satellite of centromeres (Cen-ASO). First, they showed that Cen-ASO depleted minor satellite transcripts. Second, they observed highly frequent meiotic arrest in Cen-ASO-microinjected oocytes. This arrest was associated with failure of securin degradation and depended on the spindle checkpoint activity. Live imaging and quantitative chromosome dynamics analysis showed severe chromosome alignment defects in Cen-ASO-microinjected oocytes. Notably, they frequently observed breaks at major satellite regions, which depended on bipolar forces exerted via spindle microtubules. Such centromere damages have not been described in previous studies using somatic cells, and therefore the observation described here suggests oocyte-specific requirement of minor satellite transcripts for protecting major satellite DNAs from damages. Overall, this is an interesting manuscript reporting unexpected phenotypes. However, their conclusion about the role for minor satellite transcripts should require additional experiments to be robustly supported. Especially, major concerns would be on (1) the questionable specificity of Cen-ASO and (2) lack of explanation to how minor satellite transcripts might remotely act to prevent damages at major satellite DNAs.

Major points
1) Most importantly, all the dataset came from experiments using only one oligonucleotide targeting the minor satellite transcript (Cen-ASO). Could the authors design another oligonucleotide and show that same phenotypes are observed? Alternatively, any other experimental tools that may specifically deplete minor satellite transcripts?

We thank the reviewer for this suggestion. Multiple experimental tools should be used to confirm the phenotypes. A new siRNA oligonucleotide was designed to knock down the minor satellite transcript (Details in Method). The efficiency of this siRNA was tested (Fig.S1D), and same phenotypes are observed (Fig.S1E, F), suggesting the phenotype is not an artefact generated by the ASO (indeed we used a 5MM-ASO in the original work).

2) The specificity of Cen-ASO is questionable, especially based on the images of the chromosomes of 100uM-Cen-ASO-microinjected oocytes (Figure 2A). They appear to have less-condensed chromosome arms, which may suggest off-targets. In the experiments shown in Figure 3 and 4, they used 40uM Cen-ASO conditions, in which chromosome arms appear to be morphologically normal. But can the authors exclude the possibility that invisible levels of side effects along chromosome arms induced spindle-checkpoint-dependent arrest?
Please note the off target effect described above may indeed be an on target effect as a result of the loss of centromeric RNA. It is not the focus here but there is no a priori reason to suppose it is indeed off target. It is not observed with the 5MM-ASO control, so it is not a result of simply adding ASO. As this reviewer suggested, we have stopped using 100uM to deplete centromeric RNA (See Q3) and tested lower concentration (20uM) which could also induce MI arrest (See Q4). Meanwhile, Mad2 was concentrated on isolated centromeres, which indicated that the SAC was activated by centromere damage (See Q7). Further we point out that if changes do occur in the chromatin in response to the ASO, this may not necessarily be an off-target effect, since at this point the roles of the centromeric RNAs in meiosis remain unknown.

3) Figure 1D and E - these experiments should be done in 40uM Cen-ASO conditions, instead of 100uM. It would be informative to add Reversine before meiotic resumption and compare the timing of anaphase onset between H2O+Reversine and Cen-ASO+Reversine, which would be a good test for spindle-checkpoint-independent effects of Cen-ASO.

As this reviewer suggested, Figure 1D and E were performed in 40uM Cen-ASO condition (Fig 1 F and G). The result is consistent with 100uM Cen-ASO. The timing of PBE was compared between H2O+Reversine and Cen-ASO+Reversine, no significant differences were detected (Fig 1 D and E). Therefore, The activation of SAC induced MI arrest in 40uM Cen-ASO condition.

4) There could be some side effects even at 40uM conditions. How about testing lower concentrations, such as 20uM and 10uM? In such conditions, could RNA depletion levels be correlated with the phenotypes observed?

Indeed, the 20uM Cen-ASO was tested previously. The knockdown efficiency of 20uM Cen-ASO concentration is lower than 40uM, while the RNA depletion level is correlated with oocyte MI completion rate (Fig 1 B and C).

5) Figure 3 - please test endogenous CENP-C levels by immunostaining. Quantitative data of CENP-C signals (antibodies and CenpC-mCherry) would be helpful.

The CENP-C was tested by immunostaining in 5MM-ASO and Cen-ASO injected oocytes. No difference was shown in statistical analysis (Fig 2 F and G).

6) The authors suggest that microtubule attachments to kinetochores are defective in Cen-ASO oocytes. This could be experimentally addressed by immunostaining.

The K-Mt attachment defects was detected by immunofluorescence after cold treatment (Figure 2D and E).
7) Please quantify Mad2 and/or other active spindle checkpoint markers on kinetochores in Cen-ASO oocytes. The results of 6) and 7) would help support the activation of the spindle checkpoint in these oocytes.

The SAC component Mad2 was stained in Cen-RNA depletion oocytes. Mad2 was shown concentrated on damaged centromeres but not intact centromeres (Fig 3I and J).

8) How minor satellite transcripts play a role in the integrity of major satellite DNAs? The minor satellite transcripts translocate to major satellite DNA regions to protect them? It would be ideal if the authors could experimentally address these questions, or at least discussions should be provided.

Thanks for these useful questions. We would like to do further investigations in following projects. As suggested the discussion was added to the end of this report.

9) page9: “These transcripts are abundant” - This statement should require a measurement of centromeric RNA transcripts.

We are sorry for the use of the word abundant as we have no reference point. The term is no longer used.

Minor points:
(1) Fig 1B - 100uM and 40uM labels are swapped.

We have corrected.

(2) The concentration of Reversine used is not described.

The concentration of Reversine (100nM) was added to Method.

(3) How much is the concentration of 5MM-ASO?

The concentration of 5MM-ASO (40μM) was labelled in the figures.

(4) Fig 3B - arrows do not indicate chromosomes shown in the insets.

Due to the Z-stack images, chromosomes are overlapped with each other. We have changed the arrows to squares which could make it clearer.

(5) Fig 4E - any explanations to RNase H in the text or legend?

The information on RNase H has been added into the legend of Figure 4H now.
Thanks. We have corrected these typos.
Reviewer #2 (Comments to the Authors (Required)):

In the manuscript by Wu et al., the authors describe a fascinating feature of mouse oocyte meiosis that could be implicated in contributing to the high rates of aneuploidy that occur in the female germline. Following findings from cancer cell biology, the authors examine mouse oocytes for the presence of non-coding RNAs that are made from centromeres. They find by using anti-sense oligo knockdown of these RNAs, that mouse oocytes arrest in meiosis I and frequently have “damaged” centromeres that split apart from the bivalents. Partial rescue of the MI arrest with Reversine treatment suggests that the arrest is SAC-mediated; and rescue of the splitting phenotype by reducing kinetochore-microtubule tension via monastrol treatment suggests that the RNAs provide a protective mechanism in a high tension microtubule setting. Overall, this reviewer is excited by the model presented, but my enthusiasm is reduced because of over-interpretations, small sample sizes, and lack of experimental details. These are described below:

1. The rescue with reversine is an important experiment. Currently only 19 oocytes were assessed making this underpowered to conclude that the arrest is SAC induced. Furthermore, if this experiment was performed with any replicates (it is not indicated), then the number of oocytes per experiment is extremely limited to draw a meaningful conclusion. More replicates are required. The authors show that 12/19 extrude a polar body upon reversine treatment. Why do the authors think the other 7 fail to extrude a polar body? Do they think the SAC activated in an MPS1-independent manner?

We don’t think the reviewer means underpowered in a statistical sense. In any event an underpowered study is only a problem if no statistical difference is observed. However the reviewer is correctly pointing out that it would be useful to have different doses tested. In the revised manuscript we use 40uM Cen-ASO (n=91) instead of 100uM to perform this experiment. The vast majority of such Cen-RNA KD oocytes extrude a polar body after Reversine (Fig 1C). It may be that arrest induced by 100uM Cen-ASO does induce such large amounts of DNA damage that it cannot simply be reversed by a lowering of Mps1 activity.

Does MAD2 still localize to these kinetochores? Does MAD2 get more enriched at the broken centromeres?

The SAC component Mad2 was stained in Cen-RNA depleted oocytes. Mad2 was shown concentrated on damaged centromeres but not intact centromeres (Fig 3I and J). Therefore, the damaged centromeres activated the SAC to arrest oocytes at MI.

Could a combination of Reversine + Monastrol treatment support 100% polar body extrusion? Ultimately the connection between the cell cycle defects and centromere/kinetochore defects are interesting but unfortunately not well substantiated.

As requested by another reviewer, we repeated the experiments with 40uM Cen-ASO. Most oocytes extrude the first polar body after Reversine treatment (Fig 1C). The onset of anaphase
was also compared between control and Cen-RNA depleted oocytes (Fig 1D), which indicate that the SAC activation is responsible for MI arrest.

2. Fully grown oocytes are transcriptionally silent. When do the authors speculate that these centromeres are being transcribed? It would be helpful to explain this point to the reader for cell type context.

The cell type context was added into the introduction. The RNAs are transcribed in growing oocyte (Seydoux and Braun, Cell 2006; DeJong, Gene 2006).

3. In Figure 1D it looks like securin-yfp starts to re-accumulate in the reversine treated ASO group. Is this a representative panel of images? If so, why do the authors think securin starts to accumulate again? If not representative, then the authors should consider another oocyte to show.

The reversine treatment experiments were updated for 40uM Cen-ASO and representative images were shown in Figure 1F. The re-synthesis of securin is a normal occurrence following completion of MI as it is required to protect sister chromatid cohesion in MII (Herbert et al. NCB 2003).

4. The threads of satellite DNA shown in Figure 3B remind me of findings from Zielinska AP and Schuh M in Current Biology in 2019. Can the authors speculate and connect findings here to kinetochore splitting and chromosome fibers found in the 2019 paper?

Done. We added the speculations and connections to the discussion.

5. Page 3: Balboula et al did not specifically show that "non-coding RNAs are essential for meiosis progression". Their experiments injecting RNaseA into oocytes cannot distinguish coding vs non-coding RNAs.

Thank you. We have changed our interpretation. The "non-coding" description was removed.

6. Figure 3B: I'm confused by the zoom panels. The legend indicates that the arrows correspond to the zoom, but they do not seem to correlate. Perhaps a box would be more accurate?

Due to the Z-stack images, chromosomes are overlapped with each other. That is the reason why we choose some specific slices and show them in an enlarged version. They are correlated. We have changed the arrows to square which could make it clearer. Apologises for the confusing presentation.
7. Figure 3E - what is the gray line of tubulin measuring? From pole to pole? Are these lines in 3E an average or just one representative scan?

The gray line showed the intensity of tubulin, which is measured between paired centromeres. The representative image presented intact and damaged bivalents.

8. The authors need to detail how their images were analyzed. What do they mean by “angle” in the 3D style plots? Is this a necessary measurement? It is not discussed in the results. If it is not necessary, I recommend removing these 3D style plots and presenting the data in a standard 2D mode.

We supposed that the 3D style is more efficient to show the bivalents alignment defects systematically. The detailed measurements description was updated in Fig S2 A, B, C.

9. Error bars are missing from many figures such as 1C.

Error bars are now provided in all figures.

10. The 100 and 40uM labels in Figure 1B are flipped.

Done, we apologize for the error in figure assembly.

11. P value in text for Fig 3B, C looks like a placeholder?

Thank you - data now added.

12. Insert appropriate primary literature citations for: monastrol function (page 8), SAC function (page 2), major satellite definition (page 2)

Done. The primary literature is properly cited.

Text issues:
Centromere text is written specific for describing mouse centromeres (highly ordered repeats) but is conveyed that this is a ubiquitous property of centromeres. Many organisms don’t have sequence defined centromeres. This introduction section needs to be written with more precision.

Yes. We add ‘In most organisms’ before this sentence and a reference behind.

“reversine to overcome a SAC arrest” is not precise. Reversine inhibits MPS1, the kinase that triggers recruitment of SAC MCC components. Therefore, reversing treatment prevents SAC signaling.

Yes. We have changed our description.
GV stage oocytes; GV is not a stage - it is more appropriate to say GV intact. Prophase I is a cell cycle stage.

Yes. 'GV stage oocytes' was changed to GV intact oocytes.

The manuscript should be carefully edited by the native English speaking co-authors. There are many typos, sections of awkward phrasing and grammatical errors to tend to.
Reviewer #3 (Comments to the Authors (Required)):

This paper presents a functional analysis of centromere-derived RNA transcripts during mouse meiosis. Using antisense oligonucleotides targeting the mouse minor satellite repeats, the authors find that injection of these oligos results in meiotic chromosome segregation defects due to extensive DNA damage. Based on these findings, the authors provide a model in which centromere transcription prevents DNA damage. These studies are focused on an interesting and still poorly understood topic of centromere transcription and centromere-derived transcripts. However, all of the data presented in this paper relies on their antisense targeting strategy, and I am strongly concerned about this data and their interpretations. In the absence of an orthologous approach to selectively target minor satellite-derived transcripts and the other experiments listed below, this paper is not suitable for publication in the JCB.

1. There have been a variety of models put forward describing roles for the transcription of centromere regions or roles of centromere-derived transcripts, including recent work suggesting that these transcripts don’t play a functional role. However, protecting centromere regions from DNA damage has not been a phenotype observed to my knowledge in any prior work, creating caution for the findings here. This could be related to the organism or the fact that this present study is focused on oocytes, but due to the fundamental nature of centromeres, it seems surprising that this would vary that much between other systems. Thus, instead this phenotype could be an artifact of the technical strategy used to target the centromere RNAs.

As this reviewer suggested, the centromeric DNA damage was never reported in any prior publications. Similarly, we also suggest that that the unexpected phenotype described here may result from the specific process of meiotic spindle formation and the exceptionally long duration of this quite unique cell division. Meiosis is very different from mitosis. Therefore, the centromeric RNA could have found a niche role in oocyte meiosis which helps to complete this special biological process.

To confirm the technical strategy is appropriate, we used a parallel approach, using a different depletion technology (siRNA), and targeted a different sequence in the minor satellite repeat to repeat key experiments (see Q3).

2. The simplest explanation for this data is that the antisense oligo is acting to induce the DNA damage, either by targeting to centromere regions and complexing with DNA there, or indirectly through RNA interactions. The authors provide a negative control for a similar oligo that lacks the ability to associate with minor satellite repeats due to sequence changes. However, they do not provide a positive control for a sequence that targets a different genomic repeat and a distinct long non-coding RNA. As I am concerned that the ASO oligo and this targeting strategy itself is responsible for the observed phenotypes and does not reflect a role for centromere-derived RNAs, the authors should conduct similar experiments with a sequence that recognizes a different prevalent repeat region (for example, LINEs).
Actually, in our previous research, a specific ASO target major satellite repeats was used to knocked down the transcripts, however, it does not affect MI completion (Fig S1G, H and I). We now include these data here as it serves as the control the reviewer is seeking.

3. Similar to the point above, it would be important to show a similar phenotype to that observed for their ASO strategy using an alternative targeting scheme. One possibility would be for them to use their Transcription Activator-like Effector (TALE) construct, which they show recognizes the minor satellite repeats and centromere regions. They could create a fusion between the TALE and an RNAse to locally degrade RNA in the vicinity of centromeres.

To avoid the artifact of the technical strategy, an siRNA was designed to deplete centromeric RNA. The efficiency of siRNA was tested (Fig.S1D), and the same phenotypes are observed (Fig.S1E, F).

4. In contrast to somatic cells where transcriptional inhibition will rapidly result in a variety of phenotypes including cell death, oocytes have a pool of maternal RNAs such that they don’t require ongoing transcription for viability. A recent study found that completely inhibiting transcription in oocytes (albeit from a different organism) did not result in meiotic chromosome segregation defects (PMID: 31422918). The authors should conduct similar studies to completely inhibit RNA Polymerase II, such as using Triptolide or alpha-Amanitin.

In the revision, RNA Polymerase II was inhibited by alpha-amanitin. Similar to the mentioned article, RNA transcription was not required for oocyte MI completion (Fig.2A and B).

5. DNA damage is assessed visually based on chromosome morphology. It would be helpful to have an additional marker for DNA damage, such as gamma-H2AX.

Thank you for this suggestion. Gamma-H2AX staining showed the DSBs concentrated at major satellite repeats (Fig.3 A and B).

6. As a minor point, the authors state that “Centromeric RNA was detected in oocytes at all 3 timepoints, with peak expression during MI.” However, they are not measuring expression, but instead are measuring levels, which will reflect both transcription and degradation.

Thank you for this suggestion. We have changed that to ‘peak level’.
June 14, 2021

Re: JCB manuscript #202011153R-A

Dr. Tianyu Wu  
Dr. Keith T. Jones

Shanghai Medical College of Fudan University  
NO. 131 Dong'an Road  
Shanghai 200032  
China

Dear Drs. Wu and Jones,

Thank you for submitting your manuscript entitled "Loss of centromeric RNA activates the spindle assembly checkpoint in mammalian female meiosis I." The manuscript has now been assessed by the three original reviewers. Based on their comments, I would like to invite you to submit a final revised version, if you can address the remaining important points, as outlined here.

1. Reviewer #3 raises a strong concern that the reported phenotype that you observed upon Cen-ASO is an off-target effect. To make sense of your observations, one may assume that minor satellite RNA that had been transcribed prior to oocyte maturation must stay at the centromere, and somehow protect the major satellite repeats at the pericentromeric heterochromatin. However, it is not clear if this is how you interpret the result. Furthermore, this does not seem to be consistent with reported behaviors of human centromere RNAs during mitosis - human centromere RNAs stay in cis at the transcription site (PMID 28787590). More recently, it was also reported that the majority of centromere RNA dissociate from the centromere during mitosis (PMID: 33174837). It would be important to relate your findings with these previous reports and discuss the apparent contradictions. In addition, the mechanism by which minor satellite RNA can protect major satellite DNA segments is far from clear. Can you really rule out the possibility that the phenotypes that you observed are not off-target effects of Cen-ASO? Could it be possible that Cen-ASO actually targets DNA, perhaps via DNA triplex formation, or interferes with proteins that interact with Cen-ASO? (By the way, please define "mN" and asterisks in ASO oligo sequences.) It would be important to fully discuss the caveats and limitations of this study.

2. RNA-FISH (Figure S1A and B) is a critical control experiment to validate the findings reported in this paper. There are several issues with the presentation. First, the shape of chromosomes in Cen-ASO samples look much fatter than control samples. Since the experiment was apparently performed only once, it would be important to show that the negative data of Cen-ASO samples are not due to technical artifacts that affect preservation of the sample. To address this point, it would be important to show that the result is reproducible. If the overall shape of chromosomes is reproducibly altered by Cen-ASO, this would suggest that Cen-ASO treatment would cause non-specific effects on chromosome architecture, which may explain the microtubule-dependent damage at the peri-centromeric heterochromatin. Second, the experimental conditions used for the +RNase sample are not described in the methods section. Assuming that this is RNaseA treatment, please describe the detailed procedure, including the information about the source and concentration of RNaseA.
Third, the background intensity of FISH signals is too low. Upon adjustment, I found that signals for reverse probes for +RNase condition in S1A do not match between the merged image and the single channel image (please see attached file for examples). There must be some error in the selection of the image. Please clarify this by providing your original high-resolution images.

3. Reviewer #1 raises three specific issues. Please address these points. Considering the concerns related to Cen-ASO treatment as pointed out above, I would recommend that you check if RNAi indeed leads to disappearance of RNA-FISH signals and generates DNA damage.

4. As Reviewer #2 notes, there are numerous grammatical errors throughout the manuscript (e.g., Page 4, "In addition, we were also tested ...). Many sentences can be simplified/restructured (e.g., Page 4, "It was observed that the level of forward transcripts was nearly double that of reverse transcripts (Fig. S1B; p<0.0001, t-test), therefore we treated the forward transcripts as the main centromeric RNA and focused on them in all further experiments.""). A space must be inserted between numbers and units (e.g., 40 μM, not 40μM). Since co-authors are based on UK, it is difficult for me to find an excuse to ignore these issues. Although the revision will require a substantial extension of the Discussion, I expect that the authors can create space by omitting unnecessary words.

5. I found some additional abnormal/questionable images as shown in the attached file. Please explain how these problematic images were generated. Please submit your original image files (including metadata) for review. Details of image processing procedures must also be disclosed.
   a. Fig. 1F: Even after fully increasing brightness, there are no detectable signals at 9:00 for 5MM-ASO and Cen-ASO samples, questioning if these images actually contain any cellular micrographs.
   b. Fig. 2D: Microtubule signals are artificially segmented into vertical segments.
   c. Fig. 2D: Arrowheads added below to point out "artificially" segmented boundaries.
   d. Fig. 2D: The image is supposed to contain only green and black/white signals, but red and blue signals can be found.

In summary, as pointed out by Reviewer #3, the results reported in this manuscript do not fit well with previous observations in the field. We feel that this is one of the most interesting aspects of the manuscript. However, to make extraordinary claims, the authors must present solid evidence that experiments were executed carefully and with the highest standards.

Our general policy is that papers are considered through only one revision cycle; but, in this case we are open to one additional short round of revision. However, please note that this does not constitute a commitment to accept the revised manuscript and that we may reject it if the revisions do not satisfactorily address both the reviewer comments and our concerns as outlined above.

The typical time frame for such final revisions is one month. However, we understand that measures implemented to limit the spread of COVID-19 also pose challenges to scientific researchers so please let us know if you will require more time. Please note that we expect to make a final decision without additional reviewer input upon resubmission. Along with the revised manuscript please submit a cover letter that includes a point-by-point response to the remaining reviewer comments.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact me or the scientific editor listed below at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.
Reviewer #1 (Comments to the Authors (Required)):

The revised manuscript now includes new experiments and discussions that address my concerns raised in the previous round of review. I would support publication if the following comments to their newly incorporated data could be addressed.

1) Their siRNA-mediated knockdown reduced the rate of MI completion, which nicely recapitulated the results of Cen-ASO-mediated knockdown. However, what kind of defects caused MI arrest in siRNA-injected oocytes remains unclear. Does the siRNA-mediated knockdown also recapitulate centromeric DNA damages, which was observed in Cen-ASO-injected oocytes? It seems that they indeed observed centromeric damages in siRNA-injected oocytes, as shown in Fig. S1E, but this result is not mentioned in the main text. Showing the rates of centromeric damages with statistical tests would be necessary.

2) It is impossible to judge K-Mt attachments in Fig. 2D. Magnified images of non-attached and attached kinetochores would be helpful.

3) How do they determine "damaged centromeres" in experiments Fig. 3C and Fig. 3I? Perhaps based on separation of ACA signals from chromosomes? Maybe I was confused by Fig 3C, in which they show a chromosome missing one MajSat-mClover spot, indicated by a yellow box. To me this looks like a chromosome with a damaged centromere, but the legend says the yellow box indicates a chromosome without damaged centromeres.

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

I appreciate the attention to increasing the number of oocytes used in experiments and assessing the SAC in other ways. I find the MS much improved and reports important findings. The authors need to detail all of the plasmids in their methods (a few are missing). I also urge them to copy edit as there are still many English language issues throughout (too many to list).

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

For the revised paper, the authors have conducted several additional experiments and have edited
the text to alter some of their conclusions. Although I appreciate the effort that they have made for this paper and I value the importance of studying these processes during oocyte meiosis, I continue to be concerned about the data and conclusions in this paper.

For the previous paper, the model from the authors was that active transcription at centromeres during meiosis was required for core meiotic activities and chromosome segregation. For this revised paper, they now instead propose that there is not active transcription in isolated oocytes, and instead that any RNAs present represent maternally produced transcripts that are loaded into the oocyte. The major reason that is likely responsible for this change is that they now provide data to show that RNA Polymerase II inhibition using alpha-Amanitin does not result in any of the effects or behaviors that they report. Instead of creating strong caution for the interpretation of the morpholino experiments due to the highly differing results, this caused the authors to reframe their model. This is a major change in their model, concept, and ideas, and something that gives caution for considering the interpretation of the behaviors. This is substantially different from most existing models for possible roles of centromere transcription during mitosis, in which ongoing transcription affect centromere-related process, not the transcripts themselves.

In my opinion, this substantial change in their model requires an additional level of proof related to the RNA transcripts. The idea that active and ongoing transcription at centromeres would create a defect that is localized specifically to centromere regions would make sense. However, if transcripts that are produced maternally are subsequently involved in centromere function (specifically at this region), this would require that these mature transcripts act in trans and are localized to centromeres through their associations with a specific RNA binding protein. This seems highly unlikely and it is important to note that no prior study has provided clear evidence for this behavior (the paper that they cite is very likely for nascent transcripts). This would require centromere-derived RNAs to contain specific sequences that would be recognized by protein factors, and then subsequently targeted back to centromere regions. Ideally, it would be nice to test the localization of centromere-derived RNAs using their FISH probe to assess whether alpha-Amanitin treatment and a second RNA polymerase II inhibition strategy (for example, a Cdk7 or Cdk9 inhibitor) causes a change in satellite levels at centromeres. In this case, this would suggest that there is some level of ongoing transcription (which would be interesting), but that this is not required for oocyte function, possibly highlighting an off target effect of the morpholinos.

Ultimately, I still believe that the most likely explanation is that some features of their treatments are compromising the repetitive DNA sequences present at centromere regions, likely due to a technical off-target effect.

If this paper does move forward, my strong recommendation would be for the authors to use caution in their wording and conclusions, as well as provide an extended discussion highlighting the limitations of the current study, possible issues, and alternative interpretations.

Minor points:

• The introduction strongly overstates the nature of the prior literature on centromere transcription and transcripts, and how well established these different points are. There is still a strong debate on these, and many of these statements are highly suspect.

• I disagree with the author's statement in their response letter that "Meiosis is very different from mitosis." There are of course differences, but in many cases very similar related concepts and processes occur. To invoke this to explain discrepancies with prior work does not seems appropriate, as technical issues remains the most likely explanation. If there are differences, then
there should be a clear molecular explanation for these behaviors.
Dear editor:

We thank the editor and the reviewers for their careful and considered feedback on the manuscript. Here we give a point-by-point response those comments (our responses in red; yours in black bold). We have extensively revised the manuscript throughout to improve the quality of the written English. We have not marked these changes in the text as they do not change the scientific narrative, but we have marked on the resubmission passages of text with extensive revision- where we highlight those changes specifically below.

1. Reviewer #3 raises a strong concern that the reported phenotype that you observed upon Cen-ASO is an off-target effect.

We remind the reviewer and editor, that we have used a 5 base mis-match ASO as a control. Such a mis-match is regarded as the best control for antisense. We also point to the fact that we observed the damage phenotype with respect to an entirely separate siRNA approach. This is reported in the submission. Finally, we also point to the fact that no equivalent phenotype was reported in the paper using a MajSAT ASO. Hence the response occurred for the Cen-ASO, but not the MajSAT ASO. Despite the above we acknowledge it remains feasible that the Cen-ASO, but not the 5MM-ASO, is inducing its action by a mechanism independent on the sequence it is targeting. We have revised the Summary paragraph on p 9 (highlighted) to reflect the fact that conclusions are drawn on the effects of the ASO.

To make sense of your observations, one may assume that minor satellite RNA that had been
transcribed prior to oocyte maturation must stay at the centromere, and somehow protect the major satellite repeats at the pericentromeric heterochromatin. However, it is not clear if this is how you interpret the result.

In the wording of the revised text, p 6 highlighted paragraph, we make this point clear. We include a penultimate paragraph (p 9) which outlines the interpretation.

Furthermore, this does not seem to be consistent with reported behaviors of human centromere RNAs during mitosis - human centromere RNAs stay in cis at the transcription site (PMID 28787590). More recently, it was also reported that the majority of centromere RNA dissociate from the centromere during mitosis (PMID: 33174837). It would be important to relate your findings with these previous reports and discuss the apparent contradictions.

A good point as differences does exist in the literature. The contradictory results might be explained by the use of different species and because the Cen-RNA sequence is not conserved. However, a very relevant recent investigation reports that Cen-RNAs are trans acting at centromeres in yeast (see Fig. 5, PMID: 30850541). We have put all these related results into our discussion (p10).

In addition, the mechanism by which minor satellite RNA can protect major satellite DNA segments is far from clear. Can you really rule out the possibility that the phenotypes that you observed are not off-target effects of Cen-ASO? Could it be possible that Cen-ASO actually targets DNA, perhaps via DNA triplex formation, or interferes with proteins that interact with Cen-ASO? (By the way, please define "mN" and
asterisks in ASO oligo sequences.) It would be important to fully discuss the caveats and limitations of this study.

We have extended the discussion and added the limitations of this investigation. The ASO induced centromere damage is associated with the timing of Cen-RNA decrease because the phenotype requires time for RNA knock down. Without prior incubation of Cen-ASO in GV oocytes, Cen-ASO injection does not induce the centromere damage, which indicates that an RNA decrease is associated with DNA damage (i.e. time for RNA degradation is required).

Please note that siRNAs are double strand structure, which is difficult to bind DNA.

Additionally, the artifact RNA-DNA binding should be equal to each centromere and create the same phenotype on all centromeres. However, only a few centromeres (6 per oocytes) were damaged by Cen-ASO, but not all of them, which is consistent with that the Cen-RNA level and knock down efficiency are variable on centromeres (RNA-FISH). Only a few RNA-FISH signals are invisible with Cen-ASO treatment.

The precise localization of MinSAT RNAs on pericentromeric heterochromatin has been observed and proved by at least two methods in two independent publications (FISH, PMID: 16731634; RIP, PMID: 31677973). The localization is consistent with our results, the DNA cleavage was created at just pericentromeric heterochromatin. Importantly, these consistent results were all from murine cells.

"mN" and asterisks in ASO oligo sequences are defined in Methods (p22, highlighted).
2. RNA-FISH (Figure S1A and B) is a critical control experiment to validate the findings reported in this paper. There are several issues with the presentation.

First, the shape of chromosomes in Cen-ASO samples look much fatter than control samples. Since the experiment was apparently performed only once, it would be important to show that the negative data of Cen-ASO samples are not due to technical artifacts that affect preservation of the sample. To address this point, it would be important to show that the result is reproducible. If the overall shape of chromosomes is reproducibly altered by Cen-ASO, this would suggest that Cen-ASO treatment would cause non-specific effects on chromosome architecture, which may explain the microtubule-dependent damage at the peri-centromeric heterochromatin.

The ‘much fatter’ chromosome is an overlapped image because we showed a z-stack of images. The chromosome alignment was severely affected by the loss of Cen-RNA, which creates the abnormal shape of bivalents distribution after overlap.

As suggested the RNA-FISH experiments were performed twice more: they are repeatable (see this stated in the revised paper p32). The results from these latest experiments have been used for the representative images in the Figure to show the decrease of RNA-FISH signal (see p32 Fig. S1 A,B).

Second, the experimental conditions used for the +RNase sample are not described in the methods section. Assuming that this is RNaseA treatment, please describe the detailed procedure, including the information about the source and concentration of RNaseA.
We have added the required information in methods section (see p24).

Third, the background intensity of FISH signals is too low. Upon adjustment, I found that signals for reverse probes for +RNase condition in S1A do not match between the merged image and the single channel image (please see attached file for examples). There must be some error in the selection of the image. Please clarify this by providing your original high-resolution images.

The images were not matched because the contrast of the images was not the same. As the background intensity of FISH signals is too low, we have selected some more representative images with high background intensity from our latest repeated experiments for this figure (p32 Fig. S1A).

3. Reviewer #1 raises three specific issues. Please address these points. Considering the concerns related to Cen-ASO treatment as pointed out above, I would recommend that you check if RNAi indeed leads to disappearance of RNA-FISH signals and generates DNA damage.

We have confirmed the RNAi induced Cen-RNA knock down by RNA-FISH (p32 Fig. S1I).

4. As Reviewer #2 notes, there are numerous grammatical errors throughout the manuscript (e.g., Page 4, "In addition, we were also tested ...). Many sentences can be simplified/restructured (e.g., Page 4, "It was observed that the level of forward transcripts was nearly double that of reverse transcripts (Fig. S1B; p<0.0001, t- test), therefore we treated the forward transcripts as the main centromeric RNA and focused on them in all further experiments." A space must be inserted between numbers and units (e.g.,
40 μM, not 40μM). Since co-authors are based on UK, it is difficult for me to find an excuse to ignore these issues. Although the revision will require a substantial extension of the Discussion, I expect that the authors can create space by omitting unnecessary words.

We apologize for the poor grammar in parts of the manuscript. We have extensively revised the resubmitted text and as such we hope the syntax is much better.

5. I found some additional abnormal/questionable images as shown in the attached file. Please explain how these problematic images were generated. Please submit your original image files (including metadata) for review. Details of image processing procedures must also be disclosed.

a. Fig. 1F: Even after fully increasing brightness, there are no detectable signals at 9:00 for 5MM-ASO and Cen-ASO samples, questioning if these images actually contain any cellular micrographs.

Levels of securin-GFP can decrease to background levels in oocytes as the APC is active (during either MI or MII). For example, see similar images of securin from our paper nearly two decades ago when we started imaging GFP constructs in eggs: J Cell Sci (2004) 117 (26): 6289–6296. Please see Fig 3 in that paper for the same complete loss of signal, and the reappearance several hours later. For better visualization, the contrast was increased consistently (p12). Original images are attached.

b. Fig. 2D: Microtubule signals are artificially segmented into vertical segments.

c. Fig. 2D: Arrowheads added below to point out "artificially" segmented boundaries.

d. Fig. 2D: The image is supposed to contain only green and black/white signals, but red and blue
signals can be found.

These abnormal features were not observed in original images and our AI files. Original images are attached. The image was z-stack with maximum projection (20 μm for each spindle and 1 μm intervals), only Gaussian blur (Σ=2) was processed.

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

The revised manuscript now includes new experiments and discussions that address my concerns raised in the previous round of review. I would support publication if the following comments to their newly incorporated data could be addressed.

1) Their siRNA-mediated knockdown reduced the rate of MI completion, which nicely recapitulated the results of Cen-ASO-mediated knockdown. However, what kind of defects caused MI arrest in siRNA-injected oocytes remains unclear. Does the siRNA-mediated knockdown also recapitulate centromeric DNA damages, which was observed in Cen-ASO-injected oocytes? It seems that they indeed observed centromeric damages in siRNA-injected oocytes, as shown in Fig. S1E, but this result is not mentioned in the main text. Showing the rates of centromeric damages with statistical tests would be necessary.

Yes, we have shown the damaged centromeres induced by siRNA microinjection in Fig. S1. As
suggested, the rates of centromeric damages with statistical tests were performed and shown in Fig. S1M (see p32). We have put the data in the main text (see p7 highlight).

2) It is impossible to judge K-Mt attachments in Fig. 2D. Magnified images of non-attached and attached kinetochores would be helpful.

The magnified images of non-attached and attached kinetochores have been added to Fig. 2D (see p14).

3) How do they determine "damaged centromeres" in experiments Fig. 3C and Fig. 3I? Perhaps based on separation of ACA signals from chromosomes? Maybe I was confused by Fig 3C, in which they show a chromosome missing one MajSat-mClover spot, indicated by a yellow box. To me this looks like a chromosome with a damaged centromere, but the legend says the yellow box indicates a chromosome without damaged centromeres.

Yes. The separation of centromere signals was treated as damaged centromeres. We have corrected the interpretation (see p17 Fig. 3C legend, highlighted).

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

I appreciate the attention to increasing the number of oocytes used in experiments and assessing the SAC in other ways. I find the MS much improved and reports important findings. The authors need to
detail all of the plasmids in their methods (a few are missing). I also urge them to copy edit as there are still many English language issues throughout (too many to list).

We have detailed all the plasmids in methods section (see p22, highlight).

We have worked extensively on the English in the revised submission.

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

For the revised paper, the authors have conducted several additional experiments and have edited the text to alter some of their conclusions. Although I appreciate the effort that they have made for this paper and I value the importance of studying these processes during oocyte meiosis, I continue to be concerned about the data and conclusions in this paper.

For the previous paper, the model from the authors was that active transcription at centromeres during meiosis was required for core meiotic activities and chromosome segregation. For this revised paper, they now instead propose that there is not active transcription in isolated oocytes, and instead that any RNAs present represent maternally produced transcripts that are loaded into the oocyte. The major reason that is likely responsible for this change is that they now provide data to show that RNA Polymerase II inhibition using alpha-Amanitin does not result in any of the effects or behaviors that they report. Instead of creating strong caution for the interpretation of the morpholino experiments due to the highly differing results, this caused the authors to reframe their model. This is a major change in their model, concept, and ideas, and something that gives caution for considering the interpretation of the behaviors. This is substantially different from most existing models for possible roles of centromere
transcription during mitosis, in which ongoing transcription affect centromere-related process, not the transcripts themselves.

We apologize for the easy misinterpretation that must have been made. We never meant to imply that active transcription during oocyte maturation underlay the phenomenon. We have always taken the view that the dogma that oocytes are transcriptionally quiescent is correct and we never meant to imply that our work breaks that dogma. The addition of the amanitin data in the paper simply shows that. We had always assumed that maternal stores were responsible for the phenomenon being observed. We make this clear in the text.

We do not know the scientific background of the reviewer- hence an apology here. But when we write ‘maternal stores’ we are not implying the RNA is ‘loaded’ (reviewers’ word above) into the oocyte. We are merely using the convention that oocyte stores of RNA exist from the time they were transcriptionally active. Such maternal RNA stores are well described and a known essential component of a GV oocyte.

In my opinion, this substantial change in their model requires an additional level of proof related to the RNA transcripts. The idea that active and ongoing transcription at centromeres would create a defect that is localized specifically to centromere regions would make sense. However, if transcripts that are produced maternally are subsequently involved in centromere function (specifically at this region), this would require that these mature transcripts act in trans and are localized to centromeres through their associations with a specific RNA binding protein. This seems highly unlikely and it is important to note that no prior study has provided clear evidence for this behavior (the paper that they cite is very likely for
We appreciate the RNA knowledge from this reviewer. Although the mechanism of Cen-RNA localization is not clear, the Cen-RNA is found to localize at centromeric and pericentromeric regions by RNA FISH, RIP qRT-PCR or RNA pulldown (PMID: 16731634, PMID: 31677973, PMID: 19542185). The protein (SAFB) associates with MajSAT and MinSAT RNAs at pericentromeric heterochromatin has also been reported (PMID: 31677973).

The relationship between transcription and transcripts was fully discussed in an extensive review (PMID: 2987177). Reports of centromeric RNA complexed with CENPC in the nucleolus (PMID: 17623812), soluble RNA complexes with HJURP and CENP-A (PMID: 25117489), and trans-acting RNAs in Xenopus (PMID: 27184843) and Drosophila (PMID: 25365994) are evidence in favor of roles for the transcripts not coupled to transcription. Meanwhile, a recent investigation clarifies that Cen-RNAs are trans-acting at centromeres (PMID: 30850541, Fig. 5).

This would require centromere-derived RNAs to contain specific sequences that would be recognized by protein factors, and then subsequently targeted back to centromere regions. Ideally, it would be nice to test the localization of centromere-derived RNAs using their FISH probe to assess whether alpha-Amanitin treatment and a second RNA polymerase II inhibition strategy (for example, a Cdk7 or Cdk9 inhibitor) causes a change in satellite levels at centromeres. In this case, this would suggest that there is some level of ongoing transcription (which would be interesting), but that this is not required for oocyte function, possibly highlighting an off target effect of the morpholinos.
A recent investigation posits that the scaffold attachment factor B (SAFB) cooperates with MajSAT and MinSAT RNAs to stabilize the pericentromeric heterochromatin (MajSAT DNA) in mouse cells. In this work the pericentromeric heterochromatin was maintained by phase separation which is SAFB and Cen-RNA dependent. Loss of SAFB complex induce the alteration of 3D heterochromatin organization (PMID: 31677973). This result in mitosis is consistent with ours in meiosis.

We have done our best to minimize side effects- please see revised summary paragraph (see p9).

Ultimately, I still believe that the most likely explanation is that some features of their treatments are compromising the repetitive DNA sequences present at centromere regions, likely due to a technical off-target effect.

If this paper does move forward, my strong recommendation would be for the authors to use caution in their wording and conclusions, as well as provide an extended discussion highlighting the limitations of the current study, possible issues, and alternative interpretations.

We thank the recommendation from this reviewer. We do make it plain on p9 (revised) that the interpretation is being made on the ASO data.

Minor points:

• The introduction strongly overstates the nature of the prior literature on centromere transcription and
transcripts, and how well established these different points are. There is still a strong debate on these, and many of these statements are highly suspect.

Thanks for this suggestion. We have changed our interpretation in the introduction and minimized the suspect statements. Some information and conclusions (see below) were deleted from introduction (see p3).

‘The recruitment of the chromosomal passenger complex (CPC) is also reported to be centromeric RNA dependent in both human and mouse cell lines (Smurova and De Wulf, 2018; Ferri et al., 2009; Ideue et al., 2014). The overexpression of mouse centromeric transcripts caused mitotic arrest and mis-localization of Aurora-B kinase (AURKB) (Bouzinba-Segard et al., 2006). As such centromeric RNA may be present at a critical level, above which it induces mitotic defects.’

• I disagree with the author’s statement in their response letter that "Meiosis is very different from mitosis." There are of course differences, but in many cases very similar related concepts and processes occur. To invoke this to explain discrepancies with prior work does not seems appropriate, as technical issues remains the most likely explanation. If there are differences, then there should be a clear molecular explanation for these behaviors.

We were merely making the point: MI is a reductional division involving cosegregation of a sister chromatid pair. This segregation is unique to meiosis and we feel when we are dealing with a closer examination of the MinSAT regions such a distinct in architecture is very relevant and requires us to
draw a distinction. We do agree with the reviewer however that many parallels exist (indeed the involvement of the APC in both processes is a good example).
July 19, 2021

RE: JCB Manuscript #202011153RR

Dr. Tianyu Wu
Shanghai Medical College of Fudan University
NO. 131 Dong'an Road
Shanghai 200032
China

Dear Drs. Wu and Jones,

Thank you for submitting your revised manuscript entitled "Loss of centromeric RNA activates the spindle assembly checkpoint in mammalian female meiosis I." We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Reports is < 20,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends.

2) Figures limits: Reports may have up to 5 main text figures.

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

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

5) Abstract and title: The summary should be no longer than 160 words and should communicate
the significance of the paper for a general audience. The title should be less than 100 characters including spaces. Make the title concise but accessible to a general readership.

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

7) For all cell lines, vectors, constructs/cDNAs, etc. - all genetic material: please include database / vendor ID (e.g., Addgene, ATCC, etc.) or if unavailable, please briefly describe their basic genetic features, even if described in other published work or gifted to you by other investigators. Please be sure to provide the sequences for all of your oligos: primers, si/shRNA, RNAi, gRNAs, etc. in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers/vendor identifiers (where appropriate) for all of your antibodies, including secondary.

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

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

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Reports may have up to 5 supplemental figures and 10 videos. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section. Please include one brief sentence per item. There is no need for a separate cover page for supplemental information.

11) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

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