High density of REC8 constrains sister chromatid axes and prevents illegitimate synaptonemal complex formation

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 22 February 2016

Thank you for the submission of your research manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see below, two referees acknowledge the potential interest of your findings and support publication in EMBO Reports upon substantial minor revisions. In contrast, referee #2 challenges the relevance of your data because of severe conceptual mistakes and wrong interpretation of the data. However, given the constructive and very positive comments of two referees, I would like to invite you to revise your manuscript with the understanding that all referee concerns (as detailed in their reports), including those of referee #2, must be fully addressed in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional
Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Important: All materials and methods should be included in the main manuscript file.

Regarding data quantification and statistics, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

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

REFEREE REPORTS

Referee #1:

The manuscript by Dr. Agostinho et al describes a thorough analysis of meiotic chromosome structure in wild-type mice and in mice that are mutant for several subunits of meiotic cohesin complexes. Although the Stag3, Smc1B, and Rec8 mutant mice used in the study have been previously characterized, the application of super-resolution microscopy to examine the integrity of meiotic chromosomal axes is novel and the data described reveal important insights into the relationship between meiotic cohesin complexes, sister chromatid cohesion, and synaptonemal complex (SC) assembly. In particular, it is interesting that reducing cohesin function via the Smc1B or Stag3 mutation results in localized separation of sister chromatids, and that the regions where sister chromatids separate are nevertheless strongly labeled by axial/lateral element proteins on each chromatid, and central synaptonemal complex proteins often assemble between sister chromatids. These regions of sister synapsis usually lack REC8 cohesin, but are bounded by REC8 cohesin. This is consistent with published data demonstrating that sister synapsis occurs in rec8 mutants, but further suggests that REC8 cohesin acts locally to prevent SC assembly between sisters. RAD21L and RAD21 cohesins do not seem to have this ability, since they colocalize with sites of sister separation. Indeed, although RAD21L and RAD21 associate with meiotic chromosomes and genetic evidence suggests that RAD21L is required for normal meiosis, it is unclear whether sister chromatid cohesion (SCC) mediated by RAD21L or RAD21 cohesin is important. This paper clearly shows that the mere association of a cohesin subunit with meiotic chromosomes should not be taken as evidence that the protein is involved in SCC.

The data described are of high quality, and the claims are novel and convincing. The conclusions will be of interest to researchers studying meiosis and gametogenesis as well as those studying meiotic and mitotic chromosome segregation and sister chromatid cohesion. Although other groups have applied super-resolution microscopy to studies of meiosis, the combination of advanced microscopy and in depth mutant analysis makes this paper stand out.

Although I believe that this manuscript will be of interest to the readership of EMBO Reports, I do think there is room for improvement. However, most of my suggestions are minor and aimed at improving the clarity of the manuscript rather than requesting additional data.
Major Comments:

1. First and foremost, I would note that the paper is very long and prolix, and could be shortened significantly without removing any data. This would likely help the manuscript tremendously.

2. Figure 1 shows staining of entire nuclei and magnified insets showing individual chromosomes, but the subsequent figures show only individual chromosomes. It is understandable that the authors would want to show individual chromosomes to highlight the separation of sister chromatids in various mutants, but it would be good to also show whole nuclei as in figure 1. The images of whole nuclei could be included as supplemental figures, which would allow them to be presented in a large enough format that sister separation would be obvious. This would support the claims in the text that the phenomena described are not unusual events and demonstrate that the individual chromosomes shown are representative.

3. The authors should clearly state in the figure legends what stage of meiosis is being shown. This is done for some figures, but not for all.

4. Abstract: "We show that REC8 cohesin complexes are separated from each other by a distance smaller than 15% of the total chromosome length" unless there is proof that the foci detected represent individual complexes, this is an overstatement. In the results section, the distribution of distances between REC8 foci are described, not of REC8 cohesin complexes. This is the proper way to describe the results.

Minor comments:

1. Abstract: "REC8, but not RAD21 or RAD21L, cohesin complexes flank sites that show local loss of SCC" While it is important and interesting that REC8 is often detected at the edges of these sites, I think it is equally interesting and important that REC8 is absent from these sites while RAD21 and RAD21L are detected in these regions. If possible, it would be good to include this idea in the abstract.

2. Abstract: "a high density of randomly distributed REC8 cohesin complexes suppresses the loss of SCC and illegitimate SC formation." "suppresses the loss of SCC" suggests that SCC was established in the region and then was lost, but this is not known. All that is known is that sisters are apart in that region. I would suggest rewording this to say something like, "a high density of randomly distributed REC8 cohesin complexes promotes SCC and prevents illegitimate SC formation."

3. Introduction, page 3, second paragraph: The second sentence should be changed to say, "In vertebrates, the somatic cohesin complex..." or "In mammals, the somatic cohesin complex..." since SMC1a, STAG1, STAG2, etc, are not present in all organisms.

4. Introduction, page 3, third paragraph: "The SC is a meiosis-specific structure that enables crossover recombination" it would be better to say the SC promotes or facilitates crossover recombination, since CO recombination can happen in SC mutants in budding yeast and in the complete absence of an SC in fission yeast.

5. Introduction, page 4, first paragraph: the meiosis-specific alpha kleisins RAD21L and REC8 are introduced, but no mention is made of the "mitotic" kleisin RAD21 and its association with meiotic chromosomes and potential role in meiosis. Because the meiotic localization of RAD21 is examined in this paper, it should be introduced.

6. Introduction, page 4, first paragraph: "REC8, is detected on chromosomes prior to DNA replication at the pre-leptotene stage of meiosis I and remains bound to chromosomes until the metaphase stage of meiosis I" I believe that REC8 cohesin remains bound to centromeres until anaphase of meiosis II to keep sisters together.

7. Introduction, page 4, first paragraph: "We observed local loss of SCC and aberrant SC formation along cohesed sister chromatid axes" As written, it is not clear that the loss of SCC and sister synapsis occurred at the same site.
8. Results, page 6, paragraph 3: "REC8 expression is strongly reduced in Stag3 mutant mice" This should say REC8 levels are strongly reduced, since reduced staining intensity or diminished bands on western blots do not necessarily mean that rates of transcription or translation are reduced.

9. Results, page 6, paragraph 3: "Consistently, and similarly to Rec8−/− spermatocytes, SYCP3-labeled axial structures detected in Stag3 mutant spermatocytes at a zygotene-like stage display two discernible sister chromatid axes" It appears that many centromere signals are not associated with detectable axial structures. If true, this should be mentioned, and it should be discussed how the quantification of the number of axes with SCC loss was quantified if some chromosomes did not have detectable axes.

10. Results, page 7, first full paragraph: The figure shown (Fig 1D) shows very little asynapsis and the only chromosome with obvious LLSCC is the one in the inset. Yet the numbers reported indicate an average of 6 instances of LLSCC per nucleus examined. Is this nucleus not representative? If so, a more representative nucleus with more examples of LLSCC should be shown. If the nucleus is representative of a typical Smc1B−/− nucleus, it must be the case that the example shown in the inset is an extreme example of LLSCC, and this should be acknowledged.

11. Results, page 7, second full paragraph: "...and between the separated sister chromatids of Rec8−/−, univalent 16 chromosomes..." Care should be taken here and throughout the manuscript when discussing the rec8 mutants. While sister chromatids can be resolved in rec8 mutants unlike in wild-type animals, it is clear that SCC persists and that sisters are tethered together. It is easy to read "separated sister chromatids" to mean that there are no connections between the sisters, which is not the intended meaning. This sentence could be re-written to say something like, "...were further characterized by comparing the inter axis-distances measured at these sites with the inter-axis distances between the AEs assembled along synapsed homologous chromosomes in wild-type spermatocytes and along sister chromatids of Rec8−/−, univalent chromosomes." Another example: in the legend to figure 1A, the sister chromatid axes of Rec8−/− univalent are referred to as "non-cohesed." This would suggest the absence of SCC rather than the weakening of SCC. It is clear that SCC persists, because sisters are present in pairs!

12. Results, page 7, third full paragraph. Reduced levels do not necessarily mean reduced expression.

13. Results, page 8, final paragraph: "Consistent with a role in maintaining SCC, REC8 was..." This makes it sound like REC8 is important for continued SCC once established, but not for SCC per se. Perhaps "Consistent with a role in mediating SCC" would be better. The meaning of "maintain" is also unclear in the second full paragraph on page 9.

14. Discussion: The model that REC8 cohesin suppresses sister synapsis by keeping sisters too close for SCs to assemble is intriguing. I would be interested to see how the authors envision being too close prevents SC assembly! Of course, an alternative model is that SC assembly between sisters pushes REC8 cohesin out of the zone of LLSCC to the flanking regions, given the evidence that cohesin rings can slide (e.g., in response to transcription).

Solicited comments:

1. Does this manuscript report a single key finding? YES/NO
   If YES, please describe it in one sentence.
   Yes. In some mutants with compromised cohesin function, chromosomal regions that lack REC8 cohesin exhibit separation of sister chromatids and ectopic assembly of synaptonemal complexes between sister chromatids; these regions are often flanked by REC8 cohesin.

2. Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (NO)? YES/NO
   Yes

3. Is it of general interest to the molecular biology community? YES/NO
   If YES, please say why, in a single sentence. If NO, please state which more specialized community
you feel it is aimed at (or none), in a single word or phrase.

Yes. It has only recently been shown that the long-held belief that REC8 was the only alpha-kleisin subunit of meiotic cohesin complexes was wrong, and we are only beginning to understand the functional importance of each meiotic kleisin; this article represents an important advance in our understanding.

4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longer format article (NO)? YES/NO

Yes, the data clearly support the key finding

Referee #2:

In this MS, Agostinho et al, study the role of different mammalian meiotic cohesins in sister chromatid cohesion (SCC) establishment/maintenance and SC assembly in cohesin mutants. They described in these mutants what they named local loss of SCC and aberrant SC formation along cohesed sister chromatid axes. Sites of local loss of SCC were flanked by REC8, but not, RAD21 or RAD21L cohesin complexes. Based on these observations, they propose that separation of sister chromatid axes is prevented by a high density of randomly distributed, yet closely juxtaposed REC8 cohesin complexes.

A very important point of the paper is based on what to my opinion is a misunderstanding of the concept loss of sister chromatid cohesion. As indicated by the name, this occurs when sister chromatids lose their very tight association established as replication proceeds during the S-phase. The protein complex that keeps sister chromatids joined is the cohesin complex from yeast to man. However, Agostinho et al., employ this term as a synonymous of the illegitimate synapsis that take place between sister chromatids in the arrested spermatocytes of REC8 mutants (Xu et al., Dev cell 2004). This pairing between sister chromatids give rise to the appearance of a "synapsed-like Lateral element in single univalents. However, this structure (observed as two parallel rods with the SIM) is interpreted as loss of sister chromatid cohesion. Importantly, this interpretation of the SIM images they analyze is exemplified in a schematic representation in figure 1, and to my understanding it is wrong.

The appearance of two new Lateral Elements between sister chromatids (either in a univalent or within a partially synapsed bivalent) is used as a synonymous of sister chromatid cohesion. Both of these terms should be used in a very strict manner. As a consequence the ACA staining of the centromeres do not reveal two dots (one per chromatid) as expected in the absence of sister chromatid cohesion.

In the paper by Xu et al., they firstly analyzed the structures generated in the REC8 mutants by both IF and EM. Please see the figure 3L in the paper by Xu et al., 2005.

In addition Xu et al., also detected SCP1 binding along the length of almost every univalent in zygotene-like type D (Figures 3H and 3I) and type E (Supplemental Table S4 and Supplemental S2B) nuclei. In addition Xu et al., claimed that "Despite the extensive SCP1 binding, the majority of univalents in these Rec8−/− nuclei remained clearly separated".

Since the EM has by far more resolution than the SIM, it is obvious that the structures observed in the bivalent from a WT spermatocytes are not constituted by two superimposed identical structures as exemplified in the figure 1H (left) of this MS. In addition, the local loss of SCC is not represented by the appearance of two parallel rods (AEs) positive for SYCP3.

The wrong use of the term loss of cohesion is exemplified in this incongruence: "Despite the occurrence of LLSCC close to the centromeres, these were not affected by loss of cohesion, as shown by the close association of the two ACA foci"

In other words, the term local loss of sister chromatid cohesion is to my understanding local synapsis between sister chromatids. And the term global loss of sister chromatid cohesion is
synapsis between sister chromatids in a univalent. Accordingly, the authors corroborate experimentally that this is the case since there is SYCP1 labelling in the regions where they say there is LLSCC because they are aware that synapsis between sister chromatids occurs in the REC8 mutant (as they state in the text), however they maintain the wrong concept and the wrong representation of the observation (figure 1).

Other minor errors, Rec8 is present until the metaphase stage of meiosis II and not meiosis I as stated in the text in lanes 17 to 20 in page 3.

Finally, in the paper by Xu et al., they depicted a diagrammatic representation of what is happening in the regions where interchromatid synapsis occur (Xu et al., 2005, figure 7b). This interpretation fits very well with what Agostinho et al. are observing.

On these basis, the MS by Agostinho et al. is not suitable for publication in this or other more specialized journal given the severe conceptual mistake and the wrong interpretation of the results throughout the article.

Referee #3:

Agostinho et al. have examined the chromosome axis and SC structures in meiotic cohesin mutants Rec8 KO, Smc1β KO and hypomorphic Stag3. Their super-resolution microscopic analyses (SIM and STED) clearly illuminate the previously unforeseen chromosome axis structure and the localization of different cohesin subunits on the chromosomes. Furthermore, they assessed the relationship between sister chromatid cohesion and inter-sister SC formation in STAG3 KO, where REC8-mediated cohesion is largely impaired. They conclude that a high density of REC8 deposition along the chromosome prevents illegitimate inter-sister SC formation. This study highlights the physiological importance of REC8 in preventing illegitimate inter-sister SC formation.

I reviewed a previous version of their manuscript, which was submitted to another journal. Their current manuscript has been improved, and addresses most of the concerns (in terms of the quantification data) that I raised before. Thus, the manuscript provides important insights into SC formation in meiosis and should be open to the field. The several concerns in terms of interpretation listed below should be properly addressed or rephrased before it can be considered for publication.

Comments:

(1) Page2 line 8
"Chromosome axis length" would be better rather than "chromosome length".

(2) Page6 line 28 "some degree of SCC loss....."
The authors define LLSCC as regions locally devoid of SCC in Stag3 and Smc1β-/- mutants. However, it is an open question whether splitting of sister axes (most prevalent in rec8 KO) represents a bona fide loss of SCC. Because the interpretation of the data obtained in the current manuscript relies on this premise, this should be clearly defined and appropriately stated in an early part of the manuscript. At this early stage of the manuscript, "axial opening", to which they refer (Page13 line 4, Discussion), would be more appropriate wording than LLSCC.

(3) The causal relationship between inter-sister SC assembly and axial splitting is still unclear. While most axial splitting is accompanied by inter-sister SC assembly (Fig2A quantified data), is the reverse also true? As shown in the Fig2B, illegitimate SC is recognized even at the cohered sister axis (SYCE1 on the left column, TEX12 on the right column).

(4) Page13 line10,
The citations (3, 4) should be added to (2). IP studies in (3, 4) demonstrated the physiological interaction in testis extracts rather than somatic cultured cells, where recombinant genes were transfected.

(5) Page13 line15,
The citation should be (24, 25) rather than (27), since RAD21L had not yet been identified in the study of Rec8KO by Mckay.

1st Revision - authors' response 29 March 2016

We have now finished revising our manuscript. As previously discussed we have thoroughly revised our use of the term sister-chromatid cohesion (SCC) and replaced the descriptive term used in our manuscript (LLSCC), with a different descriptive term: local separation of axial elements (LSAEs), in the Results section. Based on our results, we have then elaborated on the role of REC8 in SCC and alternative models for SCC, in the Discussion.

Furthermore, we have addressed the comments of all three referees. Our point-to-point response file includes the detailed list of responses to all the points the referees raised, and how they were addressed throughout the manuscript.

The comments from all three referees have helped us to better communicate our results and to be more precise with the interpretation of these results. We are most grateful for this.

We hope that you will find the revised manuscript ready for publication in EMBO Reports.

Response to Referees - Manuscript Number: EMBOR-2016-42030V1

Referee #1

The manuscript by Dr. Agostinho et al describes a thorough analysis of meiotic chromosome structure in wild-type mice and in mice that are mutant for several subunits of meiotic cohesin complexes. Although the Stag3, Smc1B, and Rec8 mutant mice used in the study have been previously characterized, the application of super-resolution microscopy to examine the integrity of meiotic chromosomal axes is novel and the data described reveal important insights into the relationship between meiotic cohesin complexes, sister chromatid cohesion, and synaptonemal complex (SC) assembly. In particular, it is interesting that reducing cohesin function via the Smc1B or Stag3 mutation results in localized separation of sister chromatids, and that the regions where sister chromatids separate are nevertheless strongly labeled by axial/lateral element proteins on each chromatid, and central synaptonemal complex proteins often assemble between sister chromatids. These regions of sister synapsis usually lack REC8 cohesin, but are bounded by REC8 cohesin. This is consistent with published data demonstrating that sister synapsis occurs in rec8 mutants, but further suggests that REC8 cohesin acts locally to prevent SC assembly between sisters. RAD21L and RAD21 cohesins do not seem to have this ability, since they colocalize with sites of sister separation. Indeed, although RAD21L and RAD21 associate with meiotic chromosomes and genetic evidence suggests that RAD21L is required for normal meiosis, it is unclear whether sister chromatid cohesion (SCC) mediated by RAD21L or RAD21 cohesin is important. This paper clearly shows that the mere association of a cohesin subunit with meiotic chromosomes should not be taken as evidence that the protein is involved in SCC. The data described are of high quality, and the claims are novel and convincing. The conclusions will be of interest to researchers studying meiosis and gametogenesis as well as those studying meiotic and mitotic chromosome segregation and sister chromatid cohesion. Although other groups have applied super-resolution microscopy to studies of meiosis, the combination of advanced microscopy and in depth mutant analysis makes this paper stand out. Although I believe that this manuscript will be of interest to the readership of EMBO Reports, I do think there is room for improvement. However, most of my suggestions are minor and aimed at improving the clarity of the manuscript rather than requesting additional data.

Major Comments:

1. First and foremost, I would note that the paper is very long and prolix, and could be shortened significantly without removing any data. This would likely help the manuscript tremendously.

Response:
We agree and thank the referee; we have tried to the best of our ability, to make this manuscript
more concise and easier to read, namely by thoroughly revising the Introduction and the Results sections.

2. Figure 1 shows staining of entire nuclei and magnified insets showing individual chromosomes, but the subsequent figures show only individual chromosomes. It is understandable that the authors would want to show individual chromosomes to highlight the separation of sister chromatids in various mutants, but it would be good to also show whole nuclei as in figure 1. The images of whole nuclei could be included as supplemental figures, which would allow them to be presented in a large enough format that sister separation would be obvious. This would support the claims in the text that the phenomena described are not unusual events and demonstrate that the individual chromosomes shown are representative.

Response:
As requested by the referee we have included whole nuclei images of all individual chromosomes shown in figures 2, 3, 4, 5 and 6 of this manuscript. These are included whenever possible in the respective Figures, or in Figures EV1 and EV3.

3. The authors should clearly state in the figure legends what stage of meiosis is being shown. This is done for some figures, but not for all.

Response:
As requested by the referee we have included the meiotic stage imaged, in all relevant panels, and in the relevant Figure legends.

4. Abstract: "We show that REC8 cohesin complexes are separated from each other by a distance smaller than 15% of the total chromosome length" unless there is proof that the foci detected represent individual complexes, this is an overstatement. In the results section, the distribution of distances between REC8 foci are described, not of REC8 cohesin complexes. This is the proper way to describe the results.

Response:
We have changed the sentence to: "We show that REC8 foci are separated from each other by a distance smaller than 15% of the total chromosome axis length (...)", page 2 line 7.

Minor comments:
1. Abstract: "REC8, but not RAD21 or RAD21L, cohesin complexes flank sites that show local loss of SCC" While it is important and interesting that REC8 is often detected at the edges of these sites, I think it is equally interesting and important that REC8 is absent from these sites while RAD21 and RAD21L are detected in these regions. If possible, it would be good to include this idea in the abstract.

Response:
As suggested by the referee we have have included in the abstract: “REC8, but not RAD21 or RAD21L-cohesin complexes flank sites of LSAE, whereas RAD21 and RAD21L appear predominantly along the separated sister-chromatid axes.” Page 2, lines 10-12.

2. Abstract: "a high density of randomly distributed REC8 cohesin complexes suppresses the loss of SCC and illegitimate SC formation." "suppresses the loss of SCC" suggests that SCC was established in the region and then was lost, but this is not known. All that is known is that sisters are apart in that region. I would suggest rewording this to say something like, "a high density of randomly distributed REC8 cohesin complexes promotes SCC and prevents illegitimate SC formation."

Response:
We thank the referee for pointing this out. As suggested, we have changed the sentence to: “(...) we propose that a high density of randomly distributed REC8 cohesin complexes promotes SCC and prevents illegitimate SC formation.” Page 2, lines 13-15.

3. Introduction, page 3, second paragraph: The second sentence should be changed to say, "In
vertebrae, the somatic cohesin complex..." or "In mammals, the somatic cohesin complex..." since SMC1a, STAG1, STAG2, etc, are not present in all organisms.

Response:
As suggested, we have changed the sentence to “Several meiosis specific components have been identified in mammals, (…)”. Page 3, lines 12-13.

4. Introduction, page 3, third paragraph: "The SC is a meiosis-specific structure that enables crossover recombination" it would be better to say the SC promotes or facilitates crossover recombination, since CO recombination can happen in SC mutants in budding yeast and in the complete absence of an SC in fission yeast.

Response:
As suggested by the referee we have changed the sentence to: “ (…) the synaptonemal complex (SC), a meiosis-specific structure that facilitates crossover recombination (…)”, page 3 lines 15-16.

5. Introduction, page 4, first paragraph: the meiosis-specific alpha kleisins RAD21L and REC8 are introduced, but no mention is made of the "mitotic" kleisin RAD21 and its association with meiotic chromosomes and potential role in meiosis. Because the meiotic localization of RAD21 is examined in this paper, it should be introduced.

Response:
We have, as requested by the referee, included RAD21 in the introduction, page 4 lines 17-24.

6. Introduction, page 4, first paragraph: "REC8, is detected on chromosomes prior to DNA replication at the pre-leptotene stage of meiosis I and remains bound to chromosomes until the metaphase stage of meiosis I" I believe that REC8 cohesin remains bound to centromeres until anaphase of meiosis II to keep sisters together.

Response:
We thank the referee for pointing out this mistake, as suggested we have changed the sentence to: “ (…) REC8, is detected on chromosomes prior to DNA replication at the pre-leptotene stage of meiosis I and remains bound at centromeres until the anaphase stage of meiosis II.” Page 4, lines 7-8.

7. Introduction, page 4, first paragraph: "We observed local loss of SCC and aberrant SC formation along cohesed sister chromatid axes" As written, it is not clear that the loss of SCC and sister synopsis occurred at the same site.

Response:
We have, as requested by the referee, changed the sentence to: “We detected local separation of sister-chromatid axial elements (LSAE) in univalent chromosomes of hypomorphic Stag3 and Smc1β−/− mutant spermatocytes, accompanied by illegitimate SC formation at these sites. Sites of LSAE were flanked by REC8, but not, RAD21 or RAD21L cohesin complexes.” Page 5, lines 4-7.

8. Results, page 6, paragraph 3: "REC8 expression is strongly reduced in Stag3 mutant mice" This should say REC8 levels are strongly reduced, since reduced staining intensity or diminished bands on western blots do not necessarily mean that rates of transcription or translation are reduced.

Response:
We thank the referee for point this out, as requested we have changed the sentence to “REC8 levels are strongly reduced in Stag3 mutant mice.” Page 6, line 21.

9. Results, page 6, paragraph 3: "Consistently, and similarly to Rec8−/− spermatocytes, SYCP3-labeled axial structures detected in Stag3 mutant spermatocytes at a zygotene-like stage display two discernible sister chromatid axes" It appears that many centromere signals are not associated with detectible axial structures. If true, this should be mentioned, and it should be discussed how the quantification of the number of axes with SCC loss was quantified if some chromosomes did not have detectible axes.
Response: We have, as requested by the referee clarified in the text that the axes quantified were the ones associated with centromeres, page 6 lines 26-27.

10. Results, page 7, first full paragraph: The figure shown (Fig 1D) shows very little asynapsis and the only chromosome with obvious LLSCC is the one in the inset. Yet the numbers reported indicate an average of 6 instances of LLSCC per nucleus examined. Is this nucleus not representative? If so, a more representative nucleus with more examples of LLSCC should be shown. If the nucleus is representative of a typical Smc1B-/- nucleus, it must be the case that the example shown in the inset is an extreme example of LLSCC, and this should be acknowledged.

Response: We thank the referee for pointing out that some of the univalent chromosomes in the spread shown do not have obvious local separation of sister chromatid axes, specially considering the size at which the whole nucleus is shown. We have replaced it with another nuclei with several instances of local separation of sister-chromatid axes.

11. Results, page 7, second full paragraph: "...and between the separated sister chromatids of Rec8-/-, univalent 16 chromosomes..." Care should be taken here and throughout the manuscript when discussing the rec8 mutants. While sister chromatids can be resolved in rec8 mutants unlike in wild-type animals, it is clear that SCC persists and that sisters are tethered together. It is easy to read "separated sister chromatids" to mean that there are no connections between the sisters, which is not the intended meaning. This sentence could be re-written to say something like, "...were further characterized by comparing the inter axis-distances measured at these sites with the inter-axis distances between the AEs assembled along synapsed homologous chromosomes in wild-type spermatocytes and along sister chromatids of Rec8-/- univalent chromosomes." Another example: in the legend to figure 1A, the sister chromatid axes of Rec8-/- univalent are referred to as "non-cohesed." This would suggest the absence of SCC rather than the weakening of SCC. It is clear that SCC persists, because sisters are present in pairs!

Response: We thank the referee for the suggestion. We have changed the sentence to "(…) were further characterized by comparing the inter axis-distances measured at these sites with the inter-axis distances between the AEs of synapsed wild-type homologs and the inter-axis-distances between the AEs of sister-chromatids of Rec8-/- univalents." Page 7, lines 13-16. We have also clarified the legends in Figure 1.

12. Results, page 7, third full paragraph. Reduced levels do not necessarily mean reduced expression.

Response: As pointed out by the referee we have changed the word “expression” with “levels”. Page 7, line 22.

13. Results, page 8, final paragraph: "Consistent with a role in maintaining SCC, REC8 was..." This makes it sound like REC8 is important for continued SCC once established, but not for SCC per se. Perhaps "Consistent with a role in mediating SCC" would be better. The meaning of "maintain" is also unclear in the second full paragraph on page 9.

Response: The sentence is no longer included in the results section. In an effort to clarify and correct our description of phenotypes observed we have replaced throughout the results section the definition of LLSCC with local separation of axial elements (LSAE), and avoided referring to potential implication on SCC in the results section.

14. Discussion: The model that REC8 cohesin suppresses sister synapsis by keeping sisters too close for SCs to assemble is intriguing. I would be interested to see how the authors envision being too close prevents SC assembly! Of course, an alternative model is that SC assembly between sisters pushes REC8 cohesin out of the zone of LLSCC to the flanking regions, given the evidence that cohesin rings can slide (e.g., in response to transcription).
Response:
We thank the referee for his/hers suggestion. Although we cannot exclude the sliding model, one would expect that SC components/assembly would in that case also “push” REC8 along the sister-axes of unsynapsed and yet-unsynapsed regions of zygotene autosomes leading to LSAEs. Yet, despite this being a stage when SC proteins are already loaded, LSAEs was never observed in our analysis. The model we put forward “too close to synapse” is in line with the proposed idea that REC8 might enforce a close physical association of sister-chromatids limiting potential SC binding sites of each sister-chromatid, ensuring that only one surface (on the sister-chromatid pair) is available for attachment of SC components, thus restricting SC formation to between homologs. In the absence of REC8, since the tight association of sister-chromatids is lost, binding sites for SC components on each sister chromatid become accessible, allowing illegitimate SC assembly between sister-chromatids.

Referee #2

In this MS, Agostinho et al, study the role of different mammalian meiotic cohesins in sister chromatid cohesion (SCC) establishment/maintenance and SC assembly in cohesin mutants. They described in these mutants what they named local loss of SCC and aberrant SC formation along cohesed sister chromatic axes. Sites of local loss of SCC were flanked by REC8, but not, RAD21 or RAD21L cohesin complexes. Based on these observations, they propose that separation of sister chromatid axes is prevented by a high density of randomly distributed, yet closely juxtaposed REC8 cohesin complexes. A very important point of the paper is based on what to my opinion is a misunderstanding of the concept loss of sister chromatid cohesion. As indicated by the name, this occurs when sister chromatids lose their very tight association established as replication proceeds during the S-phase. The protein complex that keeps sister chromatids joined is the cohesin complex from yeast to man. However, Agostinho et al., employ this term as a synonymous of the illegitimate synapsis that take place between sister chromatids in the arrested spermatocytes of REC8 mutants (Xu et al., Dev cell 2004). This pairing between sister chromatids give rise to the appearance of a “synapsed-like Lateral element in single univalents. However, this structure (observed as two parallel rods with the SIM) is interpreted as loss of sister chromatid cohesion. Importantly, this interpretation of the SIM images they analyze is exemplified in a schematic representation in figure 1, and to my understanding it is wrong. The appearance of two new latera]
SYCP3.

Response:
We agree with the referee as to the higher resolution achieved with EM, in that sense we would like to point out the following reference (Dietrich et al., 1992) and the quote from Xu et al., 2005, where it is used:

“Detailed ultrastructure studies of the wt murine SC indicated that the AE/LEs may be deposited along individual wt meiotic sister chromatids (Dietrich et al., 1992). Possibly, the proximity of paired sister chromatids normally renders these two AE/LE strands as a single functional unit and their dual nature is not usually evident in wt cells, but it is in Rec8-/- meiocytes and the spo76-1 mutant where sister chromatids have lost their tight association.”

In regard to the referee’s comment that “loss of SCC is not represented by the appearance of two parallel rods (AEs) positive for SYCP3 ” we would like to point out for example Zickler and Espagne, 2016:

“ (…) Although wild-type sister chromatids appear tightly conjoined in a single LE (Fig. 2A), mutants of both Rec8 and Spo76/Pds5, revealed that in certain conditions, separated sister chromatids can each build an AE/LE (Fig. 2J [34,82]). (…) Local separation of the two sister chromatids (indicated by double AEs) specifically at the sites of COs (marked by late RNs) is also seen in wild-type pachytene nuclei analyzed in EM (…)”.

As well as Figure 5 from Bannister et al., 2004 and the respective legend:

“ Loss of sister chromatid arm cohesion in Rec8mei8/Rec8mei8 spermatocytes. Mutant spermatocyte nucleus labeled with anti-SCP3 (100 objective). White box indicates area of digital enlargement (inset) illustrating precocious separation of sister chromatid arms. Shaded arrows indicate other examples of precocious separation of sister chromatid arms.”

The wrong use of the term loss of cohesion is exemplified in this incongruence: “Despite the occurrence of LLSCC close to the centromeres, these were not affected by loss of cohesion, as shown by the close association of the two ACA foci”.

Response:
We believe that by replacing the use of LLSCC with LSAEs we address the referee’s concern, yet we do not agree with what he/she calls incongruence, as our result is the expected result for this mouse model, as shown when analyzed for the first time by Bannister et al., 2004:

“ (…) In some mutant nuclei premature separation of sister chromatid arms was apparent, indicating that REC8 may be required for maintenance of sister chromatid cohesion, at least along the chromatid arms (Fig. 5, arrows and inset). Cohesion at sister centromeres appeared to be intact in mutant spermatocytes.”

In other words, the term local loss of sister chromatid cohesion is to my understanding local synapsis between sister chromatids. And the term global loss of sister chromatid cohesion is synapsis between sister chromatids in a univalent. Accordingly, the authors corroborate experimentally that this is the case since there is SYCP1 labelling in the regions where they say there is LLSCC because they are aware that synapsis between sister chromatids occurs in the REC8 mutant (as they state in the text), however they maintain the wrong concept and the wrong representation of the observation (figure 1).

Response:
Our data shows that, as in other model systems (see Zickler and Espagne, 2016 for example in Sordaria), upon loss of cohesion function separation of sister-AEs becomes apparent. We believe that this can clearly represent local loss of SCC along the chromosome arms, as stated by Bannister et al, 2004 when analyzing the mei8 REC8 mouse model. If our interpretations were to be misled as suggested by the referee and the sister chromatids to remain cohesed along the arms this would mean that RAD21 or RAD21L would maintain SCC in meiosis. The role of RAD21 in meiotic SCC has been disputed by several studies including our current manuscript, and that of Ishiguro et al., 2014. In the later Ishiguro et al, showed that the local separation of AEs observed in Rec8 KO spermatocytes at early zygotene is suppressed in later stages, and suggests that SCC in REC8 KO cells is mediated by RAD21L and SPO11 and achieved mainly by a zipper-like assembly of SC.
rather than canonical sister chromatid cohesion, in agreement with our data.

Indeed, also Xu et al. had carefully weighed in the unlikely role of RAD21 in SCC: 1) “This raises the possibility that a RAD21/SCC1-containing complex may provide SCC in Rec8−/− meiocytes. However, it has been proposed that in mitotic cells at least, cohesin forms a proteinaceous ring of diameter ~45 nm encircling sister chromatids, (...) (Gruber et al., 2003, Haering et al., 2002 and Haering et al., 2004). (...) It is difficult to envisage that the formation of an SC-like structure that spans approximately 100 nm between sister chromatids in Rec8−/− meiocytes could take place in the presence of SCC maintained by a RAD21/SCC1-containing complex. 2) “(...) The SC-like structure forming aberrantly between sister chromatids may act as a surrogate form of cohesin and bind together sister chromatids.”

Other minor errors:

Rec8 is present until the metaphase stage of meiosis II and not meiosis I as stated in the text in lanes 17 to 20 in page 3.

Response: We thank the referee for pointing out this mistake, we have changed the sentence to: “(...) REC8, is detected on chromosomes prior to DNA replication at the pre-leptotene stage of meiosis I and remains bound to centromeres until the anaphase stage of meiosis II.” Page 4 lines 7-8.

Finally, in the paper by Xu et al., they depicted a diagrammatic representation of what is happening in the regions where interchromatid synapsis occur (Xu et al., 2005, figure 7b). This interpretation fits very well with what Agostinho et al. are observing.

Referee #3

Agostinho et al. have examined the chromosome axis and SC structures in meiotic cohesin mutants Rec8 KO, Smc1β KO and hypomorphic Stag3. Their super-resolution microscopic analyses (SIM and STED) clearly illuminate the previously unforeseen chromosome axis structure and the localization of different cohesin subunits on the chromosomes. Furthermore, they assessed the relationship between sister chromatid cohesion and inter-sister SC formation in STAG3 KO, where REC8-mediated cohesion is largely impaired. They conclude that a high density of REC8 deposition along the chromosome prevents illegitimate inter-sister SC formation. This study highlights the physiological importance of REC8 in preventing illegitimate inter-sister SC formation. I reviewed a previous version of their manuscript, which was submitted to another journal. Their current manuscript has been improved, and addresses most of the concerns (in terms of the quantification data) that I raised before. Thus, the manuscript provides important insights into SC formation in meiosis and should be open to the field. The several concerns in terms of interpretation listed below should be properly addressed or rephrased before it can be considered for publication.

Comments:

(1) Page2 line 8
"Chromosome axis length" would be better rather than "chromosome length".

Response: As suggested by the referee we have changed all instances of refereeing to “chromosome length” with “chromosome axis length”.

(2) Page6 line 28 "some degree of SCC loss......"
The authors define LLSCC as regions locally devoid of SCC in Stag3 and Smc1β/−/− mutants. However, it is an open question whether splitting of sister axes (most prevalent in rec8 KO) represents a bona fide loss of SCC. Because the interpretation of the data obtained in the current manuscript relies on this premise, this should be clearly defined and appropriately stated in an early part of the manuscript. At this early stage of the manuscript, "axial opening", to which they refer (Page13 line 4, Discussion), would be more appropriate wording than LLSCC.
Response:
We thank the referee for the suggestion. In an effort to clarify and correct our description of phenotypes observed we have replace throughout the results section the definition of LLSCC with local separation of axial elements (LSAE).

(3) The causal relationship between inter-sister SC assembly and axial splitting is still unclear. While most axial splitting is accompanied by inter-sister SC assembly (Fig 2A quantified data), is the reverse also true? As shown in the Fig 2B, illegitimate SC is recognized even at the cohered sister axis (SYCE1 on the left column, TEX12 on the right column).

Response:
We agree with the referee, SC components are detected also along axis regions devoid of LSAEs, nevertheless these regions do not display tripartite SC organization, therefore we speculate that this aggregation of SC components along the axes reflects the tendency for self assembly of some SC components, as seen in over-expression assays in Cos7 cells (Costa et al., 2005; Öllinger et al., 2005; Yuan et al., 1996; Yuan et al., 1998). At present we cannot explain the nature of this association and its relationship to the inter-sister SC with a tripartite structure.

(4) Page 13 line 10,
The citations (3, 4) should be added to (2). IP studies in (3, 4) demonstrated the physiological interaction in testis extracts rather than somatic cultured cells, where recombinant genes were transfected.

Response:
We thank the referee for pointing this out; we have added references 3 and 4 to reference 2. Please note that the references numbering has changed during the revision. Page 15, line 1.

(5) Page 13 line 15,
The citation should be (24, 25) rather than (27), since RAD21L had not yet been identified in the study of Rec8KO by Mckay.

Response:
We thank the referee for pointing this out. Nevertheless, only Ishiguro et al., 2014 shows the localization of RAD21L in Rec8−/− cells. The sentence has been removed from the discussion in the current revision, and is now included as on the results section as: “In agreement with these results, the expression of RAD21L and RAD21 in Rec8−/− spermatocytes was not sufficient to prevent extensive separation of AEs (Fig 3B and Fig EV3 D, E (Ishiguro et al., 2014)). Page 9, lines 8-10.

2nd Editorial Decision
07 April 2016

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

REFEREE REPORTS

Referee #1

The revised manuscript by Agostinho et al describes a thorough analysis of meiotic chromosome structure in wild-type and cohesin mutant mice. The revisions to the manuscript included a thorough rewriting, which shortened and significantly clarified the text. Revisions were also made to the figures to show whole nuclei in addition to individual chromosomes. The changes made address all of the issues I raised after reviewing the initial submission, and I believe that the revised manuscript will be of interest to the molecular biology community and is suitable for publication in EMBO Reports.

Solicited comments:
1. Does this manuscript report a single key finding? YES/NO
If YES, please describe it in one sentence.
Yes. In some mutants with compromised cohesin function, chromosomal regions that lack REC8 cohesin exhibit separation of sister chromatids and ectopic assembly of synaptonemal complexes between sister chromatids; these regions are often flanked by REC8 cohesin.

2. Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (NO)? YES/NO

Yes

3. Is it of general interest to the molecular biology community? YES/NO

If YES, please say why, in a single sentence. If NO, please state which more specialized community you feel it is aimed at (or none), in a single word or phrase.

Yes. It has only recently been shown that the long-held belief that REC8 was the only alpha-kleisin subunit of meiotic cohesin complexes was wrong, and we are only beginning to understand the functional importance of each meiotic kleisin; this article represents an important advance in our understanding.

4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longer format article (NO)? YES/NO

Yes, the data clearly support the key finding

Referee #2

In the revised version of the MS by Agostinho et al. together with the rebuttal letter, the authors deal with the points raised by me and the other reviewers in a comprehensive and detailed way.

I would like to outline, that they have modified in the results the term loss of sister chromatid cohesion by the most appropriate Local Separation of Lateral Elements. They propose in the first paragraph of the discussion that based on the loss of REC8 in the Stag3 mutant "bubbles" in their AEs and that LSAEs are observed when the REC8 foci are more distant, the LSAEs represent true local loss of Rec8-mediated sister chromatid-arm cohesion. Although this is a non-demonstrated proposal the term now is restricted to the discussion (and also in the abstract). Importantly, the authors have modified the schematic representation of the wt Lateral elements in figure 1H. In the new corrected figure, there is a single barr (LE) instead of the two bars/rods per chromosome shown in the first version. This is a crucial point that has been corrected properly and without which there was no way to interpret the whole text. In addition, the text has been shortened and improved largely.

Thus, I would support for publication this MS.

Referee #3

The authors properly addressed all my concerns. It is now suitable for publication in this journal.
## 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 ≥ 2, 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 authorship guidelines in preparing your manuscript.

### 2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:
- A specification of the experimental system investigated (e.g., 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 or unpaired), simple p tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only. More complex techniques should be described in the methods section.
  - Are tests one- or two-sided?
  - Are there adjustments for multiple comparisons?
  - Are statistical test results, e.g., P = + value and P = -value?
  - Definition of 'center values' as median or average.
  - Definition of error bars as s.d. or s.e.m.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section on statistics, reagents, animal models and human subjects.

### B. Statistics and general methods

<table>
<thead>
<tr>
<th>Question</th>
<th>Answer</th>
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<tbody>
<tr>
<td>1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?</td>
<td>For animal studies, include a statement about sample size estimate even if no statistical methods were used. Otherwise possible we have tried to always have biological triplicates to make sure that reported phenomena are reproducible. Materials and methods, page 16.</td>
</tr>
<tr>
<td>1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.</td>
<td>For every figure, are statistical tests justified as appropriate? Materials and methods, page 16.</td>
</tr>
<tr>
<td>2.a. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?</td>
<td>Were any steps taken to minimize the effects of subjective bias when selecting analytical samples to treat (e.g. randomization procedure)? If yes, please describe.</td>
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<td>2.b. Were any steps taken to minimize the effects of subjective bias when allocating analytical samples to treatment (e.g. randomization procedure)? If yes, please describe.</td>
<td>Were any steps taken to minimize the effects of subjective bias during group allocation or when assessing results (e.g. blinding of the investigator)? If yes, please describe.</td>
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<td>3.a. For animal studies, include a statement about randomization even if no randomization was used.</td>
<td>For every figure, are statistical tests justified as appropriate? Materials and methods, page 16.</td>
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<td>4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or when assessing results (e.g. blinding of the investigator)? If yes, please describe.</td>
<td>Was the data treated as the assumptions of the test(s), e.g., normal distribution? Describe any methods used to assess it.</td>
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<tr>
<td>5.a. For animal studies, include a statement about blinding even if no blinding was done.</td>
<td>Were there any steps taken to minimize the effects of subjective bias when selecting analytical samples to treat (e.g. randomization procedure)? If yes, please describe.</td>
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<td>5.b. For animal studies, include a statement about blinding even if no blinding was done.</td>
<td>Were there any steps taken to minimize the effects of subjective bias during group allocation or when assessing results (e.g. blinding of the investigator)? If yes, please describe.</td>
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<td>6. Are there any steps taken to minimize the effects of subjective bias during group allocation or when assessing results (e.g. blinding of the investigator)? If yes, please describe.</td>
<td>Do all human and animal studies conform to relevant national and institutional guidelines? Materials and methods, page 16.</td>
</tr>
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### C. Reagents

- The data shown in figures should satisfy the following conditions:
  - Biological replicates (including how many animals, litters, cultures, etc.).
  - Justified definitions of statistical methods and measures.
  - A description of the sample collection allowing the reader to understand whether the samples represent technical or the exact sample size (n) for each experimental group/condition, given as a number, not a range.
  - An explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

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We recommend considering the MRA+ guidelines (PLOS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. Please confirm compliance.

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To show that antibodies were profiled for use in the system under study (e.g., species, provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile, e.g., Antibodies to see link at top right).

Identify the committee(s) approving the study protocol.

Deposition is strongly recommended for any datasets that are central and integral to the study. Please consider the journal's data policy. If a structured public repository exists for a given data type, we encourage the provision of links to the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad and the Proteome Archive).

Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practical, and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public controlled repositories such as Biorxiv, database for the Arts and EGA (see link at top right).

For human studies, we recommend that you follow the REMARK reporting guidelines (PLOS Biol. 8(6), e1000412, 2010). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.

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