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Kinetochore individualization in meiosis I is required for centromeric cohesin removal in meiosis II

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1st Editorial Decision 23rd Oct 2020

Thank you for submitting your manuscript on kinetochore individualization in meiosis I for our editorial consideration. We have now received reports from three expert referees, copied below for your information. I am happy to say that all reviewers consider this work both of interest and of high technical quality, and that we would therefore like to pursue this work further for EMBO Journal publication. I am therefore inviting you to prepare a revised manuscript, incorporating the various comments and suggestions raised by the referees. As some of their concerns also relate to experimental revisions, please do not hesitate to get in contact with me early during your revision in case you would like to discuss any of points raised - we are of course acutely aware of potential difficulties with lab access and experimental revision work in the present COVID-19 pandemic situation. Please note that our 'scooping protection' policy means that the publication of competing work during even an extended revision of your study would not affect our final decision on it; at the same time, our policy to allow only a single major revision round will make it important that all referee points are diligently answered to at the time of resubmission.

REFEREE REPORTS

Referee #1:

Review on EMBO Journal manuscript 106797 entitled "Kinetochore individualization in meiosis I is required for centromeric cohesin removal in meiosis II" by Katja Wassmann and co-workers.

Shugoshin-PP2A protects pericentromeric cohesin from cleavage by separase, thereby preserving sister chromatid cohesion throughout meiosis I. As a corollary, separase-dependent sister chromatid separation in meiosis II requires prior inactivation of shugoshin-PP2A and, hence, deprotection of pericentromeric Rec8. The molecular mechanism
of this deproctetion is one of the most thrilling unresolved questions of meiosis research.

Previous work in mammalian meiocytes has shown that the bi-orientation of sister kinetochores

coincides with the dissociation of shugoshin 2 (Sgol2) from pericentromeres. This gave rise to the "deprotection-by-tension" model, which essentially states that microtuble-dependent pulling forces across amphitelically attached sister kinetochores are key to shugoshin inactivation and deprotection of pericentromeric Rec8.

Using mouse oocytes as a model system, the Wassmann-lab now revisists the question of how and when pericentromeric Rec8 is rendered susceptible to separase cleavage. Their key findings are the following:

• Contrary to current belief, sister kinetochores individualize not only in MII but already in early anaphase I.
• Sgol2 leaves the pericentromere twice: The first time during late anaphase I; it then reappears in meiosis II to leave pericentromeric chromatin once more in anaphase II.
• Kinetochore individualization does not require eviction of Sgol2 from pericentromeres (which takes place later) but essentially depends on the proteolytic activity of separase.
• A pool of Sgol2 depends in its localization on Mps1 activity (but not kinetochore localization of this kinase). It is this population of Sgol2, which disappears from pericentromeres in anaphase II. (Curiously, pericentromeric localization of Sgol2 in early MII depends on Mps1 activity in MI, while the kinase is dispensable in MII). It is unlikely that degradation or removal of Mps1 from kinetochores is required for eviction of Sgol2 from pericentromeres in anaphase II. More importantly, inactivation of Mps1 is dispensable for removal of pericentromeric Rec8 and sister chromatid separation in anaphase II.
• Using an Eg5 inhibitor to induce monopolar spindles the authors show that tension is dispensable 1) for kinetochore individualization in anaphase I and 2) for removal of pericentromeric Rec8 and separation of sister chromatids in anaphase II. The latter finding strongly argues against the "deprotection-by-tension" hypothesis.
• When separase is expressed only in MII, then oocytes will undergo this second meiotic division in presence of bivalents. Gryaznova et al. show that under these conditions separase will still remove arm cohesin and trigger kinetochore individualization but, surprisingly, fails to remove pericentromeric Rec8. Consequently, bivalents separated only into dyads but not sister chromatids.
• When oocytes are tricked by overexpression of Sgol2 to undergo MII in presence of bivalents, separase is able to trigger full separation, i.e. to dismantle bivalents into sisters. Importantly, this correlates with retained kinetochore individualization in anaphase I, thus argueing that separase-dependent kinetochore individualization in MI is prerequisite for deprotection of pericentromeric cohesin in MII.

This is a great paper with several surprising observations that force us to totally rethink the mechanism of shugoshin inactivation in mammalian meiosis. The experiments are carfully controlled, and the clear-cut data fully support the drawn conclusions. I have very little to criticize but nethertheless suggest below one experiment, which - if it worked - would make this paper even stronger. However, I realise 1) that this experiment is challenging and 2) time to publish this work is pressing due to similar findings having recently been reported by the Nasmyth-group on BioRxiv. Thus, I would not make successful completion of this experiment a condition for accepting this already very fine manuscript.

Gryaznova and colleagues show that pericentromeric Sgo2 alone is not necessary for preventing precocious sister separation in meiosis II - even during an extended, CSF-mediated arrest. However, this does not answer the important question of whether pericentromeric Rec8 is still protected from separase if the protease were to be activated prematurely. (Co-expression of a separase activity sensor could be used to test for successful activation of the protease.) It would therefore be interesting to learn whether the overexpression of hypermorphic separase (Ser1121Ala or

Pro1122Ala) specifically in MII would result in premature loss of sister chromatid cohesion during a CSF arrest. If not, would pericentromeric Rec8 be removed if in addition Mps1 would be inhibited by reversine treatment from GVBD onwards?

Minor points:

I don't think that "pericentromere" is synonymous to "inner centromere" as stated on top of page 6.

CREST is not introduced as a maker for inner kinetochores when it is first mentioned on top of page 6.

It is important to illustrate in a schematic how pericentromeric versus centromeric localization of Sgol2 looks like in MI and MII. However, figure 1C does not suffice to do so and its clarity should be improved.

This sentence on page 11 could be improved: "Our data indicate that bipolar tension and eviction of pericentromeric Sgo2 are not essential for allowing sister chromatid separation by converting centromeric cohesin protection to deprotection in oocyte meiosis II."

Referee #2:

The manuscript by Gryaznova et al focuses on how protection of centromeric cohesin is removed for meiosis II. Previous work proposed that the tension produced by bipolar microtubule-kinetochore attachments of sister chromatids led to the deprotection of Sgo2 for sister chromatid separation. In this manuscript, the authors dispute this model and then also present a number of findings. The authors show that the centromeres become separated into distinct foci in anaphase I, prior to Sgo2 removal from the pericentromere. There are distinct pools of Sgo2 at the kinetochore, with Bub1 and Mps1 kinases recruiting different pools. Mps1 recruits the pool needed for the pericentromere. Mps1 must be present in meiosis I for the protection, but Mps1 degradation is not required in meiosis II for deprotection, as degradation occurs after segregation. Importantly, using a monopolar spindle, the authors show that bipolar attachments, that pull sister chromatid kinetochores apart, are not needed for deprotection. The kinetochores individualized in anaphase I. And, sisters could separate on a monopolar spindle in meiosis II. Kinetochore individualization requires separase. Providing separase back in meiosis II, permits the removal of arm cohesin, but not pericentromeric cohesin. However, kinetochores were individualized. The authors suggest that there are two steps required for pericentromeric cohesin. First, separase activity in meiosis I promotes kinetochore individualization, an event required in meiosis II.

Overall, the data are beautifully presented, well-controlled, and convincing. Although the authors do not fully present the full understanding of how Sgo2 deprotection occurs for cohesin cleavage in meiosis II, they were able to discount a leading model in the field and demonstrate several important findings:
1) Kinetochore individualization occurs in anaphase I, prior to bipolar spindle attachment.
2) The separase results are particularly intriguing in that separase activity is required in meiosis I for kinetochore individualization to set up for deprotection in meiosis II.
3) There are different pools of Sgo2 provided by Bub1 and Mps1. The Mps1 pool is needed for protection of pericentromeric cohesin.

There are a couple of points that need further clarification:
1) The section, "Is Sgo2 at the pericentromere required for cohesin protection until anaphase II onset" is not clear. I don't think the results address the stated question in the section heading. How much remaining Sgo2 at the pericentromere is present with Mps1 inhibition? It is likely that this remaining pool prevents PSSC. Is this pool further diminished with prolonged arrest? The authors state that "...pericentromeric Sgo2 alone is not necessary for precocious sister separation in meiosis II, even under prolonged arrest conditions". I am not understanding how the authors came to that conclusion with the data they presented. The authors show that with Mps1 inhibition, Sgo2 levels decrease, and there is some PSSC and this percent of PSSC does not further increase with a longer arrest. However, we do not know if there is further Sgo2 decrease during the CSF arrest.
2) The Sgo2 overexpression results, in which additional Sgo2 was present on the chromosomes and prevented some bivalents from separating but did not prevent sister chromatids from separating despite Sgo2 pericentromeric localization, suggests that there is a step of Sgo2 inactivation that is unaccounted for. The concluding statement that, "individualization in meiosis I leads to centromeric cohesin removal in meiosis II, even on bivalents" seemed insufficient to explain what was happening. Although the authors say they cannot rule out an inactivation step, they seem to not be dismissive of it. Further explanation in the discussion was also not sufficient. It is unclear to me of how kinetochore individualization could allow loss of protection in the pericentromere and even further along the chromosome arm (as some bivalents stayed together in meiosis I due to excess Sgo2 on the arms and need to be segregated in meiosis II).
3) The image in Figure 6B is not clear. What are the two green CREST foci in the center? Can the authors add a cartoon drawing of the chromosomes to show what is happening?
4) On Page 5, the following sentence is confusing, "We also exclude eviction of Mps1 and pericentromeric Sgo2 as key events mediating deprotection".
5) In Figure 1D, it would be helpful to have a key for the red (Sgo2) and green (CREST) blobs in the drawings.

Referee #3:

The MS by Gryaznova et al. deals with a controversial and classical question regarding what makes the kinetochores to change from being fused at meiosis I to be separated and susceptible to chromatid separation during meiosis II. This change is associated to the deprotection of centromeric cohesin that take place during the second meiotic division that allows the separation of sister chromatids. Two classical models have been proposed to explain the "open" susceptibility of Rec8 cohesin to be released at the anaphase II onset. The "tension model" propose that tension deprotects cohesin when chromosome congressed at metaphase II. The APC/C model proposes that APC/C coordinates deprotecton of Rec8 with separase activation. A third model from Dr. Wassmann proposes that I2PP2A (inhibitor of PP2A) counteracts protection of Rec8 in meiosis II independently of tension. In this work, the authors analyze which upstream event is required for such deprotection of centromeric cohesin by modifying the bipolar tension and reading it out by the corresponding Sgo2 displacement. By making use of genetic analysis, the authors exclude good hypotehisezed candidates that are shown not to be involved in the deprotection mecahisms such as Mps1 and pericentromeric Sgo2. Elegantly, the authors show that fusion of sister kinetochores is resolved in a Separase-dependent manner already in anaphase I and that this process is independent of bipolar tension. This assertion is validated with very well conducted genetic rescue experiments in Separase conditional mutants. Altogether, the authors show surprisingly that the "deprotection" of Rec8 during metaphase II takes place already at the final stages of meiosis I in

mammalian oocytes. This conclusion is novel and of great biological relevance and opens up new avenues to understand the obscure mechanisms governing the segregation of dyads during the second meiotic division in eukaryotes. Moreover, mechanistically the authors convincingly show that activation of separase is the molecular mechanism that "deprotect" cohesins to be cleaved by separase adding a new and surprising twist to the complex mechanisms of separase regulation and substrate cleavage.
Major concerns

Figure 1. The authors show the already known observation that kinetochores are already visible at two dots already in anaphase I. Interestingly, they show that both sgo2 and PP2A are already removed from the pericentromere at anaphase I. This occurred independently of the bipolar tension of the metaphase II. Given the relevance of the staining of Sgo2 is very weak and could be improved. It is difficult to differentiate from the figures the centromeric and pericentromeric labelling of Sgo2 and thus the resulting data analysis. It also would be positive to mention that this observation is not validated in squash spermatocytes (Gomez et al., 2006).
Figure 2A. The obtained conclusion is that the pericentromeric Sgo2 is removed when oocytes exit meiosis I and relocated when oocytes enter meiosis II to be removed again in anaphase II. The term relocated can give rise to misinterpretations. Descriptively, the results show that during the second division there is a novel labelling of Sgo2 to the chromosomes but not relocation (new loading?). By making use of a battery of tools (inhibitors, dead kinase mutants and delocalized kinase MPs1 N), essentially similar to those employed earlier by the same group in a pevious report (El Yakouebi et al. 2017), the authors conclude that there are also two pools of Sgo2 at the second division and that the MPs1-kinase activity dependent fraction of Sgo2 primarily localized to the pericentromere is removed for sister separation in meiosis II. The alternative conclusion that Sgo2 brought to the centromere in meiosis I by Mps1 may move to the pericentromere in meiosis II in an Mps1-independent manner seems less likely due to the amount of protein (labelling intensity).
Figure 3. To determine whether attachment of chromosomes in meiosis I to both poles of the bipolar spindle is a prerequisite for arm cohesin removal is an outstanding question. The results showing that in the presence of a monopolar-induced spindle sister kinetochores separated once arm cohesin was released and that cohesin removal during the second division II was also unaffected is very important and of great biological relevance. These result are in contrast with the established paradigm stating that tension-dependent removal of cohesin protection through bipolar attachment is required for centromeric cohesin cleavage in mouse oocytes. The chemical experimental model employed is more precise that nocodazole and points toward this conclusion is robust and confident.
Kinetochore individualization occurs onto sister kinetochores that have been previously mono-oriented during prometa-metaphase I. Though this process is not very well understood in vertebrates, Meikin together with Plk1 and partially through Sgo2 are known players involved in this process (Kim et al. 2015; PMID: 25533956). It would be interesting to analyze (as far as the Abs are available) how these proteins are modified when "deprotection" take place under the different conditions.

Figure 4. This is the main and outstanding molecular mechanism of the MS. Chromosomes that are structurally at metaphase I (though have passed through the cell cycle to the second division due to the loss of separase) have not however individualized their sister kinetochores. This is a surprising and exceptional result and demonstrates that separase activity (through proteolytic processing of an unknown substrate that points towards sgo2 or sgo2-dependent process) is the molecular trigger of kinetochore individualization. This result would be emphasized and discussed in more depth.
Figure 5 and 6. The genetic rescue experiment with separase / sgo2 demonstrate that point (Figure

4) and also opens up new questions: how are sister chromatids released upon sgol2 injection given the presence of high amounts of Sgo2 in the arms and centromeres in the bivalents at metaphase II?
These results would suggest that Sgo2 is not able to carry out its protective role during the second division independently on the individualization of sister kinetochores or not given that despite the localization of sgo2 at the arms in metaphase II the arm cohesion (Rec8) is also released. In fact, from my view, the critical point would be that chromosomes (bivalents) must have passed through a previous separase wave of activation for sgo2 to become incompetent in cohesin protection during a second wave of separase activation (anaphase II). In other words, Sgo2 that has passed through a previous active separase state has lost its protector function on centromeric Rec8. Otherwise, it is hard to explain how separase is able to release arm Rec8 and centromeric Rec8 after overexpressing sgo2.
The conclusion raised: "In conclusion, individualization of sister kinetochores in meiosis I leads to centromeric cohesin removal in meiosis II, even on bivalents" is too descriptive and does not consider the Sgo2 overexpression results. In this same sense, and remarkably, the authors show that bivalents that goes through meiosis II in the absence of separase release only their arm Rec8 but not centromeric Rec8 when separase is reintroduced. This observation, that if Separase is absent in meiosis I but present in meiosis II removes arm cohesin instead of centromeric cohesin in meiosis II, is of utmost relevance. In support of it, when Bivalents are transplanted from meiosis I into cytoplasm from meiosis II they behave as if they were in meiosis I (Ogushi et al., 2020). Altogether, authors should emphasize it strongly.
In fact, the authors discuss and do not exclude that inactivation of sgo2 would contribute to the cleavage of cohesin in meiosis II, though they pay special emphasis in that the event that determines whether cohesin can be cleaved during the second meiotic division is sister kinetochore individualization. From my point, this is more likely a consequence than a cause. The results point towards separase cleavage of substrates (Sgo2?) as the cause of cohesion deprotection.

Here is our point-by-point reply to the issues raised by the reviewers:

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• When oocytes are tricked by overexpression of Sgol2 to undergo MII in presence of bivalents, separase is able to trigger full separation, i.e. to dismantle bivalents into sisters. Importantly, this correlates with retained kinetochore individualization in anaphase I, thus argueing that separase-dependent kinetochore individualization in MI is prerequisite for deprotection of pericentromeric cohesin in MII.

This is a great paper with several surprising observations that force us to totally rethink the mechanism of shugoshin inactivation in mammalian meiosis. The experiments are carfully controlled, and the clear-cut data fully support the drawn conclusions. I have very little to criticize but nethertheless suggest below one experiment, which - if it worked - would make this paper even stronger. However, I realise 1) that this experiment is challenging and 2) time to publish this work is pressing due to similar findings having recently been reported by the Nasmyth-group on BioRxiv. Thus, I would not make successful completion of this experiment a condition for accepting this already very fine manuscript.

First, we sincerely thank this reviewer for his/her positive comments and enthusiasm. It is also a pleasure to hear that he or she considers the research questions we are addressing in this manuscript as interesting as we do!

Gryaznova and colleagues show that pericentromeric Sgo2 alone is not necessary for preventing precocious sister separation in meiosis II - even during an extended, CSF-mediated arrest. However, this does not answer the important question of whether pericentromeric Rec8 is still protected from separase if the protease were to be activated prematurely. (Co-expression of a separase activity sensor could be used to test for successful activation of the protease.) It would therefore be interesting to learn whether the overexpression of hypermorphic separase (Ser1121Ala or Pro1122Ala) specifically in MII would result in premature loss of sister chromatid cohesion during a CSF arrest. If not, would pericentromeric Rec8 be removed if in addition Mps1 would be inhibited by reversine treatment from GVBD onwards?

Whether there is still some protection in meiosis II preventing Rec8 cleavage if separase were active in an untimely manner before fertilization is indeed a key question, to which we do not have the answer yet. (I want to mention that this is also not the main message of our manuscript.) But the suggestion to overexpress Ser1121Ala separase to obtain active separase in CSF-arrested oocytes (without requiring APC/C activation) is an excellent one. As proposed by this reviewer, we have therefore expressed this mutant, which is Cyclin B-Cdk inhibition resistant, in meiosis II. But like in meiosis I (Touati et al, Cell Reports 2012), also in meiosis II Ser1121Ala separase is not active to cleave Rec8 prior to anaphase onset in oocytes, and no precocious sister chromatid segregation was observed, unlike in mitosis (Hellmuth et al, Nature 2020). Importantly, addition of Reversine from GVBD onwards did not change the outcome of the experiment (except for acceleration of meiosis I, missegregations and some PSCS due to loss of Mps1 in meiosis I). Nevertheless, for the reasons below we prefer not to draw any conclusions from this experiment for our manuscript at this stage:

We could not perform the biosensor assay for separase (Nikalayevich et al, Methods Mol. Biol. 2018) in oocytes expressing hypermorph separase, because this is a technically very challenging experiment, requiring double injections of metaphase II oocytes, yet achieving sufficient expression of separase, and checking for expression of the constructs and/or cleavage of the sensor by imaging three fluorescence tags simultaneously (2 for the biosensor and one for separase). Concerning the experiment proposed by this reviewer, overexpression of Ser1121Ala separase may indeed allow to overcome inhibition by Securin, but unfortunately, it is already quite difficult to express separase by mRNA injection in mouse oocytes and in our hands, impossible to overexpress.

Securin was suggested to be the main inhibitor of separase in oocyte meiosis II (Nabti et al. Dev Biol. 2008), even though Securin knock-out mice are fertile (Wang et al, Mol. Endocr. 2001) and Securin protein levels are much lower in metaphase II, compared to metaphase I (Marangos et al, Nat. Cell Biol. 2008, Nabti et al. Dev Biol. 2008). Securin may keep separase in check in the absence of Sgo2-Mad2 and Cyclin B1-Cdk inhibition in meiosis II. To address unambigiously whether there is indeed no protection in meiosis II we would therefore need to do the experiment proposed by this reviewer in oocytes derived from securin knock-out mice, and then use our biosensor assay to check for separase activity. However, we think this is beyond the scope of our manuscript, the main conclusion being that separase cleavage activity for kinetochore individualization in meiosis I is required to be able to remove centromeric cohesin in the following meiosis II division.

Minor points:

I don't think that "pericentromere" is synonymous to "inner centromere" as stated on top of page 6.

We agree, this phrase has been removed.

CREST is not introduced as a maker for inner kinetochores when it is first mentioned on top of page 6.

This is now included in the revised version at the beginning of the results section.

It is important to illustrate in a schematic how pericentromeric versus centromeric localization of Sgol2 looks like in MI and MII. However, figure 1C does not suffice to do so and its clarity should be improved.

We agree with this reviewer that it was not very clear from our scheme to understand what we call centromere or pericentromere, and why. To clarify the exact localization of pericentromeric chromatin relative to our stainings we have performed additional stainings. Chromosome spreads in metaphase of meiosis I and II were stained with an antibody recognizing Histone H3 K9 tri-methylation, a mark for pericentromeric heterochromatin (Nakagawa et al, Current Genetics, 2019), to better illustrate the different regions. The result is included in the revised version of the manuscript (Fig 1C and EV1A). We define a region within pericentromeric heterochromatin staining with hardly visible pericentromeric chromatin and less Hoechst or Propidium iodide DNA staining (EV1A, and B) between the two sister chromatids as "chromatid junction",corresponding to the region where centromeric Rec8 and Sgo2 are localized in meiosis II. We include a new scheme in Figure 1C, and the different regions where Sgo2 is localized in metaphase II are labeled in Figure EV2A, where we now show 3-D rendering of the different pools of Sgo2 in meiosis II. An additional scheme for the quantifications is now included in Figure EV2B.

This sentence on page 11 could be improved: "Our data indicate that bipolar tension and eviction of pericentromeric Sgo2 are not essential for allowing sister chromatid separation by converting centromeric cohesin protection to deprotection in oocyte meiosis II."

We have reformulated this phrase which - we agree- was hardly comprehensible.

Referee #2:

The manuscript by Gryaznova et al focuses on how protection of centromeric cohesin is removed for meiosis II. Previous work proposed that the tension produced by bipolar microtubule-kinetochore attachments of sister chromatids led to the deprotection of Sgo2 for sister chromatid separation. In this manuscript, the authors dispute this model and then also present a number of findings. The authors show that the centromeres become separated into distinct foci in anaphase I, prior to Sgo2 removal from the pericentromere. There are distinct pools of Sgo2 at the kinetochore, with Bub1 and Mps1 kinases recruiting different pools. Mps1 recruits the pool needed for the pericentromere. Mps1 must be present in meiosis I for the protection, but Mps1 degradation is not required in meiosis II for deprotection, as degradation occurs after segregation. Importantly, using a monopolar spindle, the authors show that bipolar attachments, that pull sister chromatid kinetochores apart, are not needed for deprotection. The kinetochores individualized in anaphase I. And, sisters could separate on a monopolar spindle in meiosis II. Kinetochore individualization requires separase. Providing separase back in meiosis II, permits the removal of arm cohesin, but not pericentromeric cohesin. However, kinetochores were individualized. The authors suggest that there are two steps required for pericentromeric cohesin. First, separase activity in meiosis I promotes kinetochore individualization, an event required in meiosis II.

Overall, the data are beautifully presented, well-controlled, and convincing. Although the authors do not fully present the full understanding of how Sgo2 deprotection occurs for cohesin cleavage in meiosis II, they were able to discount a leading model in the field and demonstrate several important findings:

1) Kinetochore individualization occurs in anaphase I, prior to bipolar spindle attachment.

2) The separase results are particularly intriguing in that separase activity is required in meiosis I for kinetochore individualization to set up for deprotection in meiosis II.

3) There are different pools of Sgo2 provided by Bub1 and Mps1. The Mps1 pool is needed for protection of pericentromeric cohesin.

We thank this reviewer for his/her positive and constructive comments on our manuscript and appreciating the novelty of our study.

There are a couple of points that need further clarification:

1) The section, "Is Sgo2 at the pericentromere required for cohesin protection until anaphase II onset" is not clear. I don't think the results address the stated question in the section heading. How much remaining Sgo2 at the pericentromere is present with Mps1 inhibition? It is likely that this remaining pool prevents PSSC. Is this pool further diminished with prolonged arrest? The authors state that "...pericentromeric Sgo2 alone is not necessary for precocious sister separation in meiosis II, even under prolonged arrest conditions". I am not understanding how the authors came to that conclusion with the data they presented. The authors show that with Mps1 inhibition, Sgo2 levels decrease, and there is some PSSC and this percent of PSSC does not further increase with a longer arrest. However, we do not know if there is further Sgo2 decrease during the CSF arrest.

The reviewer is correct in saying that we do not address whether Sgo2 is required for cohesin protection until anaphase II onset. We have changed the section title to "Is Mps1-dependent Sgo2 required for cohesin protection in metaphase II ?" because we are analyzing the impact of loss of Sgo2 depending on Mps1.

The reviewer is also correct in stating that we did not address whether Sgo2 levels decrease during prolonged CSF-arrest. We have included these data in the revised manuscript (Fig.

2F). We found that there is no decrease of Sgo2 upon prolonged CSF-arrest, and even a small increase with time. We have not addressed this point in the original version of the manuscript because we did not see any PSCS in CSF-arrested oocytes that was happening in meiosis II. The PSCS that we observed in Reversine treated oocytes (we had to treat oocytes from entry into meiosis I onwards) was already happening in meiosis I such as we had shown previously (El Yakoubi et al, Nat Comm. 2017), and not during CSF-arrest. We thought that prolonged CSF-arrest in the presence of Reversine should reveal additional PSCS if the Mps1-dependent pool of Sgo2 were required for protection in meiosis II. This was not the case, hence we concluded that Mps1 kinase activity is not required for cohesin protection in meiosis II. However, this data nevertheless does not allow us to conclude that there is no more protection of centromeric cohesin in meiosis II. Centromeric cohesin protection in meiosis II may still require Sgo2, brought there by some other mechanism than the one depending on Mps1 kinase activity (or on Bub1 kinase activity, because oocytes expressing only a kinase-dead mutant of Bub1 do not show PSCS in meiosis II either, hence we also do not think that Bub1-dependent H2A phosphorylation would be essential).

2) The Sgo2 overexpression results, in which additional Sgo2 was present on the chromosomes and prevented some bivalents from separating but did not prevent sister chromatids from separating despite Sgo2 pericentromeric localization, suggests that there is a step of Sgo2 inactivation that is unaccounted for. The concluding statement that, "individualization in meiosis I leads to centromeric cohesin removal in meiosis II, even on bivalents" seemed insufficient to explain what was happening. Although the authors say they cannot rule out an inactivation step, they seem to not be dismissive of it. Further explanation in the discussion was also not sufficient. It is unclear to me of how kinetochore individualization could allow loss of protection in the pericentromere and even further along the chromosome arm (as some bivalents stayed together in meiosis I due to excess Sgo2 on the arms and need to be segregated in meiosis II).

We agree with this reviewer that our data point to another step in regulating Sgo2, either to activate or to inhibit it. We can also imagine that Sgo2's protective role at the centromere is cell cycle dependent and can take place only in meiosis I, and that Sgo2 inactivation in meiosis II is not required, or occurs in a manner that does not involve its delocalization. In response to this reviewer and reviewer 3 we have added a paragraph on these issues to the discussion.

3) The image in Figure 6B is not clear. What are the two green CREST foci in the center? Can the authors add a cartoon drawing of the chromosomes to show what is happening?

We have added a cartoon and a better explanation that should allow the reader to better distinguish the bivalents we try to show here. The two green dots in the middle are background signals, we have included an image of the whole spread to better show this.

4) On Page 5, the following sentence is confusing, "We also exclude eviction of Mps1 and pericentromeric Sgo2 as key events mediating deprotection".

We have reformulated this sentence.

5) In Figure 1D, it would be helpful to have a key for the red (Sgo2) and green (CREST) blobs in the drawings.

A legend has been added to all the chromosome schemes for quantifications throughout the manuscript.

Referee #3:

The MS by Gryaznova et al. deals with a controversial and classical question regarding what makes the kinetochores to change from being fused at meiosis I to be separated and susceptible to chromatid separation during meiosis II. This change is associated to the deprotection of centromeric cohesin that take place during the second meiotic division that allows the separation of sister chromatids. Two classical models have been proposed to explain the "open" susceptibility of Rec8 cohesin to be released at the anaphase II onset. The "tension model" propose that tension deprotects cohesin when chromosome congressed at metaphase II. The APC/C model proposes that APC/C coordinates deprotecton of Rec8 with separase activation. A third model from Dr. Wassmann proposes that I2PP2A (inhibitor of PP2A) counteracts protection of Rec8 in meiosis II independently of tension. In this work, the authors analyze which upstream event is required for such deprotection of centromeric cohesin by modifying the bipolar tension and reading it out by the corresponding Sgo2 displacement. By making use of genetic analysis, the authors exclude good hypotehisezed candidates that are shown not to be involved in the deprotection mecahisms such as Mps1 and pericentromeric Sgo2. Elegantly, the authors show that fusion of sister kinetochores is resolved in a Separase-dependent manner already in anaphase I and that this process is independent of bipolar tension. This assertion is validated with very well conducted genetic rescue experiments in Separase conditional mutants. Altogether, the authors show surprisingly that the "deprotection" of Rec8 during metaphase II takes place already at the final stages of meiosis I in mammalian oocytes. This conclusion is novel and of great biological relevance and opens up new avenues to understand the obscure mechanisms governing the segregation of dyads during the second meiotic division in eukaryotes. Moreover, mechanistically the authors convincingly show that activation of separase is the molecular mechanism that "deprotect" cohesins to be cleaved by separase adding a new and surprising twist to the complex mechanisms of separase regulation and substrate cleavage.

We thank this reviewer for his/her positive comments on our manuscript.

Major concerns

Figure 1. The authors show the already known observation that kinetochores are already visible at two dots already in anaphase I. Interestingly, they show that both sgo2 and PP2A are already removed from the pericentromere at anaphase I. This occurred independently of the bipolar tension of the metaphase II. Given the relevance of the staining of Sgo2 is very weak and could be improved. It is difficult to differentiate from the figures the centromeric and pericentromeric labelling of Sgo2 and thus the resulting data analysis. It also would be positive to mention that this observation is not validated in squash spermatocytes (Gomez et al., 2006).

The observation that the two kinetochore dots are separated at anaphase I onset is novel and has not been demonstrated before, to the best of our knowledge. Unfortunately, without a reference I am not sure which study the reviewer is referring to. A separation of sister kinetochore dots in oocytes is visible in anaphase I in Kim et al, Nature 2015 (extended data figure 8c, control oocytes), but it is not discussed as something happening specifically in early anaphase I. Nevertheless, we have now added this reference to the revised version of the manuscript.

Separation of sister kinetochores in oocytes has been shown in human prometaphase I oocytes, and shown to increase with maternal age (Zielinska et al, Elife 2015). In this case the separation is on average 1,7μm, hence by a factor of 5 higher than what we observe here, and importantly, does not occur at anaphase I onset but seems to illustrate a weakness specific to human female meiosis. In mouse oocytes kinetochore dots can appear separated

with age as well, due to precocious cohesin removal, but again this happens in early meiosis I and not at anaphase I onset (Lister et al, Current Biol. 2010, Chiang et al. Current Biol. 2010). We have added a sentence describing this phenomenon with the corresponding references in the revised manuscript. In male meiosis, a certain separation of the CREST dots is visible in telophase I (Gomez et al, Embo Reports 2007, Figure 2), but again, the authors do not discuss this small separation of sister kinetochores and also, the attachment status of kinetochores is not shown. Hence, we prefer to not further discuss this issue in male meiosis as to my best of knowledge this has not been addressed.

The staining of Sgo2 (and PP2A) is strong in metaphase I and weak in late anaphase I because there are only very low levels of Sgo2 or PP2A left (see also quantifications for Sgo2 in Fig 1F). In Figure 1A we do not distinguish early or late anaphase I, but judging from the fact that there are 20 dyads in one pool, this is a late anaphase I, hence there is hardly any Sgo2 and PP2A left. The point of Fig. 1A was to show that sister kinetochores are separated even though attachments are still monopolar at this stage (Fig 1B). In Fig. 1E, Sgo2 stainings are strong in metaphase I and early anaphase I, and hardly detectable in late anaphase I and this is confirmed by quantifications. These stainings are inherently difficult to perform in oocytes, and catching oocytes in early anaphase I is mainly a question of luck, therefore I do not think we can improve the staining anymore. I also want to insist on the fact that we are analyzing endogenous Sgo2 (and PP2A) in oocytes here (and we think that this is also the strength of our data), unlike most publications with images of exogenously expressed GFP-Sgo2 and hardly ever images of PP2A.

Figure 2A. The obtained conclusion is that the pericentromeric Sgo2 is removed when oocytes exit meiosis I and relocated when oocytes enter meiosis II to be removed again in anaphase II. The term relocated can give rise to misinterpretations. Descriptively, the results show that during the second division there is a novel labelling of Sgo2 to the chromosomes but not relocation (new loading?). By making use of a battery of tools (inhibitors, dead kinase mutants and delocalized kinase MPs1 N), essentially similar to those employed earlier by the same group in a pevious report (El Yakouebi et al. 2017), the authors conclude that there are also two pools of Sgo2 at the second division and that the MPs1-kinase activity dependent fraction of Sgo2 primarily localized to the pericentromere is removed for sister separation in meiosis II. The alternative conclusion that Sgo2 brought to the centromere in meiosis I by Mps1 may move to the pericentromere in meiosis II in an Mps1-independent manner seems less likely due to the amount of protein (labelling intensity).

We agree and have removed the term "relocated".

Figure 3. To determine whether attachment of chromosomes in meiosis I to both poles of the bipolar spindle is a prerequisite for arm cohesin removal is an outstanding question. The results showing that in the presence of a monopolar- induced spindle sister kinetochores separated once arm cohesin was released and that cohesin removal during the second division II was also unaffected is very important and of great biological relevance. These result are in contrast with the established paradigm stating that tension-dependent removal of cohesin protection through bipolar attachment is required for centromeric cohesin cleavage in mouse oocytes. The chemical experimental model employed is more precise that nocodazole and points toward this conclusion is robust and confident.

We appreciate the reviewer's positive comment. We have decided to use STLC to induce monopolar spindles and address attachment status as well as Rec8 removal. Nevertheless, we have also done the experiment with nocodazole in meiosis II oocytes in the past and obtained the same result (not included in the manuscript), showing that in oocytes, no attachments at all are required for removal of centromeric cohesin. (Hence, intrakinetochore stretch is not required either).

Kinetochore individualization occurs onto sister kinetochores that have been previously mono-oriented during prometa- metaphase I. Though this process is not very well understood in vertebrates, Meikin together with Plk1 and partially through Sgo2 are known players involved in this process (Kim et al. 2015; PMID: 25533956). It would be interesting to analyze (as far as the Abs are available) how these proteins are modified when "deprotection" take place under the different conditions.

We are not stating that deprotection takes place in anaphase I, when kinetochore individualization occurs, but we state that kinetochore individualization is a prerequisite for deprotection in the following meiosis II. Our data do not allow us to conclude that there is no more protection of centromeric cohesin at the moment kinetochore individualization occurs. We think it is unlikely that deprotection occurs at the same time as the kinetochore individualization is observed, because separase would still be active and consequently, cleave centromeric cohesin. We have insisted more on this issue in an additional paragaph in the discussion to better clarify this issue.

Hence, we would not know when the modifications of Meikin or Plk1 this reviewer refers to are supposed to take place. It is also not clear which modifications we should analyse (phosphorylations? localization? cleavage?) and which different conditions. Unfortunately, we have no access to Meikin antibodies. It is indeed attractive to speculate that Meikin is a substrate of separase (such as proposed by Maier et al., BioRxiv 2020) which may get cleaved at anaphase I onset after Rec8 cleavage on arms, and it is Meikin cleavage that leads to the kinetochore individualization we observe here. However, endogenous Meikin is present on kinetochores throughout anaphase I and it is only in meiosis II that Meikin localization to kinetochores is strongly reduced (Kim et al, Nature 2015), hence I do not think that we can address this question through simple staining with Meikin antibodies. It is just as likely that there is another pool of Rec8 that is cleaved in anaphase I, such as proposed by the K. Nasmyth group, and we actually prefer this hypothesis, because its removal would coincide with the time of kinetochore individualization. Concerning Plk1, this kinase is involved in multiple cell cycle aspects at the metaphase-to-anaphase transition (APC activation, checkpoint silencing, mono-orientation and protection of centromeric cohesin, Meikin localisation,...). Plk1 is involved in kinetochore mono-orientation together with Meikin, and hence localized to kinetochores throughout meiosis I (Kim et al, Nature 2015). Again, antibody staining alone will not allow us to obtain useful conclusions to the potential role of Plk1 in allowing or preventing kinetochore individualization. The role of Plk1 in cleavage of Rec8 is highly controversial (in in vitro cleavage assays Plk1 activity was shown to be required for Separase dependent cleavage of Rec8 (Kudo et al., JCS 2009), but in vivo the inhibition of Plk1 in late prometaphase I oocytes leads to loss of protection, indicating that Plk1 activity counteracts centromeric Rec8 cleavage (Kim et al., Nature 2015)). We think that all these questions are very interesting, but clearly beyond the scope of this manuscript at this stage.

Figure 4. This is the main and outstanding molecular mechanism of the MS. Chromosomes that are structurally at metaphase I (though have passed through the cell cycle to the second division due to the loss of separase) have not however individualized their sister kinetochores. This is a surprising and exceptional result and demonstrates that separase activity (through proteolytic processing of an unknown substrate that points towards sgo2 or sgo2-dependent process) is the molecular trigger of kinetochore individualization. This result would be emphasized and discussed in more depth.

We are satisfied that this referee considers the fact that bivalents in meiosis II in oocytes devoid of separase are found with fused kinetochores as an exciting finding. We think it is even more striking that oocytes then only remove arm cohesin and not centromeric cohesin in meiosis II. To better insist on this result we have added schemes of chromosome figures in

Fig 4D and 5B, and additionally a scheme of kinetochore individualization occuring in anaphase II when separase is absent in meiosis I (Fig 5B).

Figure 5 and 6. The genetic rescue experiment with separase / sgo2 demonstrate that point (Figure 4) and also opens up new questions: how are sister chromatids released upon sgol2 injection given the presence of high amounts of Sgo2 in the arms and centromeres in the bivalents at metaphase II? These results would suggest that Sgo2 is not able to carry out its protective role during the second division independently on the individualization of sister kinetochores or not given that despite the localization of sgo2 at the arms in metaphase II the arm cohesion (Rec8) is also released. In fact, from my view, the critical point would be that chromosomes (bivalents) must have passed through a previous separase wave of activation for sgo2 to become incompetent in cohesin protection during a second wave of separase activation (anaphase II). In other words, Sgo2 that has passed through a previous active separase state has lost its protector function on centromeric Rec8. Otherwise, it is hard to explain how separase is able to release arm Rec8 and centromeric Rec8 after overexpressing sgo2.

The referee is right in suggesting that something must have changed on Sgo2 during meiosis I, as it cannot bring about protection in meiosis II. We can also speculate that cleavage of Rec8 occurs differently in meiosis II than in meiosis I, and cannot be prevented by Sgo2 anymore once kinetochores are individualized. Localization of Sgo2 to the centromere region in meiosis II is not enough to protect centromeric cohesin, hence cohesin cleavage cannot be explained by localization or reduction of Sgo2 localization alone. We prefer the hypothesis that Sgo2 needs to be modified to bring about protection or to become unable to protect cohesin. Also in response to reviewer 2 we have added a new paragraph in the discussion to discuss the implications of our findings for Sgo2 regulation in oocytes.

The conclusion raised: "In conclusion, individualization of sister kinetochores in meiosis I leads to centromeric cohesin removal in meiosis II, even on bivalents" is too descriptive and does not consider the Sgo2 overexpression results. In this same sense, and remarkably, the authors show that bivalents that goes through meiosis II in the absence of separase release only their arm Rec8 but not centromeric Rec8 when separase is reintroduced. This observation, that if Separase is absent in meiosis I but present in meiosis II removes arm cohesin instead of centromeric cohesin in meiosis II, is of utmost relevance. In support of it, when Bivalents are transplanted from meiosis I into cytoplasm from meiosis II they behave as if they were in meiosis I (Ogushi et al., 2020). Altogether, authors should emphasize it strongly.

In fact, the authors discuss and do not exclude that inactivation of sgo2 would contribute to the cleavage of cohesin in meiosis II, though they pay special emphasis in that the event that determines whether cohesin can be cleaved during the second meiotic division is sister kinetochore individualization. From my point, this is more likely a consequence than a cause. The results point towards separase cleavage of substrates (Sgo2?) as the cause of cohesion deprotection.

At this point we observe that kinetochore individualization by separase in meiosis I is a prerequisite for centromeric cohesin removal in meiosis II. We state that an unknown substrate must be cleaved by separase for this individualization to occur, hence the individualization is a consequence of separase's cleavage activity. (Protease-dead separase does not bring about kinetochore individualization). In our study we have not analyzed post-translational modifications of Sgo2, potential cleavage of Sgo2 or other candidate proteins by separase, or whether hypothetical Sgo2 cleavage by separase occurs before kinetochore individualization (if Sgo2 cleavage were the reason for kinetochore individualization, how would centromeric cohesin remain protected in anaphase I until separase is shut off?).

Hence, we prefer to not include speculations about this without any experimental proof in our manuscript at this stage. The failure of Sgo2 when overexpressed to protect centromeric cohesin in meiosis II even though it can protect arm cohesin at least partially in meiosis I, clearly shows that crucial knowledge about Sgo2 regulation to protect centromeric cohesin is still missing. These questions cannot be addressed by overexpression or localization studies alone, and require more experiments using biochemical approaches in other meiotic model systems as they are not feasible in mouse oocytes.

1st Revision - Editorial Decision 15th Jan 2021

Thank you for submitting your revised manuscript for our consideration. It has now been reviewed once more by referees 1 and 3, who are both satisfied with the revisions and recommend publication. Following a final round of minor modification to address the presentational comments of referee 1, as well as several editorial issues listed below, we shall therefore be happy to accept the study for The EMBO Journal!

REFEREE REPORTS

Referee #1:

Re-Review on EMBO Journal manuscript 106797 entitled "Kinetochore individualization in meiosis I is required for centromeric cohesin removal in meiosis II" by Katja Wassmann and co-workers.
In the revised version, Gryaznova and colleagues have added additional data and further improved on the clarity of the manuscript, thereby adequately addressing most points of criticism previously raised by the referees including my own few reservations. Therefore, I now happily recommend publication of this beautiful and impressive study in the EMBO Journal.
Despite this enthusiasm, I outline some further points below. This constructive criticism does not regard the content of the manuscript but is merely intended to improve this already fine manuscript even further in style of presentation and writing.
The authors have added several illustarting and helpful cartoons. It would be even nicer if:
- all cartoons would be uniform in style,
- the sister chromatids would be discernible due to slightly different coloring (see attached example, which will be sent to editor by email),
- cohesin rings would not be shown to embrace two entire condensed chromatids but rather such that the cartoons match the actual staining (see attached example again).

Replace
"...that for up to now Rec8 phosphorylation as a requirement for cleavage by Separase in vivo has only been demonstrated in yeast and C. elegans..."
by
"...that Rec8 phosphorylation as a requirement for cleavage by Separase in vivo has so far only been demonstrated in yeast and C. elegans..."
"When analysing the localisation of endogenous Sgo2 in mouse oocytes, we and others have found that Sgo2 is localized to the centromere region of paired sister chromatids ("dyads") also in meiosis II, where centromeric cohesin has to be cleaved and Sgo2's protective role is not required (Chambon et al., 2013b, Lee et al., 2008)."
Would "...must no longer be active" be better than ..."is not required"?
Replace
"...First, bipolar tension applied on sister kinetochores in meiosis II, but not meiosis I, was suggested to move Sgo2-PP2A-B56 far enough away from Rec8 at the pericentromere holding sister chromatids together to allow its phosphorylation (Gomez et al., 2007, Lee et al., 2008)...."
by
"...First, bipolar tension applied on sister kinetochores in meiosis II, but not meiosis I, was suggested to move Sgo2-PP2A-B56 far enough away from remaining cohesion-mediating Rec8 at the pericentromere to allow its phosphorylation (Gomez et al., 2007, Lee et al., 2008)...."
"However, when we performed chromosome spreads at the metaphase to anaphase transition of meiosis I, we observed that already in anaphase I sister kinetochores became visible as two separate dots (Fig 1A and B),..."
Figure 1B does not show chromosome spreads.
Replace
"...To determine whether bipolar tension in meiosis II was indeed the trigger to remove protection, we asked at what time during the transition from meiosis I to meiosis II removal of Sgo2 and PP2A

from the region where sister chromatids are connected and which we call "chromatid junction" (Fig 1C and EV1A) was observed...."
by
"...To determine whether bipolar tension in meiosis II was indeed the trigger to remove protection, we asked at what time during the transition from meiosis I to meiosis II Sgo2 and PP2A were removed from the region where sister chromatids are connected and which we call "chromatid junction" (Fig 1C and EV1A)...."
"Unexpectedly, both Sgo2 and PP2A were removed from this region inbetween sister chromatids in anaphase I, whereas Sgo2 and PP2A co-localizing with CREST at the centromere persisted in anaphase I (Fig 1A and C)."
Figure 1C does not show this.
"For this, we asked how Sep-/- oocytes segregate bivalents when rescued with exogenously expressed Separase only in meiosis II, by injecting CSF-arrested oocytes with mRNA coding for wild type Separase. Injected metaphase II-arrested Sep-/- oocytes were activated to undergo anaphase II and then examined by chromosome spreads to address whether chromosomes or sister chromatids were separated (Fig EV4B)."
Split up into two sentences.
Middle of page 14:
Interestingly, in S. cerevisiae it has been shown that Sgo1 still protects centromeric Rec8 in meiosis II, and degradation of Sgo1 is indeed necessary for anaphase II onset (Arguello-Miranda et al., 2017, Jonak et al., 2017). But our results make it rather unlikely that the decision of whether to cleave or not to cleave centromeric Rec8 in meiosis II is due to Sgo2 or Mps1 de-localization or degradation in oocytes. Since inhibition of Mps1 kinase activity and the resulting loss of Sgo2 from the chromatid junction in meiosis II does not further influence chromatid segregation, we think that the function of Sgo2 which is visible at the chromatid junction and dependent on Mps1, is not essential for meiosis II under normal conditions.
This argument contains a flaw in logic: That "inhibition of Mps1 kinase activity and the resulting loss of Sgo2 from the chromatid junction" does not result in precocious sister separation does not show that centromeric Rec8 is not protected by Sgo2 in metaphase II. Thus, the authors cannot rule out that - similar to the situation in yeast - shugoshin still needs to be inactivated at the metaphase-to-anaphase transition of M II.
"In mitosis, Separase is tightly inhibited by securin binding, phosphorylation by Cyclin B/Cdk1 (Stemmann et al., 2006), and...."
Phosphorylation is necessary but not sufficient for separase inhibition. Following phosphorylation, the Cdk1-Cyclin B1 complex has to stably bind to separase to repress its proteolytic activity.
Page 15: I don't think it is correct to state "that chiasmata lead to co-orientation of sister kinetochores".
Page 15: "Crucially though, bivalents with fused kinetochores separate into chromosomes whereas bivalents with individualized kinetochores segregate into sister chromatids."
Page 16: " Loss of Sgo2 in Sgo2 knock-out oocytes results in separation of sister chromatids instead of chromosomes in meiosis I (Llano et al., 2008), ..."
Given that chromatids are also chromosomes, I recommend replacing "chromosomes" with "dyads".
"According to our data, this unknown Separase substrate does not seem to be protected by pericentromeric Sgo2, because its loss due to Mps1 inhibition does not lead to precocious sister kinetochore individualization and overexpression of Sgo2 does not prevent it."
Unclear what "its" and "it" refer to: unknown separase substrate, Sgo2 or sister kinetochore individualization?

Referee #3:

The authors have addressed all the concerns raised in my previous report. The point by point response is very well argued and the few changes requested have been incorporated in the MS including a new diagrammatic representation. From my point, the MS in this present form should be directly accepted for publication in EMBO J.

Referee #1:

Re-Review on EMBO Journal manuscript 106797 entitled "Kinetochore individualization in meiosis I is required for centromeric cohesin removal in meiosis II" by Katja Wassmann and co-workers.
In the revised version, Gryaznova and colleagues have added additional data and further improved on the clarity of the manuscript, thereby adequately addressing most points of criticism previously raised by the referees including my own few reservations. Therefore, I now happily recommend publication of this beautiful and impressive study in the EMBO Journal.
Despite this enthusiasm, I outline some further points below. This constructive criticism does not regard the content of the manuscript but is merely intended to improve this already fine manuscript even further in style of presentation and writing.
The authors have added several illustarting and helpful cartoons. It would be even nicer if:
- all cartoons would be uniform in style,

We have adjusted the cartoons so they are uniform in style (schemes of chromosomes with schemes to explain how quantifications were done).

- the sister chromatids would be discernible due to slightly different coloring (see attached example, which will be sent to editor by email),

It is true that in meiosis, sister chromatids are often (but not always) depicted in two distinct colour shades to indicate that they have recombined prior to meiosis I. However, we do not think that this information is essential to understand the main message of the manuscript, namely the need for individualization of sister kinetochores in anaphase I for centromeric cohesin removal in meiosis II. Adding two distinct shades for each of the sisters adds more information with the risk of confusing the reader, and dilute the information we consider as essential.

- cohesin rings would not be shown to embrace two entire condensed chromatids but rather such that the cartoons match the actual staining (see attached example again).

Cohesins are often shown as rings to visualize their cohesive function, hence we chose this kind of representation. It is true that this does not exactly correspond to the appearance of Rec8 staining on chromosome spreads, where a dotted signal inbetween sister chromatid arms is observed. As suggested by this reviewer we have therefore changed our schemes accordingly, to better reflect the reality of our stainings.

Replace
"...that for up to now Rec8 phosphorylation as a requirement for cleavage by Separase in vivo has only been demonstrated in yeast and C. elegans..."
by
"...that Rec8 phosphorylation as a requirement for cleavage by Separase in vivo has so far only been demonstrated in yeast and C. elegans..."

We do not agree with the semantic comment of this reviewer, as "for up to now" implies that the same may hold true for higher eukaryotes, it just hasn't been shown yet.

"When analysing the localisation of endogenous Sgo2 in mouse oocytes, we and others

have found that Sgo2 is localized to the centromere region of paired sister chromatids ("dyads") also in meiosis II, where centromeric cohesin has to be cleaved and Sgo2's protective role is not required (Chambon et al., 2013b, Lee et al., 2008)."
Would "...must no longer be active" be better than ..."is not required"?

We only show that the Mps1-dependent pool of Sgo2 has no protective function under unchallenged conditions. At this point we do not know whether Sgo2's protective role has some function on dyads in meiosis II under specific conditions (e.g. leaky Separase inhibition), hence I prefer not to use the term "must not longer be active". In my opinion, with our current knowledge this term would apply only to anaphase II.
(please, see also (*) below)

Replace
"...First, bipolar tension applied on sister kinetochores in meiosis II, but not meiosis I, was suggested to move Sgo2-PP2A-B56 far enough away from Rec8 at the pericentromere holding sister chromatids together to allow its phosphorylation (Gomez et al., 2007, Lee et al., 2008)...."
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"...First, bipolar tension applied on sister kinetochores in meiosis II, but not meiosis I, was suggested to move Sgo2-PP2A-B56 far enough away from remaining cohesion-mediating Rec8 at the pericentromere to allow its phosphorylation (Gomez et al., 2007, Lee et al., 2008)...."

I think the sentence we are using is easier to understand.

"However, when we performed chromosome spreads at the metaphase to anaphase transition of meiosis I, we observed that already in anaphase I sister kinetochores became visible as two separate dots (Fig 1A and B),..."
Figure 1B does not show chromosome spreads.

The corresponding phrase has been changed.

Replace
"...To determine whether bipolar tension in meiosis II was indeed the trigger to remove protection, we asked at what time during the transition from meiosis I to meiosis II removal of Sgo2 and PP2A from the region where sister chromatids are connected and which we call "chromatid junction" (Fig 1C and EV1A) was observed...."
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"...To determine whether bipolar tension in meiosis II was indeed the trigger to remove protection, we asked at what time during the transition from meiosis I to meiosis II Sgo2 and PP2A were removed from the region where sister chromatids are connected and which we call "chromatid junction" (Fig 1C and EV1A)...."

We have changed the sentence accordingly.

"Unexpectedly, both Sgo2 and PP2A were removed from this region inbetween sister chromatids in anaphase I, whereas Sgo2 and PP2A co-localizing with CREST at the centromere persisted in anaphase I (Fig 1A and C)."
Figure 1C does not show this.

This has been corrected.

"For this, we asked how Sep-/- oocytes segregate bivalents when rescued with exogenously expressed Separase only in meiosis II, by injecting CSF-arrested oocytes with mRNA coding for wild type Separase. Injected metaphase II-arrested Sep-/- oocytes were activated to

undergo anaphase II and then examined by chromosome spreads to address whether chromosomes or sister chromatids were separated (Fig EV4B)."
Split up into two sentences.

I think the sentence reads better like this. Obviously, if proof reading by the Journal agrees with this reviewer on making two sentences we will do so.

Middle of page 14:
Interestingly, in S. cerevisiae it has been shown that Sgo1 still protects centromeric Rec8 in meiosis II, and degradation of Sgo1 is indeed necessary for anaphase II onset (Arguello-Miranda et al., 2017, Jonak et al., 2017). Since inhibition of Mps1 kinase activity and the resulting loss of Sgo2 from the chromatid junction in meiosis II does not further influence chromatid segregation, we think that the function of Sgo2 which is visible at the chromatid junction and dependent on Mps1, is not essential for meiosis II under normal conditions.
This argument contains a flaw in logic: That "inhibition of Mps1 kinase activity and the resulting loss of Sgo2 from the chromatid junction" does not result in precocious sister separation does not show that centromeric Rec8 is not protected by Sgo2 in metaphase II. Thus, the authors cannot rule out that - similar to the situation in yeast - shugoshin still needs to be inactivated at the metaphase-to-anaphase transition of M II.

We agree with this reviewer that we cannot rule out protection of centromeric Rec8 by Sgo2. This is why we do not want to change the phrase in (*), and we also have the following statement on page 15 in the manuscript: "But although Mps1-dependent Sgo2 is not essential in meiosis II, our data does not provide evidence that there is no protection at all any more, as it may only become essential under conditions where separase control is impaired. We can speculate that other inhibitory mechanisms exist, such as additional inhibitors or activators of separase, or posttranslational modifications of Sgo2 or Rec8 itself that are required for cleavage of centromeric Rec8 in meiosis II."

We agree that the fact of having removed the pool of Sgo2 depending on Mps1 and not observing a phenotype does not show that this Sgo2 does not need to be inactivated if it is there. Hence, to avoid confusion, we have removed the phrase: "But our results make it rather unlikely that the decision of whether to cleave or not to cleave centromeric Rec8 in meiosis II is due to Sgo2 or Mps1 de-localization or degradation in oocytes.", and reformulated the paragraph.

"In mitosis, Separase is tightly inhibited by securin binding, phosphorylation by Cyclin B/Cdk1 (Stemmann et al., 2006), and...."
Phosphorylation is necessary but not sufficient for separase inhibition. Following phosphorylation, the Cdk1-Cyclin B1 complex has to stably bind to separase to repress its proteolytic activity.

We have simplified this phrase: "In mitosis, separase is tightly inhibited by Cyclin B/Cdk1 and securin (Stemmann et al., 2006), and an inhibitor composed of Mad2-Sgo2 (Hellmuth & Stemmann, 2020)", as the exact mechanism of Separase inhibition by Cyclin B1/ Cdk1 is not the main focus of this manuscript.

Page 15: I don't think it is correct to state "that chiasmata lead to co-orientation of sister kinetochores".

It has been shown that chiasmata promote biorientation, at least in yeast (Hirose et al., Plos Genetics 2011, Sakuno et al., Dev. Cell 2011), and univalents attach in a bipolar manner in mouse oocytes (see References in Herbert et al, CSH Persp. Biol. 2015). But as attachments undergo several detachment/re-attachment cycles on chiasmata-bearing chromosomes in mouse oocytes (Kitajima et al, Cell 2011) before achieving correct mono-polar attachments,

chiasmata are just one factor among others (such as kinetochore fusion and error correction) promoting monopolar attachment in oocytes, hence we have reformulated this sentence.

Page 15: "Crucially though, bivalents with fused kinetochores separate into chromosomes whereas bivalents with individualized kinetochores segregate into sister chromatids."
Page 16: " Loss of Sgo2 in Sgo2 knock-out oocytes results in separation of sister chromatids instead of chromosomes in meiosis I (Llano et al., 2008), ..."
Given that chromatids are also chromosomes, I recommend replacing "chromosomes" with "dyads".

We have changed the text accordingly.

"According to our data, this unknown Separase substrate does not seem to be protected by pericentromeric Sgo2, because its loss due to Mps1 inhibition does not lead to precocious sister kinetochore individualization and overexpression of Sgo2 does not prevent it."
Unclear what "its" and "it" refer to: unknown separase substrate, Sgo2 or sister kinetochore individualization?

We have clarified this phrase.

Referee #3:

The authors have addressed all the concerns raised in my previous report. The point by point response is very well argued and the few changes requested have been incorporated in the MS including a new diagrammatic representation. From my point, the MS in this present form should be directly accepted for publication in EMBO J.

We thank this reviewer for his/her positive comment on our revised manuscript and are happy that he/she thinks our manuscript can now be published in the Embo Journal.

Accepted **21st Jan 2021**

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Wassmann
Journal Submitted to: Embo Journal
Manuscript Number: 2020-106797

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical method was used to predetermine sample size. Sample size was determined for every experiment according to standars in the field to obtain statistically significant data. No pre-sepcified effects were detected.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The animals required to be sacrified were depending on the amount of oocytes obtained per animal. Enough oocytes to obtain statistically significant results were obtained from the samllest number of animals possible.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples or animals were excluded from analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Investigators were blinded to sample allocation were appropriate and possible.
For animal studies, include a statement about randomization even if no randomization was used.	Animals of specific genotypes were used only to obtain the cells required for our study, hence no randomization was applied. No experiments were performed on the animals themselves.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	When comparing results, investigators were blinded. Multiple investigators assessed the data to exclude bias to quantify data.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done, as no experiments on the animals were performed.
5. For every figure, are statistical tests justified as appropriate?	Statistical tests are justfied for every figure, in the legend and the Material and Methods section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Mann–Whitney U test was used for statistical analysis, non-normal distribution was assumed but was not formally tested.
Is there an estimate of variation within each group of data?	The standard errors or standard deviations are represented together with the means on each graph.
Is the variance similar between the groups that are being statistically compared?	Not always.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The following primary antibodies were used at the indicated concentrations: human CREST serum auto-immune antibody (Immunovision, HCT-100, at 1:50), mouse monoclonal anti-PP2A C subunit clone 1D6 Alexa Fluor 488 conjugate (Sigma-Aldrich, 05-421-AF488, 1:50), polyclonal rabbit anti-Sgo2 antibody (gift from José Luis Barbero, 1:50), rabbit polyclonal CenpA antbody (Cell Signaling, #2048S, 1:50),rabbit polyclonal anti Histone H3K9me3 Chip-grade antibody (Abcam, ab 8898, 1:200), rabbit anti-REC8 (gift from Scott Keeney, 1:50), mouse monoclonal anti-α-tubulin (DM1A) coupled to FITC (Sigma-Aldrich, F2168, 1:100), and polyclonal rabbit anti-Mps1 (gift from Hongtao Yu, 1:50). Secondary antibodies were used at the following concentrations: donkey anti-human CY3 (709-166-149, Jackson Immuno Research, 1:200), donkey anti-human Alexa Fluor 488 (709-546-149, Jackson Immuno Research, 1:200), donkey anti-mouse CY3 (715-166-151, Jackson Immuno Research, 1:200), donkey anti-rabbit CY3 (715-166-152, Jackson Immuno Research, 1:200), donkey anti-rabbit Alexa Fluor 488 (711-546-152, Jackson Immuno Research, 1:200), donkey anti-mouse Alexa Fluor 488 (715-546-150, Jackson Immuno Research, 1:200), donkey anti-human Alexa Fluor 647 (709-606-149, Jackson Immuno Research, 1:200), donkey anti-rabbit Alexa Fluor 647 (711-606-152, Jackson Immuno Research, 1:200), donkey anti-mouse Alexa Fluor 647 (715-606-150, Jackson Immuno Research, 1:200).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	n.a.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mus musculus was used to obtain oocytes for in vitro culture. Mice were maintained under temperature, humidity and light controlled conditions in a conventional mouse facility, with food and water access ad libitum. Adult CD-1 mice were purchased (Janvier, France). C57BL/6 mice of the indicated genotypes (Conditional Mps1deltaN and Bub1KD mice, El Yakoubi et al, 2017) were bred in our animal facility.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The project was submitted to ethical review according to the French law for animal experimentation (authorization B-75-1308), and kept under the authorization C75-05-13 at the UMR7622, IBPS, Paris France.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance with Arrive guidelines for reporting.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n.a.
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