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Meiotic Cohesin STAG3 is Required for Chromosome Axis Formation and Sister Chromatid Cohesion

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Transaction Report:

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

1st Editorial Decision 11 December 2013

Thank you for submitting your manuscript on meiosis in STAG3-deficient mice for our editorial consideration. We have now received the comments of three expert referees, which you will find copied below for your information. While the reviewers acknowledge the potential interest and importance of your findings, they are not convinced that all of the main conclusions and interpretations are decisively supported by the presented experimental evidence. In addition, they indicate that some further extension, including complementary approaches, would be required to make this study a compelling candidate for publication in a broad general journal such as this one. In light of these concerns, I am currently not in the position to predict whether this study may ultimately become suitable for acceptance, but would nevertheless offer you an opportunity to address the referees' concerns by way of a revised version of this manuscript. I do however have to point out that it is our policy to allow only a single round of major revision, and that it is therefore important to diligently respond to all points raised at this stage. When preparing a revision, please also make sure to carefully edit and proofread the manuscript before resubmission. Finally, please remember that your letter of response to the referees' comments will form part of the Review Process File, and therefore be available online to the community.

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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Thank you for the opportunity to consider this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

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REFEREE REPORTS:

Referee #1:

The authors constructed STAG3 KO mice and found that the chromosomal axis structures characteristic of meiotic chromosomes are completely lost in this strain. These data suggest that meiotic cohesin is required for axis formation and that STAG3 is the sole (or major) SCC3 subunit required for meiosis. Consequently, both male and female STAG3 KO mice show perturbed progression of meiosis, and exhibit an infertile phenotype. Notably, even in the absence of axial elements, meiotic recombination is initiated, but DSB repair is impaired (shown by the accumulation of DMC1 signals). Furthermore, because of the loss of meiotic cohesin, sister chromatid cohesion (examined by the number of ACA and RAP1 signals) is also impaired, while the telomere structures are preserved.

Although STAG3 was identified as early as 2000 (Pezzi N. 2000), its function has remained elusive. Therefore, the characterization of STAG3 KO mouse performed here may be important for understanding this meiosis-specific cohesin subunit in mammals. However, some of the technical terms and interpretations of the cytological observations are not always appropriate and may be misleading. These points should be properly addressed before consideration for publication in this journal.

Major point #1
The authors used the term 'early pachynema' or 'early pachytene-like' to represent some of the STAG3 KO spermatocytes. This term is highly misleading because "pachytene" is defined by the completion of synapsis. The definition of cell stages based on cell distribution in the seminiferous tubules (Fig. 2a and Fig. S2) is not applicable for mutant testes because meiotic progression itself is delayed or arrested. Considering that axial elements are mostly undetectable or highly fragmented, and SCP1 loading is severely impaired in the absence of STAG3 (Fig. 1 and 3), it should be termed 'leptotene-like' as previously used in reference to REC8 and RAD21L double KO spermatocytes. In fact, the overall phenotype of STAG3 KO seems nearly identical to that of REC8 and RAD21L double KO (Llano E. 2012). These points should be carefully considered and described in the manuscript.

Major point #2
Although the authors previously reported that the meiotic cohesin mutant, SMC1b KO, shows structural defects in telomeres (Caroline Adelfalk. 2009), the current study reports that STAG3 KO spermatocytes show normal telomere structures. Therefore, they suggest that SMC1b plays a role in telomere structure independent of STAG3. This interpretation is odd for the following reasons:

1) There is no high-resolution picture presenting telomere structures in WT and STAG3 KO; therefore the telomere defects cannot be inspected properly.
2) In SMC1b KO spermatocytes, telomere shortening and aberrations are observed only from the zygotene toward the pachytene stage (Caroline Adelfalk. 2009), while STAG3 KO spermatocytes arrest in the 'leptotene-like' stage (see Major point #1). Therefore, it is difficult to assess the function of STAG3 in the telomere structure because STAG3 KO spermatocytes arrest at an earlier stage than telomere defects should appear. Moreover, it has already been shown that STAG3 (but not STAG1 and STAG2) is the sole partner of SMC1b in spermatocytes (Lee J. 2011). These results strongly argue against the authors' conclusion.
3) The authors showed that telomeres are aberrantly aggregated in STAG3 KO (Fig. 4B); this observation is inconsistent with the statement that " telomere structure is not affected".

If the authors want to insist that residual SMC1b protein might work at telomeres even in the absence of STAG3, they have to show the co-localization of SMC1b and telomere markers in the absence of STAG3. Otherwise, they should remove the statement that "telomere structure is not

affected" in STAG3 KO.

Major point #3
The authors state that residual cohesin complexes exist in STAG3 KO (Fig5, Page10) and assume that cohesin complexes containing STAG1/STAG2 support cohesion (page14, discussion). Since those images in Fig. 5 are too faint, it is hard to evaluate whether the signals indeed represent cohesin complexes (SMC3, SMC1 , SMC1 , RAD21, RAD21L). It is very possible that the faint signals represent the immuno-staining background or non-specific aggregation of proteins. The authors should examine whether those dotty 'cohesin' signals indeed perfectly co-localize with each other.

Minor comments
1) Cite "Figure 1B" in the text in the proper site.
2) In Figure 3C, too many redundant images are shown for STAG3 KO. One or two representative images would be sufficient.
3) In Figure 5A, a more dispersed SYCP3 pattern should be shown for STAG3 KO.
4) Why mention stage II-III of STAG3 KO in the text without showing data in Supplemental Figure 2?

Referee #2:

In the current study, Winters et al. demonstrate that the meiosis-specific cohesin subunit STAG3 is essential for meiosis. This conclusion comes from the analyses of meiocytes from STAG3 KO mice, which are viable but infertile. By means of immunofluorescence staining, the authors characterize the effects of the Stag3 deficiency and show that it is required for formation of the synaptonemal complex (SC) and for processing DSBs.

Previous results from the Jessberger lab and other labs have shown that mice KO for meiosis-specific cohesin subunits Smc1beta, Rec8 and Rad21L are also infertile. Moreover, Llano et al (2012) showed that while spermatocytes lacking Rad21L or Rec8 arrest at a zygotene-like stage with fragmented AEs and some partially synapsed lateral elements, double KO show amore dramatic phenotype, with complete loss of the SC and little, if any, staining of Smc1beta and Stag3. Rec8, Rad21L and Smc1beta had been also shown to associate preferentially with Stag3 (Lee & Hirano 2011). Thus, the critical role of Stag3 in meiosis does not come as a surprise. In order to merit publication in EMBO J, additional data would be required.

1. Evaluation of different cohesin subunits in the STAG3 KO by immunofluorescence is difficult to judge (Figure 5) and comparisons with the wildtype are further complicated for the inability to stage the spermatocytes properly. The authors could perform a biochemical fractionation of nuclei and immunoprecipitations from nuclear extracts with different cohesin antibodies in wt and KO cells. This could give an idea of the relative abundance of the different cohesin complexes.

2. The data supporting defects in telomere and centromere cohesion are not convincing. Counting ACA spots in the images shown in Figure 4A or RAP1 spots in 4B seems rather difficult. Combining staining with ACA and Aurora B, may help identify true centromere signal. FISH with probes from subtelomere or arm regions could also provide a better idea of the state of sister chromatid cohesion.

Minor points
- Discussion is too long. It would be important to contrast the results from this study with those previously published for other KO and put forward a model for the specificities of the different complexes.
- Since the paper is about STAG3, the authors could say something in introduction about the differences in sequence between the STAG1/2 and STAG3.

Referee #3:

Cohesins are essential structural components of the chromosomes essential for correct segregation at

cell division. The subunits constituting the cohesion ring vary with some meiotic components overlapping with mitotic components and some being unique. In particular the predominant STAG protein expressed in meiosis is STAG3. This paper reports the effects of a homozygous null allele on meiosis in (predominantly) male mice. The mice are infertile (both sexes) with an arrest at stage IV tubules in the testis. Most significantly chromosome axes are not formed; as a result synaptonemal complexes are also not formed. This is a very important finding.

Although the phenotypes reported are consistent with this being a null allele of STAG3 the only data presented to support this is the rather unconvincing Western blot in Fig1 B. Do the authors have other data to support their assumption that there is no use of the splice acceptor site at exon2? Some mRNA data might help.

The authors correctly point out that the classic cytogenetic staging of meiotic prophase is not possible in this, as in other mutants. They then have two sections of the results (Meiotic arrest ... and Deficient ...) where they state this but as a result these sections are difficult to read and ramble around the topic. Could the authors simply state once that prophase staging is not possible on the usual basis then define at the start their view of what constitutes and early and late stage cell? Also please validate this staging with an established marker of progression e.g. H1t, or by the frequency of spermatocytes with these SCP3 patterns in juvenile mice e.g. 13dpp vs. 16dpp. Images in Fig2C with more restricted H2AX claimed to occur in "late pachytene" are not consistent with the "late pachytene" SCP3 patterns proposed i.e. some images have little H2AX and also very little SCP3. The paragraph about co localisation of SYCP1 and 3 seems to me to indicate no significant association which is supported by the hormad paragraph. Could these not be combined into a single sentence combined with a warning that these are proteins known to form aggregates in cells such as polycomplexes?This simplification could also be applied to the discussion which in general is complex to read and tends to obscure the important point that STAG3 is required for axis formation by speculating too much about cohesion complexes.

Based on analysis of telomeres and centromeres the authors show that some sister cohesion is maintained in the absence of STAG3 at these rather special chromatin sites. Whilst I realise that these are easy to assay using antibodies it would be useful to know if this finding applies to the bulk of loci using in situ hybridisation. Analysis of paired centromere morphology would also be of interest to indicate whether cohesion is loosened at centromeres which remain paired (see Chiang 2010).

Remaining cohesion between chromosomes does suggest that cohesin complexes remain in the mutant, however cohesin IF is not convincing of cohesin complex presence. Western blot for chromatin-associated cohesin subunits and colocalisation of "dotty" IF signals would help. Similarly the diffuse HORMAD1 staining is not convincing as genuine signal and should be supported by WB for chromatin associated protein.

Minor points
"Gamma" symbol should be used in figures
DAPI channels can be removed from images where not discussed; overcomplicate merges
Graphical plotting of some data would be of use e.g. beeswarms for ACA foci

1st Revision - authors' response 30 March 2014

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Response to Referees

We very much thank the referees for their insightful comments, which helped in improving this manuscript.

We have included substantial new, additional data in this revised version. The new data include
- mRNA analysis of Stag3 expression in wt and mutant mice (Fig. 1B)
- analysis of DMC1 foci in young males (day 11 and 13 pp; Fig. S4)
- immuno precipitation data showing the presence of cohesin proteins in wt and mutant spermatocytes (Fig. 6A)
- differential salt extraction of cohesins to show loose vesus tight chromatin association by this additional method (Fig. 6B)
- telo-FISH in addition to anti RAP1 staining of telomeres (Fig. S7).

In addition we have edited many sections of the manuscript and have revised some interpretations. Despite having added new data, we have also shortened the paper, including the discussion.

The following provides details on the revision according to the individual reviewers' comments.

Referee #1

1. We thank the referee for pointing out the difficulties in staging mutant spermatocytes. We have modified our description such that we are differentiating between staging based on chromosome structure and staging based on tubular development. Chromosome structure indicates a leptotene-like stage as the referee writes, and we are now using this term throughout the manuscript. We have eliminated the term pachytene-like. However, staging according to tubular development, i.e. presence of cells of particular stages within a given tubule, is possible. Despite the meiotic arrest, which happens at stage IV, development of tubules up to this stage occurs largely unperturbed as the presence of the respective cell associations in adult tubules shows, and as also the progression of the first wave of meiosis in young males shows. Overall, our conclusion is that tubular cell development continues until stage IV, but chromosome structures progress not beyond a leptotene-like stage. This also fits to our current understanding of the checkpoint in stage IV, which eliminates cells that develop until stage IV although they harbor major chromosomal aberrations. The progressive compaction of gH2AX signals (Fig. 2C) is also consistent with cell development along with tubular development up to stage IV. We have modified the 2nd paragraph of the Results section accordingly. We retain a modified first section of the Discussion, where we had already pointed out the problems in staging.
   We are also comparing the STAG3 deficiency phenotypes with the REC8 RAD21L double-deficiency now at several instances in the manuscript (first mentioned on p. 5; comparisons on p. 14, 15, 16, 17).

2. We agree with the referee that our interpretation of the telomere phenotype seen in Stag3 deficient spermatocytes was premature. We indeed have to consider the development of Stag3 deficient spermatocytes furthest to a chromosome stage that is leptotene-like, irrespective of whether the tubules may further develop. The Smc1b-deficient spermatocytes develop further to almost complete synapsis with axes that are only half as short as wt axes. Thus, we have altered our interpretation correspondingly (p. 10 and 15/16). We have also added new Telo-FISH data, which confirm the absence of large aberrations such as extensions and bridges, which were easily seen in case of Smc1b-deficiency. We are not convinced though that STAG3 is the only SA partner of SMC1b, since minor complexes such as those possibly present at only a certain chromosomal location are hard to detect. Also, our new IP data suggest association of SMC1b with SA1 and SA2. The clustering of telomeres is not just aberrant aggregation as the reviewer notes, but is a normal process that occurs in wt as well in late leptotene/early zygotene.

Minor Comments
1) Figure 1B is now cited at the proper site (p. 6) in the newly designed Figure 1, which contains new data.
2) We feel that it is important to show several examples of the magnified images of SYCP3/SYCP1 co-staining in Fig. 3, since the localization varies. In some instances there is clearly overlap indicated by yellow color, in others there is none, or it is combined.
3) We are now showing a cell that shows more of the typical small SYCP3 spots
4) Stages II/III are included in Figure 2, many other stages are shown in Figure S2.

Referee #2

This referee points out that one may have expected a phenotype as dramatic as that of the STAG3 deficient mice presented here, since the Rec8 Rad21L double-knockout (DKO) (Llano et al, 2012) shows a very similar phenotype, and these proteins as well as SMC1b were shown to preferentially associate with STAG3. While indeed one may have expected a substantial phenotype, this was not so clear and remained to be shown: The protein association data (co-IPs, co-expression etc) differ substantially in different publications (e.g. compare Lee and Hirano, 2011 with Guiterrez-Caballero et al., 2011). It was also uncertain whether, for example, SA1 or SA2 could compensate for loss of STAG3. Indeed the STAG3 deficiency phenotype differs from that of the DKO, for example in the continued presence of cohesins in absence of STAG3. We have substantiated these data now with co-IPs (Fig. 6). In the DKO (figure 4 of Llano et al., 2012), there were no SMC1b, STAG3, and very little if any SMC3 visible, although only IF images are shown (and although some RAD21 signals are evident). Another significant difference is that Llano et al claim that REC8 RAD21L are not required for sister chromatid cohesion. We see loss of centromeric and telomeric cohesion in

absence of STAG3. Llano et al did not specifically analyze centromeres and telomeres though. All this (and more) needed clarification.

1. We agree with the referee that conclusions based only on the IF data on cohesins in mutant spermatocyte spreads are difficult. Thus, we performed immuno precipitation using anti SMC1β to ensure precipitating meiotic cohesin complexes only from total testis extracts. The results (Fig. 6) show the presence of SMC1β, SMC3, REC8 and also SA1 in IPs from mutant extracts. RAD21 and STAG3 were absent. This data confirms the presence of cohesin complexes in absence of STAG3. We also liked to know whether the cohesins are chromatin-associated and therefore preformed differential salt extraction from testis chromatin using increasing salt concentrations for extraction (Fig. 6). These experiments also confirmed the presence of cohesins on mutant chromatin. The presence of SA1 suggests that meiotic cohesin complex(es) other than STAG3-based ones exist. One may even speculate about increased SA1 presence in absence of STAG3.

2. We do not agree that the staining for centromeres using the well-established ACA yields ambiguous results. In fact, the wt controls show a specific signal only at one end of the spermatocyte chromosomes and the expected number of signals (Fig. 4A). Likewise, the ACA signals in the mutant are clearly distinguishable, localize to DAPI-intense heterochromatic regions, are associated with the very short axes where such axes exist, and cluster as expected. The black-and-white images very clearly show distinct spots that can be counted (Supplemental Fig. 5).
The anti RAP1 signals marking telomeres were a bit more variable in intensity, although they also localized specifically to each end of the wt chromosomes (Fig. 4B), clustered in some cells and were clearly distinct. Considering our previous data showing telomere deficiencies in SMC1b deficient spermatocytes, we are now also showing telomere FISH data (Fig. S6), which confirm the RAP1 staining.

Minor points
- We have shortened the Discussion. Since we have now included IP data, we are also addressing the cohesin complex compositions more extensively. Still, we feel it is to early for a comprehensive model about the presence and roles of the various distinct cohesin complexes that exist in mammalian meiocytes.
- Since the STAG3 protein itself was described many years ago, we do not like to repeat the description of the protein sequence. In Pezzi et al., 2000, the three proteins were aligned and compared.

Referee #3

1. Following the request by this reviewer, we have now included mRNA RT-PCR data (Figure 1B), which show the absence of the diagnostic transcript in the Stag3$^{ko/ko}$ mice. We have in addition now included a new set of data on cohesin complexes.

Here, co-IPs were performed and show the presence of several cohesin subunits in the Stag3$^{ko/ko}$ testes and the complete absence of STAG3 (Fig. 6A).

2. We have tried to streamline the sections on staging. In particular, we are distinguishing between staging according to chromosome structure and according to tubular development. We are now also including data from juvenile mice as the referee suggested, i.e. from mice at day 11, 13, and 15 pp. The data confirm our assessment that chromosomally the cells are in a leptotene-like stage and developmentally they may reach stage IV of the tubular epithelial cycle. We have also focused the Discussion in this regard. We like to maintain, however, our Discussion of cohesin complexes, since we have added new data (co-IP) which shows the existence of cohesin complexes in the mutant.

3. We have extended our analysis of telomeres by using FISH (Fig. S6). These data affirm our earlier conclusion about partial loss of telomeric sister chromatid cohesion. To analyze the fine structure of the centromeres, however, is very problematic given the highly compact SYCP3 dots or miniature axes. We do not see a way to decipher interkinetochore distances at such highly compact and abnormal chromosome-dots – as nice as this suggestion is.

4. As outlined above, we have added co-IP data to more convincingly show the existence of cohesin complexes. We have also added data from differential salt extractions (Fig. 6B) to show that the cohesin proteins are indeed tightly chromatin-associated, which confirms the IF data.

5. We have corrected the use of “gamma” in the images and have reduced DAPI channels. We still like to keep DAPI as it allows to assess the extend of chromatin (and thus the edge of the spread nucleus) and chromatin domains. The ACA foci are clearly visible as distinct foci, and so we think that counting them and providing the numbers is appropriate.

2nd Editorial Decision 07 April 2014

Thank you for submitting your revised manuscript on mice deficient for the STAG3 meiotic cohesin subunit. It has now been reviewed once more by the original referees 1 and 2, and I am pleased to inform you that they both consider the study significantly improved and now in principle suitable for publication in The EMBO Journal. As you will see, referee 2 still requests a few specific clarifications and modifications to the presentation, which I would like to invite you to briefly address during a final round of minor revision.

REFEREE REPORTS:

Referee #1:

The authors properly addressed my concerns. Therefore, I recommend to publish this paper in EMBO J.

Referee #2:

I find the current version of the manuscript improved and most of my criticisms addressed. I support its publication in EMBO Journal if the authors clarify a couple of questions regarding the new Figure 6.

If I understand correctly, the goal of the salt extraction experiment in Fig6B is to show that there are cohesin complexes bound to chromatin in meiotic Stag3 KO cells. The immunoblot with Smc1b antibodies suggests that this is indeed the case. My trouble comes with the immunoblot with Rec8 and Rad21L:

1. In Fig6A, the nuclear extracts from Stag3 KO testis have clearly reduced amounts of Rec8. In Fig 6B, however, there seems to be more Rec8 in any of the three fractions shown for Stag3 KO than in the corresponding fractions from the wild type. Is most of the Rec8 present in wt cells leaking? I am not sure that this immunoblot demonstrates the "looser association to chromatin of Rec8". On the other hand, the results of the Rad21L immunoblot are not commented but they seem to argue that the tightly bound complexes containing Smc3-Smc1b are those formed with Rad21L and SA1/2.

2. It would be useful to indicated in Figure 6A which are specific/unspecific bands (as in Figure 1 for Stag3) and also include MW reference on the left, as in Figure 6B.

In the Discussion of these data, the authors write

"We observed a rather weak but clear signal for SA1 and SA2 co-precipitating with SMC1 in wt, and these signals were enhanced in absence of STAG3. One may speculate that in the absence of STAG3, expression of SA1 and SA2 is upregulated or its stability is enhanced through association with cohesin complexes typically associated with STAG3"

1. At least for SA1, the immunoprecipitation with Smc1b antibody looks pretty good to me!

2. The levels of SA1 and SA2 seem to be identical in wt and KO testes , as judged by the western blot in Figure 6A (input, last 2 lanes). Thus, I think that a more likley explanation for the larger recovery of SA1 and SA2 subunits in the immunoprecipitates of Scm1b from KO cells is that SA3 has more affinity and competes better for binding to Smc1b-containing complexes than SA1 or SA2. Only when SA3 is not available, SA1 and SA2 can bind as well.

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Finally, the Discussion is still too long. I suggest the authors to eliminate the paragraph "SMC1 - based complexes are most clearly observed in early prophase I and..." since they discuss data from other papers, not their own.

2nd Revision - authors' response 08 April 2014

Thank you very much for provisionally accepting our paper! We are very happy to provide the final version, which was revised according to the points raised by referee No. 2 and your points.

In particular, we have

- inserted an improved immunoblot for REC8 in Figure 6B in response to point #1 of referee #2; the new blot fits perfectly to data shown in Fig. 6A and much better represents the four independent experiments done
- labeled the unspecific band and inserted molecular mass markers in Figure 6A as requested by referee #2
- edited the Discussion by adding a sentence on the alternative explanation for increased STAG3 co-precipitation with cohesin (p. 17 of the Discussion)
- shortened the paragraph "SMC1a-based..." of the Discussion (p. 17/18), but have not eliminated the entire paragraph. We think it is very important to compare our results with data of others. In fact, one referee earlier asked for such comparison.
- have uploaded the original blots of Figures 1C and 6A, 6B as "figure source data".

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