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NSMCE2 suppresses cancer and ageing in mice independently of its SUMO ligase activity

Ariana Jacome, Paula Gutierrez-Martinez, Federica Schiavoni, Enrico Tenaglia, Paula Martinez, Sara Rodriguez, Emilio Lecona, Matilde Murga, Juan, Mendez, Maria A Blasco & Oscar Fernandez-Capetillo

Corresponding author: Oscar Fernandez-Capetillo, CNIO

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Transfer note:

PLEASE NOTE that this manuscript was transferred to The EMBO Journal after review at another journal, and that the original referee reports and referee identities had confidentially been available to the EMBO Journal editors in this case.

Authors' transfer cover letter and point-by-point-response 16 April 2015

Thanks for your interest on our work, and to work with us in defining a work-plan for the review process. I am pasting a detailed point-by-point response to the reviewers comments, which you can use as you see fit.

Besides the point by point review, I here wanted to share with you the specific changes that I propose we could do in a reasonable timeframe (2 months?), to get your feedback on whether you consider them sufficient to move forward.

Looking forward to hearing from you.

Summary of specific experiments to be done: REF1

1. We could perform on DNA combing experiments to illustrate how NSMCE2 deletion impacts on DNA replication.
2. We could further report on chromosome segregation phenotypes observed on NSCME2 deleted B cells or MEF.
3. We could quantify NSMCE2 foci in NSMCE2-SD cells by High Content Microscopy.

REF2

1. We can provide a figure illustrating that NSMCE2 foci do NOT colocalize with a DNA break marker (i.e. 53BP1) in response to irradiation.
2. Provide additional blots to illustrate NSMCE2 deletion upon tamoxifene exposure.
3. We can provide a new dataset regarding SCE levels, in which we show that the increased SCE that is observed on NSMCE2 deleted cells, depends on MUS81. This finding, I hope, is rather substantial.

REF3

1. Provide the genetic analysis of MUS81 and NSMCE2 doubly deleted MEF to address the concern about "mechanism". These data show that the increased SCE of NSMCE2 depends on MUS81, providing a basis for the increased recombination rates.
2. Provide examples of wt mice treated with Cre, to show that the Cre does not accelerate ageing in mice. Along with our findings, this is the same mouse used previously by Eric Brown (Ruzankina et al Cell Stem Cell 2007), which also did not note any "ageing" effects of the Cre expression.
3. Provide an IP that shows that the SMC5/6 complex forms even in the absence of NSMCE2.
4. We could provide, if needed, a figure of NSMCE2 foci in response to MMC and cisPt.

In addition to the experiments, we could extend in the discussion of our phenotypes and in the description of the tumor types, as requested by the reviewers.

NOTE: Reviewers 2 and 3 want more in vitro SUMOylation assays. Please read my comments to the reviewers. If this it what it takes to communicate our work, we´ll do it, but I honestly cannot see how a RING domain without one of the metal coordinating Cysteines can work.

1st Editorial Decision 06 May 2015

Thank you for sending us your point-by-point response to the referee comments. I have taken a look at it and I have also discussed it with with my colleagues. We find your response very reasonable and would like to ask you to revise accordingly. Regarding the issue of the in vitro SUMOylation assays - we do think that it would help to have this analysis included. How much effort does it take to do the experiments? I am happy and available to discuss this issue further.

Thank you for considering us - I look forward to seeing the revised version

1st Revision 31 July 2015

I am here submitting the revised version of our manuscript entitled "NSCME2 suppresses cancer and ageing in mice independently of its SUMO ligase activity" for its consideration in The EMBO Journal.

I want to thank your interest in this work from our lab, which I believe is a herculean effort from our

group in characterizing the functions of the SMC5/6 complex in mammals using mouse models. As I mentioned by email, it has taken us a long time to come with the revised version since we really tried hard to develop a SUMOylation assay to look an endogenous NSMCE2 activity.

Unfortunately this has failed and we can only detect NSMCE2-dependent SUMOylation upon overexpression of both NSMCE2 and SUMO, data I personally believe should be interpreted with caution. We have now nevertheless completed all the experiments that I drafted in my letter to you

regarding our plan for the review, and the manuscript has significantly strengthened as a result. The new data on DNA combing requested by ref #1, which show that NSMCE2 is dispensable for DNA replication should be particularly clarifying for the field given the confusion around the potential roles of this complex. As a whole I do believe that this work should be very informative for those with an interest on SMC complexes and DNA replication and repair in general.

I hope you share that our manuscript meets the high standards for novelty and quality that you expect and help us communicate our work at The EMBO Journal.

Point-by-Point-Response

Reviewer #1

The manuscript by Jacome and colleagues focuses on NSMC2E2 in mice. The SUMO ligase NSMC2E2 is a subunit of the Smc5/6 complex (Smc5/6), which in turn belongs to the family of SMC complexes. Eukaryotic SMC complexes also include the cohesin and condensin complexes, and while these two complexes are relatively well explored, the role of Smc5/6 is more uncertain. Smc5/6 has been most extensively studied in yeast, where it has been shown to control recombination, having a crucial role in the resolution of recombination intermediates between sister chromatids during both mitotic and meiotic DNA repair. More recent investigations also couple Smc5/6 function to replication, chromatid entanglements, topoisomerase 2 and chromosome segregation in the absence of recombination, which might reflect the essential role of the complex. Some studies have also been performed in mammalian cell culture, and in general, these support a role for Smc5/6 in recombination and maintenance of chromosome structure. There is one investigation on the effect of loss of Smc5/6 function in mice published so far, and this concluded that Smc6 is essential for embryonic development, but the reason(s) for this lethality was not explored in detail (Ju et al., DNA repair, 2013). Reduced levels of NSMCE2 have also been shown to lead to chromosome missegregation in human patients, and developmental defects in both human patients and zebrafish (Payne et al, JCI, 2014). Importantly, however, no clear connection between Smc5/6 function, cancer and premature aging has been reported.

In the manuscript under review the authors find that loss of Nsmce2 leads to embryonic lethality after 2.5 dpc. Further in vitro analysis of embryos lacking NSMCE2 (shown by immunostaining) provides results that indicate that the lethality is caused by impaired chromosome segregation. Analysis of heterozygous Nsmce2-/+ mice indicates that the gene is a haploinsufficient tumor suppressor, and Nsmce2-/+ primary cells are shown display increased levels of mitotic recombination and chromosome missegregation. Using a conditional Cre-lox knockout strategy the authors also show that induction of Cre from 14.5 dpc leads to dwarfism (similar to human patients and zebrafish with reduced levels of NSMCE2) and, likely (see point 11 below), chromosome bridges at anaphase. Induction of Cre in MEFs is shown to lead to increased recombination, higher levels of sister chromatid exchanges, and cell death. Induction of loss of Nsmce2 in mice from weaning and onwards leads to premature aging and death. Several phenotypes of these mice are similar to those of Bloom patients, and the authors therefore compared the effect of double and single KO of Blm and Nsmce2 in mice B cells. The results show a synthetic lethal interaction between the two genes, accompanied by higher levels of sister chromatid exchanges and aberrant nuclear structure.

In contrast to the strong effects of an Nsmc2 knockout, knock in of sumo-ligase defective mutant is shown to have little, if any, effect on mouse development.

In addition to the above, the authors also use immunofluorescence to investigate NSMCE2 chromosomal distribution in spermatocytes and on somatic chromosomes in the absence and presence of ionizing irradiation, MMS or TOPO2A inhibitors. These binding patterns are compared with the localization of a meiotic marker for chromosome synapsis (SCP3), and with that of BRCA1. Collectively these results suggest that NSMCE2 bind structures that accumulate after replication

and/or inhibition of chromatid disentanglement, but not directly to DNA breaks. Comparison between NSMCE2 and BLM localization after MMS treatment also give support the idea that the two proteins work in separate pathways.

The observation that NSMCE2 deficiency triggers cancer development and aging is new, and advances the field of Smc5/6 research substantially. It also provides a starting point for the deciphering of recently reported, but less well explored, links between Smc5/6 and human cancer development. Based on this, and the overall high quality of methods, results, presentation and discussion, I consider that the manuscript could merit publication in xxxxxx.

We thank this reviewer for his/her appreciation of our work.

However, to strengthen the conclusions drawn, and correct a few shortcomings in the presentation and discussion of the results, the following questions/issues need to be addressed:

1. With the inducible Cre-lox system in hand, a more detailed analysis of the effect of loss of NSMCE2 could be performed, aiming to further clarify the underlying reason for the observed phenotypes. Based on the authors' discussion the effect of Nsmc2 knockout on replication could be tested (by DNA combing). It could also be investigated if anaphase bridges accumulate in specific (repetitive) regions of chromosomes.

We have now used this system to evaluate any putative role of the SMC5/6 complex on DNA replication. To this end, we have obtained the expert help of the group of Juan Mendez, expert on DNA combing. We have now included a new main figure on DNA fiber analyses that shows that the absence of NSMCE2 does not affect origin firing or fork rate (Fig 7). Given all the uncertainties around the functions of the SMC5/6 complex, I think this data, even if negative, would be rather informative for the readers of our work and hopefully clarifying for the field. We thus believe these data significantly strengthen the paper and thank the reviewer for bringing this up.

At this point we do not know whereas anaphase bridges happen preferentially around some specific sites. I must note, however, that the major segregation defect that we find in these mutants is not anaphase bridges but rather the accumulation of micronuclei (see, for instance, the new Fig S6 or Fig S11). From searching the literature, this is the mutant (NSMCE2-deficiency) where I have seen the highest spontaneous increase in micronuclei ever reported. At this point we do not know if these micronuclei land preferentially on certain sites, although as the reviewer notes repeated sequences will be a place to look for in the future given the rest of our observations.

2. The relation between tumor suppressor and anti-aging role of NSMCE2 should be discussed in more depth. As it stands now, the reason behind the premature aging, and its relation to the observed chromosome instability phenotypes, is left mostly uncommented.

We have now extended on our discussion of the mouse phenotypes. The paper was already quite lengthy and overpopulated with data (even more now after the extra data from the review), which is why we were conservative in our discussions.

3. Since many of the results are based on the new NSMCE2 antibody developed by the group, figure S1a should depict the entire western blot and not merely "the area of interest" as now.

We now show the full blot.

4. At some places in the text and figure legends it is not always clear if a "NSMCE2 focus" is MMS-induced or not. This should be clarified.

Done.

5. The non-overlapping pattern of NSMCE2 and BRCA1 within a MMS induced (?) focus is indeed interesting as the authors state. But what does it indicate? I find no comment on this in the manuscript.

We do not know what this pattern means. We added this simply because it is reminiscent of other non-overlapping foci described previously in the literature (e.g. 53BP1 and BRCA1) and thought could be of interest or provide ideas to some of the readers. But we have eliminated it in the current version for clarity.

6. The condensed chromosome phenotype that should be displayed in figure 2e is not easy to see. This should be improved. The frequency of hyper-condensation, as well as other irregularities in chromosome/nuclear structure, could also be quantified.

Figure 2e represents IF data on 2.5 dpc embryos and only wants to reveal that these embryos show major segregation defects, which is consistent with the heterogeneous cell sizes that can be observed in these embryos (Figure 2c). This is a rather challenging protocol and the observation made is qualitative (wild type embryos never show such figures). In addition, the segregation problems depicted in figure 2e are consistent with the many yeast papers reporting segregation problems on smc5/6 mutants, and with the additional figures where we show segregation problems (anaphase bridges on embryonic thymuses, micronuclei in MEF or colon, massive mis-segregation on B cells...).

To further quantify segregation problems on NSMCE2 deleted mouse cells we have now performed a complete analysis of the appearance of intercellular DNA bridges, micronuclei and major segregation defects that can be observed in NSMCE2 deleted fibroblasts. We have also included examples of each case to further document the phenotypes that can be observed in these cells (new Fig S6).

In addition, from the analysis of NSMCE deleted B cells that we used for DNA combing, it is also evident that, while NSMCE2 deleted cells replicate fine, they accumulate cells with >4n DNA content (new Fig 7). This becomes catastrophic when BLM is also deleted, as already shown in the previous version. All in all, increased recombination rates and deficient segregation are the two main phenotypes observed on NSMCE2 deleted cells.

7. The remaining residual activity detected when using SUMO1 could indeed be due to endogenous NSMCE2 in the HEK293 cells. There is no need to add claims down the line that "others used this allele as SUMO ligase dead so...". It does not strengthen the case, rather the opposite.

I can only agree with this reviewer... The only reason why I added this extra information was to, hopefully, be conclusive enough. One would have thought that mutating one of the Cys that coordinates the metal binding of the SP-RING domain (plus a catalytic His) is considered sufficient proof of killing the activity of a UQ or SUMO ligase. The fact that the sequence is conserved in yeast, and this very same mutation had been used in numerous top publications should have helped (I hoped) to strengthen this point. But what I hoped was sufficient proof, was not, and reviewer 2 and 3 requested further SUMOylation assays. We have now invested quite some time on this. However, after several independent approaches in these last 3 months, we have been unable to develop an assay that looks at the activity of endogenous (not overexpressed) NSMCE2 in wt and SD cells. We can still detect NSMCE2-dependent SUMOylationy but only when overexpressing huge amounts of both SUMO and NSMCE2. In this context, my opinion is that the relevance of *in vitro* SUMOylation assays should be taken with a pinch of salt. At this point, I prefer to state by our original claim that the mutant mice are SUMOylation defective. Nevertheless, and to contemplate the possibility that some SUMO ligase activity still exists (although I can hardly see how with an unstructured RING domain), we now have introduced the following sentence: "*Hence, and whereas we cannot formally discard that some residual activity remains on Nsmce2*$^{SD/SD}$ *cells, our data reveal that the SUMO ligase activity of NSMCE2 is largely dispensable in mice*."

8. MMS induction of NSMCE2 foci in cells expressing wild type or SD NSMCE2 should be quantified (and possibly compared to levels of BRCA1 foci) to further certify that no subtle effect of the SD mutation has been overlooked.

We have now quantified MMS (and MMC) induced NSMCE2 foci by High Content Microscopy, which again fails to detect any significant difference between the number of these foci in MEF from wild type or SD mice (new Fig S4).

9. The results obtained concerning the SUMO activity should be discussed in the context of what has been reported for this issue in human patients (Payne et al).

The data reported in the human patients carrying mutations on NSMCE2 cannot be formally linked to SUMOylation. The SUMO inactivating mutation reported by *Payne et al*, also leads to severely reduced protein levels of NSMCE2, so that it cant be said that the effects had anything to do with SUMO or was simply due to the hypomorphism. We now mention this on the discussion.

10. On page 9 it is stated that the decreased life span is associated with higher incidence of tumors How much higher? What type of tumors?

We have now added a panel on this Figure (Fig 4B) that details the incidence and type of tumors found on NSMCE2 heterozygous mice. The most interesting thing is that the appearance of tumors (i.e. pancreas or liver) that are hardly ever seen in wild type animals, or the increased frequency of

mice showing several tumors at the time of death. The broader spectrum of tumors is a frequent observation in mice with increased recombination rates.

11. Is the higher level of anaphase cells displayed in Figure S5 really signs of chromosome bridging? Or are they due to the accumulation of cells in mitosis? The bridges are not that obvious, at least when there is no wild type anaphase to compare to.

Our interpretation is that the embryonic thymocytes presented in Fig S5 (now Fig S8) accumulate at various stages in mitosis because of segregation problems (as shown throughout the manuscript), some of which present bridges. Hence the figure just wants to illustrate mitotic arrest (reminiscent of what is observed on 2.5 day embryos), rather than focusing only on the bridges. We have changed the text accordingly. We have also included a zoomed-in inset on the figure that illustrating one example of *bona fide* anaphase bridges that can be found on NSMCE2-deficient embryonic thymuses.

12. With reference to figure S6 it is stated that "the strategy was efficient as seen by the loss of NSMCE2 expression in all tissues analyzed". Since expression still is detected in some tissues (heart, brain) "loss" appears as too strong.

I have now tuned down the text to reflect the variability in the extent of NSMCE2 depletion.

13. The second sentence in the introduction, presenting SMC protein structure, is difficult to follow.

The sentence has been rewritten for clarity.

14. An "at" on page 8, just before a reference to figure 2c, should be removed.

Thanks, removed.

15. On page 14, second paragraph, "works" should be replaced with "studies" or alike.

Corrected.

16. Hydroxyurea, an the lower middle part of page 16, is misspelled.

Corrected.

17. In figure legend 5 it is stated that "Of note...stain present NSMCE2 expression, indicative of ..." The sentence is a somewhat hard to read, and where is the data this sentence refers to?

We have removed this sentence. Nevertheless, to explain why we included this, we grew some of the colonies of OHT-treated NSMCE2-lox/lox cells and invariably found that they express NSMCE2. In every growing cell tested, including cancer cells, NSMCE2 is essential.

18. The authors might want to refer to, and discuss the content of, Gómez et al, Journal of Cell Science, 126, 4239-4252, 2013.

Done.

Reviewer #2

The manuscript describes the phenotypes of a number of mouse lines with alterations in the gene encoding NSMCE2. This protein is a part of the SMC5/6 complex, with a putative role in chromosome dynamics. This is a useful study, because there are not other mouse studies of this protein. The results are interesting, and potentially provocative. For example, the conclusion that SUMO ligase activity of NSMCE2 is inessential contradicts a previous report by Potts and Yu (2005). The discovery of a strong progeria phenotype in NSMCE2-deficient mice is remarkable.

It could be argued that the manuscript is somewhat descriptive, and I had several technical questions about the work. The writing is usually quite clear, although there are a number of typographic mistakes and idiosyncratic phrases that could be fixed.

Thanks for understanding our study as useful, interesting and potentially provocative. I am rather certain that describing that the SUMO ligase activity is largely dispensable for mammalian lifespan, that NSMCE2 works independently of BLM or the cancer and ageing phenotypes observed in our mice would indeed be rather informative for the researchers in the field.

My major outstanding questions are summarized below:

1. I'm interested to know why the authors choose the nomenclature 'NSMCE2'. Although clearly formally correct, would it not be more conventional, and convenient to potential readers to call it 'Nse1' or 'MMS21' or 'Nse1/MMS21'? The risk is that people searching for papers about MMS21 won't find this paper.

Ourselves, we never use NSMCE2 when talking about MMS21. However, NSMCE2 is the actual name of the protein in mammals, so out of rigor this is the only name we can use. We have added MMS21 within brackets in the abstract, so that this problem does not happen and that our work reaches everyone interested in MMS21/NSMCE2.

2. The idea that NSMCE2 does not localize to break sites is interesting, but could use direct verification by staining for double-strand breaks. For example, do gH2AX staining on spermatocytes to show that NSMCE2 does not colocalize with breaks. Show % colocalization of NSMCE2 and gH2AX / 53BP1 in untreated MEFs and MEFS with IR, MMS, PARPi, ICRF-193 treatment.

During meiosis, this is clear. In spermatocytes NSMCE2 maps to the core of the XY chromosomes, while H2AX marks the whole sex body but the core (this is an area where I worked in the past, i.e. Fernandez-Capetillo et al Dev Cell 2003). Hence, in meiosis, they clearly do not colocalize. The fact that they do not colocalize with breaks in mitotic cells was also somehow implicit in Figure 1E, since NSMCE2 foci are not induced by ionizing radiation, but H2AX foci are. Nevertheless, to make this point clear, we now have added a panel with examples in Figure 1F showing that NSMCE2 does not colocalize with IR- induced ãH2AX foci, a bona fide marker of DSB.

3. I have some concerns with the SUMO-mutant NSMCE2. Fig S3b shows sumoylated NSMCE2 in the SD mutant. The author's contend that this represents endogenous NSMCE2, but it is also possible that the SD mutant is a hypomorph. These point mutations may inactivate NSMCE2 in yeast, but this is not fully compelling for the mammalian system given the presence of an NSMCE2-SUMO1 band. Given the possibility that the SD mutant retains E3 SUMO ligase activity, the conclusion that this enzymatic activity is dispensible is unsafe. The authors should tag their exogenous NSMCE2 to test what the SUMOylated species is. (Also, several of the labels in Fig. S3 are too small to read clearly.) 4. The other problem with this experiment, which is somewhat harder to deal with, is that autoSUMOylation in vitro may not be a good assay for SUMO ligase activity in cells. A better experiment might be to test the SUMO status of the putative substrate, Scc1. Either way, this is quite an important point, because we need to be quite sure that NSMCE-SD is a true enzymatic null before concluding that the enzymatic activity is dispensable.

For this, I can only re-state my response to ref 1, point 7. To answer this question properly/definitively, we have invested quite some time in trying to look at the activity of endogenous NSMCE2 rather than the overexpressed one by doing IP-followed by SUMOylation assays. Unfortunately all of our attempts have failed and as before we can only get NSMCE2 SUMOylation assays to work when we artificially overexpress NSMCE2 and SUMO to very high levels. My take is that the relevance of these assays has to be taken with a pinch of salt. Structurally, killing a Cysteine (and a His) from the metal coordinating domain should be definitive enough. From the many publications using RING mutants (both for SUMO or UQ), I know of no cases where mutating one of the metal- coordinating Cys from a RING domain does not kill the activity of the enzyme. The RING domain needs this Cys to coordinate the metal binding or otherwise there is essentially no domain (it is unstructured). Notwithstanding the fact that the sequence around this Cys (and His) are conserved from yeast studies, and the same mutation has been used repeatedly in top tier publications as a SUMOylation deficient strain.

In any case, to be conservative, we now have introduced the following sentence: : "*Hence, and whereas we cannot formally discard that some residual activity remains on Nsmce2*$^{SD/SD}$ *cells, our data reveal that the SUMO ligase activity of NSMCE2 is largely dispensable in mice*."

5. For the NSMCE2 GT/+ animals, the reduced lifespan and tumor incidence could arise from a dosage effect, or from a neomorphic effect of the GT allele. This is a problem with gene-trap alleles, because they might express a toxic truncated product. I don't think the conclusion that Nsmce2 is a haploinsufficient tumor suppressor is therefore completely safe.

This concern applies to any of the numerous GT alleles that have been ever reported in the DNA repair field (or in many others). Actually, it also applies to many of the conditional KO mice that have been ever made, since many of them do express truncated parts when the floxed exon is on the middle of the gene. With the antibodies we have we cannot detect a truncated product, but of course being a purist absence of evidence is not evidence of absence. To consider this option, we now explicitly mention this possibility in the discussion.

6. It's not clear what kind of antibody the authors used to stain NSMCE2. The methods mentions that polyclonal and monoclonal antibodies were generated. Which of these were used for the experiments, and what is the specificity of the monoclonal antibodies? Is it possible to use a monoclonal for the N terminus of NSMCE2 to test for the expression of a truncated protein product in NSMCE2 GT/+ cells?

The monoclonal antibody only recognizes human NSMCE2, and the polyclonal one only works in mouse cells. All experiments using mouse cells use the polyclonal antibody, and the few showing human cells use the monoclonal one. We now more clearly specify this in the methods section. Regarding specificity, the polyclonal one shows no staining in NSMCE2 KO cells, tissues or embryos, and the western band disappears. The monoclonal band (human) disappears by WB and IF when NSMCE2 is depleted by siRNA. This information was actually already on the paper. Regarding the expression of a truncated product, since we used GST-NSMCE2 (full length) and we do not know where the antibodies bind, we unfortunately cannot formally address that question.

7. What kind of tumors arise in NSMCE2 GT/+ animals? Is it just lymphoma and sarcoma, as indicated in the legend to Figure 4? Or other types as well? Note: Fig 4a does not show tumor incidence (as suggested by the text), just lifespan.

We have now added a panel on this Figure (Fig 4B) that details the incidence and type of tumors found on NSMCE2 heterozygous mice. The most interesting thing is that the appearance of tumors (i.e. pancreas or liver) that are hardly ever seen in wild type animals, or the increased frequency of mice showing several tumors at the time of death. The broader spectrum of tumors is a frequent observation in mice with increased recombination rates.

8. The loading control for Figure 5b is questionable. I can't see clear bands.

We have now provided another blot with a proper loading control. The conditional allele works very nice/consistently on all systems tested (B cells, MEF, etc...)

9. Regarding Figure 5, the authors suggest, "Interestingly, cells showing residual amounts of NSMCE2 foci presented large amounts of BRCA1 foci, indicative of increased recombination." This is confusing. The cells that have NSMCE2 foci are those that did not delete exon 3, right? If that is the case, these cells should just be WT and there should be no phenotype. Please clarify.

Since NSMCE2 foci do not appear in every cell, if we had chosen cells with no NSMCE2 foci as examples of deletion, we would be “cheating”. Out of rigor, we preferred to select those cells with very small NSMCE2 foci, which never exist on wild type cells, as cells that are strongly depleted of NSMCE2. Nevertheless, to avoid confusion, we have eliminated this sentence and simply state that NSCME2 deletion leads to increased BRCA1 foci in MEF (which is consistent with the increased recombination rates)

10. Regarding Figure 5E, I have several questions: a. How many metaphases were scored? And how many times was the experiment done? What statistical test was used? I see 27 data points for the +OHT column, which does not seem like very many. I would aim to score at least 50 per experiment.

SCE experiments were performed several independent times per condition, all of which showed the same trend. As for the number of metaphases, the reviewer should bear in mind that these cells grow very poorly, which limits the number of metaphases we normally can count. Nevertheless, the collective analysis of these data is clear, and consistent with previous literature, in that NSMCE2 deletion leads to increased recombination. We also repeated the experiments in MEF by precisely looking at SCE events at telomeres, which showed the same trend of increased recombination. The difference is rather notorious one would say (please take into account that these cells are not exposed to any genotoxic, these are spontaneous SCE events). In addition, this observation was also recapitulated in B cells, as shown in Fig 7.

To further develop this part, we have now crossed *Nsmce2* cKO animals with MUS81-KO mice. These dataset shows that, like previously shown for BLM-deficient cells by the group of S West

(Wechsler et al Nature 2011), the increase in SCE observed in NSMCE2- deficient cells is MUS81 dependent (new Fig S5). This, I believe, is very informative, since it provides an explanation of how SCE is initiated (through MUS81-dependent breakage), and further suggests a similarity between the functions of NSMCE2 and BLM. Altogether, I hope you share that the increased recombination phenotype that is observed in NSMCE2 mutant mouse cells is sufficiently documented in this MS.

b. If OHT deletion is not compatible with proliferation, as indicated in 5c, it is quite challenging to do a reliable SCE assay. Growth in BrdU medium would tend to select against NSMCE2-deficient cells.

Actually, agreed... The data shown in these figures could easily be under-representative of the actual recombination values. However, I must say, that whereas NSMCE2 deletion is essential for MEF viability, cells can grow several cycles without MMS21 before arresting. This is consistent with yeast data, and with the findings on many other chromosome segregation mutants. Cells attempt to segregate, fail, generate DNA damage, etc... until they finally arrest. In fact, we have now carefully analyzed DNA replication in NSMCE2- deficient B cells by DNA combing, which has allowed us to show that NSMCE2 is actually dispensable for the process of DNA replication. Given the uncertainties around the SMC5/6 complex functions in mammals, I believe these data will be very informative for the readers of this MS, so that I have decided to include it as part of the main figures (new Fig7).

In any case, in short, agreed. It is very likely that the increase in recombination is higher than actually shown due to inherent limitations of the system.

c. Is it possible that there is a bimodal distribution in the +OHT group, i.e. cells that have an elevated rate of SCEs, and cells that have a normal level of SCEs?

It is possible, since some cells might not have deleted NSMCE2 or still had some before entering this mitosis. Unfortunately there is not an easy way to address which SCE metaphases are WT or NSMCE2 deleted.

d. There is a risk that the elevated risk of SCEs is coming from Cre expression, as opposed to NSMCE2 ablation. To exclude this possibility, the authors should use UQ.Cre- ERT2; Nsmce2+/+ cells with OHT treatment.

In the SCE experiments performed for the review on the role of MUS81 we have now included wt cells in our analyses to evaluate the potential impact of Cre expression on the SCE phenotype (Fig S5). These data unambiguously show that NSMCE2 deletion increases SCE events in mouse cells.

11. The authors suggest that NSMCE2 deletion causes pathology similar to Bloom Syndrome patients, but BS patients always get tumors. The mice do not. That's a big difference. I would de-emphasize the phenotypic similarity with Bloom Syndrome, and cite a reference to clinical literature for what phenotypes are shared.

At no point are we stating that NSMCE deletion leads to Bloom Syndrome, but just wanted to raise the point that some of the phenotypes observed on NSMCE2 deleted animals have also been reported in BS. Comparing mouse phenotypes with human Syndromes is always far stretched, and particularly on this case given that BLM deficiency has a different impact on mice and humans. BS patients get tumors but BLM deficient mice (like NSMCE2 deficient mice) are not born.

In any case, together with the many reports in yeast that illustrate similar phenotypes in Smc5/6 and Sgs1 mutants, we just want to make the point that several of the pathologies we find upon deletion of NSMCE2 in adult mice are reminiscent to those found in BS.

12. The synthetic lethal relationship between BLM and NSMCE2 is intriguing, but slightly underdeveloped, and arguably tangential to the rest of the manuscript. I would suggest to take Figure 7 out of this manuscript, work out the mechanism, and publish it as a separate report.

Here, I beg to disagree. Sgs1 and Mms21 are also synthetic sick in yeast, yet the essential nature of the mutation severely limited the analysis of the reasons behind this phenotype. With the advantage of B cell cultures, we provide several key mechanistic insights to this phenomenon that could not be made from yeast studies: (a) that the synthetic lethality is due to catastrophic segregation (the FACS phenotype is rather spectacular), (b) that BLM and NMSCE2 suppress recombination independently and (c) that BLM and NSMCE2 foci do NOT colocalize. For those working on the SMC5/6 complex knowing that BLM and NSMCE2 localize to different foci should be very informative, and already provides an explanation for their independent activities (they both suppress recombination,

but at different places). All in all, I believe that this is important information for the readers and will be appreciated in this MS. I would prefer to leave it here, which I believe is the generous thing to do anyway.

13. The authors should comment further on the relationship of their results regarding the importance of NSMCE2 enzymatic activity with those published by Potts & Yu (2005). There appears to be a significant discrepancy here.

We are aware that our work (and others') are in a significant discrepancy with studies by Potts and Yu. Their work has repeatedly shown that MMS21 promotes recombination, particularly at telomeres (NSMB paper 2007). Not sure where the reason for the discrepancy is. One possible interpretation is that their studies where performed using siRNA knockdowns which might have an impact on cell growth and, if cells were to replicate less, then they would also be less prone to recombination. I must note, however, that not only us but all the literature available on the SMC5/6 complex using clean genetics

supports that the complex suppresses recombination. MMS21 was actually discovered more than 30 years ago by the fact that it was a hyper-recombinant mutant (23-fold). We are in agreement with this classical study, and the many others that came afterwards showing increased SCE on SMC5/6 mutants from several organisms including mice or chicken DT40 cells. Given the 2007 NSMB paper, we specifically looked at telomeres and found that, in contrast to the previous report, inter-telomeric recombination was higher on NSMCE2-deleted cells. It could of course also be that they are using human cells, and that in humans the SMC5/6 complex works differently to yeast, chicken or mouse. If possible I would prefer not to discuss much on this matter on the paper, I think the fair thing to do is to present our findings and let the scientific community judge the different datasets available.

14. The abstract is somewhat perfunctory- consider revision.

OK, I have now rewritten the abstract (but take my word that it was not perfunctory, it went from multiple rounds of thoughts/versions, and that was the best I could come with...). PS: I had to look up in Google what perfunctory means :)

Reviewer #3

NSMCE2 suppresses cancer and ageing in mice independently of its SUMO ligase activity By A. Jacome et al.

In this manuscript the authors study the function of the SMC5/6 complex in mammals. The role of this complex in genome maintenance has been investigated already in yeast, chicken and mice. This said, in this study the authors make use of 3 different, well developed NSMCE2 mouse models which show that NSMCE2, but not its SUMO ligase activity, is essential for mouse development. Interestingly, the NSMCE2 GT/+ mice show a reduced lifespan and a higher incidence of tumors, indicative that NSMCE2 is a haploinsufficient tumor suppressor. NSMCE2 GT/+ cells showed an increase in sister chromatid exchange (SCE). Finally, the authors use a conditional knock-out approach which showed progeroid features resembling Bloom's syndrome. Deletion of both Blm and Nsmece2 showed the effect of depletion of NSMCE2 to be independent of BLM. In line with this, deletion of BLM and NSMCE2 results in a further increase of the SCE, which might suggest that BLM and NSMCE2 recognize different substrates. In line with this BLM and NSMCE2 are not present in the same MMS-induced foci.

We thank this reviewer for appreciating our efforts with our mouse models, and for considering that we have developed our studies well.

Overall comments

a. The manuscript presents a carefully executed study on the function of NSMCE2 in mice, which shows that NSMCE2 suppresses accumulation of SCE through a mechanism independent of its SUMO ligase activity and distinct from that of BLM. This raises directly important questions: (1) what is the mechanism by which NSMCE2 exerts its function, (2) what are the different substrates to which BLM and NSMCE2 bind upon genotoxic damage and (3) what is the function of the SUMO

ligase activity of NSMCE2. The paper would gain significantly more impact when answers to these crucial questions would be provided.

We thank the reviewer for saying that our work is carefully executed. The reviewer here acknowledges that our work shows that NSMCE2 suppresses SCE independently of its SUMO ligase activity, and that it does so independently of BLM. We are happy enough if he/she judges that the work is good enough to show that (which I believe it does). These statements, backed by genetic work of many years, should in itself be rather interesting for the scientific community, particularly for those working on the SMC5/6 complex. This has been a confusing complex ascribed to many functions (DNA repair, cohesion...). I hope our data helps to illustrate that the complex plays a minor role (if any) on the repair of DNA breaks, or in DNA replication (see our new combing data, Fig 7), but is essential to suppress recombination and to facilitate segregation. This, I hope, will be clarifying for the field. Notwithstanding our work describing that NSMCE2 is haploinsufficient for cancer suppression, or that NSMCE2 deleted animals develop symptoms that are also found on Blooms Syndrome. Collectively, I hope he/she shares that these results significantly improve our understanding of the functions of the SMC5/6 complex in mammals.

The reviewer then comes with three questions: How does NSMCE2 work, what are its key substrates and how do they differ from those of BLM (Do we even known what are BLM substrates *in vivo*?). These are, of course very relevant questions. Unfortunately, many essential questions around this complex are sill without answers but I hope our works helps to clarify some of them.

Regarding targets: in the past, some papers have been reported in mammals using RNAi and overexpressed NSMCE2/SUMO assays on the discovery of NSMCE2 targets (e.g. (1) Potts and Yu, NSMB 2007. NSMCE2 sumoylates telomeric proteins to **promote** recombination at telomeres; or (2) Wu et al Genes Dev 2012, NSMCE2 sumoylates cohesins to promote cohesion). However, in striking contrast, and in agreement with all the good genetic data available from yeast, we find that recombination is not only not decreased but actually increased on NSMCE2 deleted cells. We also do not see any cohesion defects on NSMCE2 deleted metaphases (which we have avoided to comment to avoid further discrepancies). Identifying real targets of NSMCE2 will demand the development of assays looking at the activity of endogenous proteins without overexpression of SUMO, something we have tried in these last months but that is not a trivial endeavor. I must admit that I am not enthused in the quest for its targets given the mild (if any) phenotype of SUMOylation deficient cells and mice.

Regarding "mechanism": understanding how does the SMC5/6 complex work if of course a key remaining question, for which admittedly we do not have an answer yet. In the present version we have added 3 new pieces of data which I hope help in our understanding of this complex: (1) DNA combing which shows that the complex is dispensable for DNA replication (new Fig 7), (2) an IP that shows that the SMC5/6 complex can form in the absence of NSMCE2 (new Fig S12) and (3) a new genetic cross with MUS81 that shows that the increased SCE rates observed on NSMCE2 deleted cells depend on MUS81 (new FigS5). Whereas we still don't know how the complex works, these data will help in understanding what are (or are not) the functions of the SMC5/6 complex, and further reinforce that the main role of the complex is in suppressing recombination and not on DNA replication or DSB-repair. I hope this new data, together with the rest of the MS, are now considered sufficiently interesting to be able to communicate our findings.

b. The cancer and accelerated aging phenotypes are not characterized in a detailed manner and therefore remain somewhat superficial.

We are sorry if it came like that and we have now extended to some extent in the discussion of these phenotypes. The reviewer should bear in mind that we had to do significant text-gymnastics to fit all of these efforts on one paper. In this manuscript we are presenting 3 new unpublished models (the first ones) on NSMCE2 (genetrap, conditional KO, SUMO mutant), genetic crosses with BLM and MUS81, embryo analyses, DNA replication, etc, etc, etc...

c. In addition, CRE expression in the Nsmce2 conditional mutants might cause increased DNA breakage because of the high expression of CRE endonuclease, which may trigger enhanced cell death in the NSMCE-deficient cells and thereby cause intrauterine dwarfism and features of accelerated aging. This possibility should be investigated.

We thank the reviewer for mentioning this since this led us to identify an error in the previous version of the MS. We are actually well aware of the potential effects of the Cre, which is why ALL of our experiments (in this and all projects using Cre expressing mice in our lab) are done

comparing cells and mice that express Cre, and that they only differ on NSMCE2 status (+/+ vs lox/lox). This was already indeed indicated in our previous Figure 5B, but we made a mistake in Fig 6. We are sorry for this mistake and have corrected the labeling and properly explained what is being compared in each case.

Of note, we have never observed pro-ageing features of OHT induction when using the UQ.CreERT2 allele. Neither did the providers of this allele in Eric Brown's lab report in their own experiments (Ruzankina et al 2007).

Specific comments:

- What is the status of the SMC5/6 complex upon NSMCE2 deletion, is the complete complex disturbed or partially functional?

We have already permormed IPs on NSMCE2 deleted cells. Surprisingly, the SMC5/6 complex does form even in the absence of NSMCE2, which of course raises the question of why NSMCE2 deletion is essential. At this point, unfortunately, we simply don't know why, but we believe the data do help in our understanding of this complex.

- The SUMO activity of the NSMCE2 SD should be confirmed either in an in vitro assay, or in a cellular assay in which no endogenous NSMCE2 is present.

For this, I can only re-state my response to ref 1, point 7. To answer this question properly/definitively, we have invested quite some time in trying to look at the activity of endogenous NSMCE2 rather than the overexpressed one by doing IP-followed by SUMOylation assays. Unfortunately all of our attempts have failed and as before we can only get NSMCE2 SUMOylation assays to work when we artificially overexpress NSMCE2 and SUMO to very high levels. My take is that the relevance of these assays has to be taken with a pinch of salt. Structurally, killing a Cysteine (and a His) from the metal coordinating domain should be definitive enough. From the many publications using RING mutants (both for SUMO or UQ), I know of no cases where mutating one of the metal- coordinating Cys from a RING domain does not kill the activity of the enzyme. The RING domain needs this Cys to coordinate the metal binding or otherwise there is essentially no domain (it is unstructured). Notwithstanding the fact that the sequence around this Cys (and His) are conserved from yeast studies, and the same mutation has been used repeatedly in top tier publications as a SUMOylation deficient strain.

In any case, to be conservative, we now have introduced the following sentence: : "*Hence, and whereas we cannot formally discard that some residual activity remains on Nsmce2*$^{SD/SD}$ *cells, our data reveal that the SUMO ligase activity of NSMCE2 is largely dispensable in mice*."

- Fig 1, some of the immunofluorescence panels will be difficult to see for readers (also relevant for other figures).

We have now provided additional panels to illustrate the nature of NSMCE2 foci.

- In dividing cells TOPO2A inhibition can finally result in the onset of DSBs. To strengthen the link between focal NSMCE2 localization and replication stress the authors could test DNA-crosslinking agents such as MMC or CisPt.

TOPO2A inhibition was not used to strengthen the link between NSMCE2 foci and replication stress, but rather to show that, besides reagents that generate replication stress, topological constrains can also recruit NSMCE2. This is informative given the works of Camila Sjogren and others showing genetic interactions between SMC5/6 and topoisomerase mutants. I can't exclude that the so-called catalytic inhibitors also generate DNA breaks in dividing cells, but I must note that these treatments were short enough so that cells did not yet divide. Regarding the use of additional reagents, we now also show that NSMCE2 foci form in response to MMC in addition to MMS and PARP inhibitors.

- Fig 2, what is the genetic background of the mouse mutants used in the study? Since genetic background can be critically important specifically regarding cancer incidence and aging-related diseases, relevant for this study the authors should use for proper comparisons uniform genetic backgrounds for all mutants.

Mice used in this study came from a mixed C57BL/6-129/Sv genetic background, which is in part due to the crosses with additional lines to perform genetic studies. In addition, the phenotypes (i.e.

ageing) are qualitative enough that I honestly do not mind on to what extent they would vary from background to background. We now include background information in the methods part. Repeating all of our experiments on pure genetic background, whereas obviously better, would now take a huge amount of time, which I hope the reviewer finds not necessary given all the data provided.

- The experiment shown in fig 3D should also be performed with the other genotoxic agents used in fig 1E.

We have now quantified MMS (and MMC) induced NSMCE2 foci by High Content Microscopy, which further strengthens the fact that the numbers of NSMCE2 foci do not vary between MEF from wild type or SD mice (new Fig S4).

- Fig 4. Please provide information on the type of tumors and their incidence as well as on the number of tumours investigated for the retention of the wt NSMCE allele. Were tumours chromosomally unstable?

We have now added a panel on this Figure (Fig 4B) that details the incidence and type of tumors found on NSMCE2 heterozygous mice. The most interesting thing is that the appearance of tumors (i.e. pancreas or liver) that are hardly ever seen in wild type animals, or the increased frequency of mice showing several tumors at the time of death. We also specify the number of tumors (7) that we analyzed for the retention of the wt allele. Regarding chromosomal stability, we unfortunately did not establish tumor cell lines for chromosome stability studies. We could I perform array CGH analyses but this experiments are time and fund-costly and not sure how much mechanistic advantage will we gain from these analyses. The broader spectrum of tumors is a frequent observation in mice with increased recombination rates, which helps us strengthen the main message of the manuscript and we thus thank the reviewer for bringing this up.

- Fig 4b, are there mutations detected in the wt NSMCE2 allele in these tumors?

We have not sequenced the tumors. I guess the question goes to whether the remaining allele is defective. I can only say that all of the data provided in this MS, and all of the previous data in yeast, show that nullyzygosity is not viable for SMC5/6 complex members. We cannot even get immortalized MEF that can sustain growth without NSMCE2. I guess there is little doubt at this point on whether cells can survive without NSMCE2. The complex is absolutely essentials in all organisms tested. For instance, in yeast, whereas HR is dispensable, SMC5/6 complex deletion mutants are absolutely inviable. We are now on the quest of suppressors of the lethality, but this work is still on its early days.

- Fig 5. and the premature aging features. A potential complicating factor in the Ub.CreERT2/Nsmce2lox/lox experiments in the notion that upon tamoxifen-induced CRE expression the genome will be incised by the endonuclease not only at the NSMCE2 locus to inactivate the gene but also with lower efficiency at other sites causing DSBs which may cause genome instability specifically when the NSMCE2 protein is depleted. This phenomenon may also complicate the interpretation of the animal studies, causing increased cell death due to enhanced DNA breaks derived from the CRE expression.

As mentioned above, this was actually an error in the previous version and we always compare Cre expressing mice and cells in all figures of our MS (mice, B cells or MEF). Of note, and along with our findings, this is the very same mouse used previously by Eric Brown (Ruzankina et al Cell Stem Cell 2007), which also did not note any “ageing” effects of the Cre expression.

- Fig. 7a information on spleen size and weight including statistics should be added.

This figure was only meant to illustrate a general observation; the rest of the figure provides further details on the synthetic lethality, which I hope is sufficiently documented (Fig 7b, f, and particularly d and e should be clear enough, now Fig8).

Overall characterization of the accelerated aging phenotype is not very detailed.

Explained above. I hope the reviewer empathizes with the significant text-gymnastics that we had to do to describe all of this work and models in one manuscript.

- The discussion is rather speculative

The speculation was mostly (I guess) due to the discussion of ideas around joint molecule dissolution and resolution pathways. I did that because all genetics data coming from yeast is indicating such a role for the SMC5/6 complex. However, JM cannot be “seen”, and the field is not

trivial to discuss upon (we do not yet know what kind of "joint DNA molecules" are recognized by SMC5/6, or even BLM, for that matter). In the current version I have decreased the emphasis on JM dissolution-resolution pathways and extend on the analysis of cancer and ageing phenotypes as requested above.

Typo's:
- Figure 1C is described as figure 1B in figure legends, BLM is misspelled as BML on page 12. Legend: "(1μm 1hr)"should be (1μM 1hr)".

Thanks, corrected.

- Specify what is meant by "unresolved DNA links"(pag 7).

This was meant to integrate many DNA (topological intertwines, stalled forks, late intermediates of recombination...), which keep sister chromatids (or homologous) chromosomes linked together. It is a general statement used by those working on the SMC5/6 complex to refer to "DNA-based" linkages, *vs* "protein-based" links (i.e. chromatids linked by excessive cohesion). I have nevertheless removed it in the current version.

- Page 8, line 7 word missing after "size at" (presumable "size at 36 hrs")

Corrected.

- Page 10, line 2 from bottom: "... cellularity ...".

Corrected.

- Page 11, line 4 "... tamoxifen ...".

Corrected.

- Page 16, middle "... excision ...".

Corrected.

2nd Editorial Decision 20 August 2015

Thank you for submitting your revised manuscript to The EMBO Journal. It has now been re-reviewed by an expert referee, who had also served as reviewer of the earlier version at the previous journal. I am happy to inform you that this referee is by and large satisified with the revisions, and we shall therefore be happy to proceed with publication after a final round of minor revision, in which I would like to ask you to clarify the various remaining specific points mentioned by the referee (noting that the experiments discussed at the very end of the report should only be considered suggestions for future research).

The only remaining caveat that may need some further discussions revolves around the dispensability of NSMCE2 SUMO ligase activity. As you will see, the referee raises an interesting possibility, for which you will probably have some insighful thoughts/arguments, and possibly even some available data on damage sensitivity, that could answer this caveat - I would be happy to briefly discuss this further prior to resubmission of the ultimate version.

Once these remaining minor points will have been clarified, we should be able to swiftly proceed with acceptance and production of the final version! I look forward to hearing from you.

REFEREE REPORTS:

Referee #1:

I find that the authors have appropriately addressed all my concerns, but still want to raise the following issues.

Based on the experiments performed on mice and cells expressing the SUMO ligase-dead mutant Nsmce2SD (presented in figures 3, S3 and S4), the authors claim that the SUMO activity of SMC5/6 is largely dispensable in mice. The issue with this is that the in vitro analysis (Figure S3) indicates residual SUMOylation activity of the mutated NSMCE2. I agree with the authors that this most likely is due to the presence of endogenous wild type NSMCE2, and that the mutations they introduce are expected to disrupt the SUMO-activity (due to structural reasons). I also find that the sentence "Hence, and whereas we cannot formally discard that some residual activity remains on Nsmce2SD/SD cells, our data reveal that the SUMO ligase activity of NSMCE2 is largely dispensable in mice", appropriately highlight the reaming uncertainty.

Yet again, with the small but still remaining uncertainty in mind, the title of the manuscript could be considered to be too strong. Possibly the situation could be somewhat clarified if the authors analyze whether Nsmce2SD/SD cells are hypersensitive to MMS or other DNA damaging agent. The reason for this comes from the observation that also yeast manages without the SUMO activity, but are killed by a deletion of the NSE2 gene (yeast NSMCE2). Importantly however, yeast cells lacking the SUMO activity are highly sensitive to MMS (and other agents). If also Nsmce2SD/SD mice cells are found to be hypersensitive, it would give strong support to the notion that the SUMO activity is disrupted. It could of course be argued that a repair/recombination function for the SUMO activity should be detected as a phenotype in mice (where recombination is essential for normal development etc), but possibly a subtle defect can be missed without (high levels) of exogenous damage. And even though MMS and MMC-foci formation is normal in Nsmce2SD/SD, DNA damage repair/ cell survival might not.

Figures 3 C and S12, the figure legends seem to be wrong, and it becomes uncertain if the level of immuno-precipitated SMC6 was investigated (which is essential to address the stability of the complex). Moreover, it would have been valuable to check the levels of SMC5 in the IP, to be able to compare the efficiency of the precipitation.

Are the cells shown in Fig 5D treated with MMS or not? The text and the figure legend do not agree on this.

Figure S5: something seems to be wrong with the labelling in this figure. If not, the results do not make sense and are not what stated in the text.

The authors wrongly refer to fig 6D on top of page 12, should be 6E.

In the discussion page it is stated that NSMCE2 "foci form almost invariably around pericentromeric heterochromatin (Fig 1, 3, 5; Supplementary Fig S2, S13)". Even though there is no reason to doubt that this is the case, there are no direct results supporting this in the figures (no co-staining with centromeric markers as an example). Since this observation is interesting, it could be valuable to perform such experiment(s).

2nd Revision 24 August 2015

I am here submitting the revised version of our manuscript entitled “NSCME2 suppresses cancer and ageing in mice independently of its SUMO ligase activity” for its publication in The EMBO Journal.

As you can see in my response to the referee, I have changed the text to include some further discussion on the potential role of the SUMO ligase activity in the context of DNA damage. I want to thank your interest into this work of our laboratory, which I honestly believe is a solid and comprehensive piece of solid data that should help clarify much of the “noise” around SMC5/6 and especially about the role of the SUMO ligase NSMCE2.

Point-by-Point-Response:

Reviewer #1

I find that the authors have appropriately addressed all my concerns, but still want to raise the following issues.

Thanks for acknowledging that we have appropriately addressed the previous concerns. I here respond to the remaining comments.

Based on the experiments performed on mice and cells expressing the SUMO ligase-dead mutant Nsmce2SD (presented in figures 3, S3 and S4), the authors claim that the SUMO activity of SMC5/6 is largely dispensable in mice. The issue with this is that the in vitro analysis (Figure S3) indicates residual SUMOylation activity of the mutated NSMCE2. I agree with the authors that this most likely is due to the presence of endogenous wild type NSMCE2, and that the mutations they introduce are expected to disrupt the SUMO-activity (due to structural reasons). I also find that the sentence "Hence, and whereas we cannot formally discard that some residual activity remains on Nsmce2SD/SD cells, our data reveal that the SUMO ligase activity of NSMCE2 is largely dispensable in mice", appropriately highlight the reaming uncertainty.

Yet again, with the small but still remaining uncertainty in mind, the title of the manuscript could be considered to be too strong. Possibly the situation could be somewhat clarified if the authors analyze whether Nsmce2SD/SD cells are hypersensitive to MMS or other DNA damaging agent. The reason for this comes from the observation that also yeast manages without the SUMO activity, but are killed by a deletion of the NSE2 gene (yeast NSMCE2). Importantly however, yeast cells lacking the SUMO activity are highly sensitive to MMS (and other agents). If also Nsmce2SD/SD mice cells are found to be hypersensitive, it would give strong support to the notion that the SUMO activity is disrupted. It could of course be argued that a repair/recombination function for the SUMO activity should be detected as a phenotype in mice (where recombination is essential for normal development etc), but possibly a subtle defect can be missed without (high levels) of exogenous damage. And even though MMS and MMC-foci formation is normal in Nsmce2SD/SD, DNA damage repair/ cell survival might not.

Regarding the SUMO ligase activity, I understand that for the yeast community the lack of phenotype of these mice might be surprising, but bear in mind that there is also a very clear difference in yeast between the absence (lethal) or SUMO deficiency (alive) MMS21 mutants. It is certainly possible that in mice there might be compensation by additional SUMO ligases (like it has been shown in yeast in the context of DNA damage), or simply that the contribution of this SUMO ligase activity is very modest in unchallenged situations. We have actually done some experiments in the NSMCE2 SUMO ligase mutant cells, and we did observe some minor phenotypes in these cells (i.e. increased numbers of micronuclei). However, I do not want to help building a confounding message regarding the importance of this activity in mammals. As a mouse biologist, the fact that SUMO deficient mice live exactly as wild type animals is the most definitive proof to substantiate our actual claim (that the SUMO ligase activity is largely dispensable for mouse lifespan). Of note, we even crossed SUMOylation deficient animals with cancer prone models and saw no impact (although these data are too preliminary since we stopped the crosses after –disappointingly for us- seen no impact). Take my word that we worked really hard to see relevant phenotypes in these mice, and failed.

In any case, since absence of evidence is not the same as evidence of absence, I do not want to be dogmatic and am happy to leave the door open. To this end, I have now introduced the following sentence in the manuscript to state that we cannot rule out that other phenotypes might emerge in the presence of DNA damage.

"Of note, whereas the SUMO ligase activity of NSMCE2 is dispensable for mouse lifespan and overall health in unchallenged conditions, it remains to be seen whether it becomes limiting in the context of DNA damage."

Figures 3 C and S12, the figure legends seem to be wrong, and it becomes uncertain if the level of immuno-precipitated SMC6 was investigated (which is essential to address the stability of the complex). Moreover, it would have been valuable to check the levels of SMC5 in the IP, to be able to compare the efficiency of the precipitation.

Thanks for spotting this; we have now corrected the legends. These data are meant to be qualitative rather than qualitative. Regarding SMC5, with the choice of antibodies available for mammalian SMC5 we could not use the same for the IP and WB, but the fact that the IP was done with SMC5 antibodies should cover that we are looking at the complex. Finally, with the level of precision offered by the IP we can only state that the SMC5/6 complex does form in both NSMCE2-deleted or SUMO ligase deficient cells, and would prefer to avoid stating whether there is a little bit more or less.

Are the cells shown in Fig 5D treated with MMS or not? The text and the figure legend do not agree on this.

Yes, they are treated; the legend has been changed to include the treatment conditions.

Figure S5: something seems to be wrong with the labelling in this figure. If not, the results do not make sense and are not what stated in the text.

Indeed, the labeling of the genotypes was incorrect. We have now corrected this in the present version. Thanks for spotting this.

The authors wrongly refer to fig 6D on top of page 12, should be 6E.

Corrected.

In the discussion page it is stated that NSMCE2 "foci form almost invariably around pericentromeric heterochromatin (Fig 1, 3, 5; Supplementary Fig S2, S13)". Even though there is no reason to doubt that this is the case, there are no direct results supporting this in the figures (no co-staining with centromeric markers as an example). Since this observation is interesting, it could be valuable to perform such experiment(s).

In mouse cells, pericentric heterochromatin clusters into DAPI bright spots that are easily seen by microscopy. The referred figures illustrate the fact that NSMCE2 foci always form abut these DAPI-rich spots. We could have used antibodies like H3K9me3 or any other that mark pericentric heterochromatin, but the results would have been identical since these antibodies in mammalian cells show a 1:1 correlation with DAPI rich spots. We believe these results are consistent with previous yeast genomic efforts that mapped

SMC5/6 complex members around repeated sequences. A similar bias might happen in mammalian cells. We are currently using the tools described here (e.g. NSMCE2 conditional knockout cells) to perform a proper and comprehensive analysis of the genomewide distribution of NSMCE2 in mouse cells, but these studies will not be ready soon and will demand further efforts from our lab.