THE EMBO JOURNAL

In vivo reduction of RAD51-mediated homologous recombination triggers aging but impairs oncogenesis

Gabriel Matos-Rodrigues, Vilma Barroca, Ali Muhammad, Elodie Dardillac, Awatef Allouch, Stephane Koundrioukoff, Daniel Lewandowski, Emmanuelle Despras, Josée Guirouilh-Barbat, Lucien Frappart, Patricia Kannouche, Pauline Dupaigne, Eric Le Cam, Jean-Luc PERFETTINI, Paul-Henri Romeo, Michelle Debatisse, Maria Jasin, Gabriel Livera, Emmanuelle Martini, and Bernard Lopez
DOI: 10.15252/embj.2022110844

Corresponding author(s): Bernard Lopez (bernard.lopez@inserm.fr) , Emmanuelle Martini (emmanuelle.martini@cea.fr)

Review Timeline:

Submission Date:	3rd Feb 22
Editorial Decision:	23rd Mar 22
Revision Received:	6th Jun 23
Accepted:	21st Aug 23

Editor: Hartmut Vodermaier

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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dr. Bernard S Lopez
Institut Cochin
Reproduction and Cancer
24 rue St Jacques
Paris 75014
France

23rd Mar 2022

Re: EMBOJ-2022-110844
In vivo inactivation of RAD51-mediated homologous recombination leads to premature aging but not to tumorigenesis

Dear Bernard,

Thank you for submitting your manuscript on in vivo effects of SMRAD51 expression to The EMBO Journal. I sent it to three expert referees, who have now returned the below-copied comments. As you will see, the reviewers acknowledge the potential interest of this work and its results, but also raise a number of substantive concerns that would need to be decisively addressed before publication may be warranted. In my view, the following issues would be key for providing sufficiently definitive support for the main conclusions:
- Strengthening the evidence for absence of tumorigenesis upon SMRAD51 expression, e.g. by utilizing mouse models with decreased tumor latency or p53 deficiency [refs 1 & 2]
- Deeper investigation of the aging/inflammation-related phenotypes (and how they may originate from HR deficiency) on the hematopoietic system and on rapidly-dividing tissues [ref 2]
- Conclusively confirming that SMRAD51 expression abolishes HR (and A-EJ, SSA) in the murine system (MEFs) and better defining its role in fork protection [refs 1 & 3]
In addition, all referees note that experimental descriptions and presentations are presently insufficient to properly assess the results, and that a revised manuscript would require significantly more careful preparation in various aspects.

Should you be able to satisfactorily address these concerns, as well as the various more specific points listed in the reports, we would be interested in considering a revised manuscript further for EMBO Journal publication. I realize that this may not be trivial and possibly require significant further time and effort, and therefore also cannot make strong commitments regarding eventual acceptance at this stage; however, in light of the comments and our follow-up discussions with the referees, I do feel that incorporation of the above suggestions would be necessary to make the study a more compelling candidate for a publication in a broad general journal like this one.

Since it is our policy to allow only one single round of major revision, it would be helpful to delineate what could (and what could not) be done already during the early stages of your revision work. I am therefore inviting you and your co-workers to carefully consider the reports, and to email me a tentative point-by-point response detailing how the various points raised in all three reports might be addressed/answered, which may serve as the basis for further discussion via email or call. We could also discuss publication of a less extensive revision in another EMBO Press title (such as EMBO reports or Life Science Alliance), in case this should be an option for you, as well as extensions of the default three-months revision period.

Further information on preparing and uploading a revised manuscript can be found below and in our Guide to Authors. Thank you again for the opportunity to consider this work for The EMBO Journal, and I look forward to hearing from you in due time.

With kind regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

*** PLEASE NOTE: All revised manuscript are subject to initial checks for completeness and adherence to our formatting guidelines. Revisions may be returned to the authors and delayed in their editorial re-evaluation if they fail to comply to the following requirements (see also our Guide to Authors for further information):

1) Every manuscript requires a Data Availability section (even if only stating that no deposited datasets are included). Primary

datasets or computer code produced in the current study have to be deposited in appropriate public repositories prior to resubmission, and reviewer access details provided in case that public access is not yet allowed. Further information: embopress.org/page/journal/14602075/authorguide#dataavailability

2) Each figure legend must specify
- size of the scale bars that are mandatory for all micrograph panels
- the statistical test used to generate error bars and P-values
- the type error bars (e.g., S.E.M., S.D.)
- the number (n) and nature (biological or technical replicate) of independent experiments underlying each data point
- Figures may not include error bars for experiments with n<3; scatter plots showing individual data points should be used instead.

3) Revised manuscript text (including main tables, and figure legends for main and EV figures) has to be submitted as editable text file (e.g., .docx format). We encourage highlighting of changes (e.g., via text color) for the referees' reference.

4) Each main and each Expanded View (EV) figure should be uploaded as individual production-quality files (preferably in .eps, .tif, .jpg formats). For suggestions on figure preparation/layout, please refer to our Figure Preparation Guidelines: http://bit.ly/EMBOPressFigurePreparationGuideline

5) Point-by-point response letters should include the original referee comments in full together with your detailed responses to them (and to specific editor requests if applicable), and also be uploaded as editable (e.g., .docx) text files.

6) Please complete our Author Checklist, and make sure that information entered into the checklist is also reflected in the manuscript; the checklist will be available to readers as part of the Review Process File. A download link is found at the top of our Guide to Authors: embopress.org/page/journal/14602075/authorguide

7) All authors listed as (co-)corresponding need to deposit, in their respective author profiles in our submission system, a unique ORCiD identifier linked to their name. Please see our Guide to Authors for detailed instructions.

8) Please note that supplementary information at EMBO Press has been superseded by the 'Expanded View' for inclusion of additional figures, tables, movies or datasets; with up to five EV Figures being typeset and directly accessible in the HTML version of the article. For details and guidance, please refer to:
embopress.org/page/journal/14602075/authorguide#expandedview

9) Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be clearly noted in the figure legend and/or the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure. Finally, we generally encourage uploading of numerical as well as gel/blot image source data; for details see: embopress.org/page/journal/14602075/authorguide#sourcedata

Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 21st Jun 2022:
Link Not Available

If you choose to alternatively have this study further considered by another EMBO Press publication, please use the following hyperlink to directly transfer the manuscript, optionally with inclusion of referee reports and identities:
Link Not Available

Referee #1:

The Manuscript by Matos-Rodrigues et al. describes a mouse that overexpresses a hybrid form of RAD51, which contains the N terminus of the yeast RAD51. The allele is dox inducible and its induction in the three-month-old mice resulted in phenotypes associated with aging, including increased serum levels of pro-inflammatory cytokines, suppression of bone marrow function accompanied by extramedullary hematopoiesis, and premature death. Expression of the allele in younger mice (P12-14) resulted in loss of progenitor cells in multiple tissues, high inflammatory response, and death in less than three weeks. The cellular phenotypes associated with this allele in MEFs included increased DNA damage, decreased replication speed, fork asymmetry, but apparent presence of fork protection. The experimental work is fine, but I have major issues with the authors conclusions that lack of HR in their model leads to lack of tumorigenesis. The text needs to be re-written to de-emphasize absence of tumorigenesis and less emphasis on the lack of HR in the mutant.

1.The authors never show absence of HR. The assays shown in Appendix Figure 1 shows partial HR deficiency. The

sensitivities to olaparib and MMC are also very mild. Without proper controls of RAD51 KD and BRCA2 KD, which I suspect would lead to a much greater sensitivity, they cannot say how much HR is missing. They should also test HU sensitivity. Cells should be very HU sensitive if they lack HR.

2. The authors are surprised that they do not see tumorigenesis. However, previous work with conditional BRCA2-/- mice indicates that only after p53 is deleted, the phenotype of tumorigenesis is revealed EMBO J. 2007 Jun 6;26(11):2732-42. Without p53, the phenotype of BRCA2 deficiency is degenerative in nature. Their studies in young mice are still postnatal. If they wanted to seriously test that mutant as a tumor suppressor, they would have to delete it specifically in tissue of interest during embryogenesis or just after birth when they are still proliferative, and they would have to do it in p53-/- background.

Appendix Figure S3 shows that one out of the six mice developed SCC. SCCs are characteristic of FA patients so maybe RAD51is a tumor suppressor after all?

3.They use the lack of nascent strand degradation phenotype as indicating that the aging phenotypes are due to HR deficiency ("Therefore, these data confirmed that the phenotypes generated by SMRAD51 expression resulted from HR deficiency rather than the HR-independent role of RAD51.") There is a distinct possibility that the reason they do not see nascent fork degradation is because the mutant does not support fork reversal. This has to be tested because the phenotypes might be secondary to lack of reversal and not lack of HR.

4. Another issue (related to above) is poor explanation of what the hybrid RAD51 is. There are three bands upon expression of the mutant allele. The authors only indicate that they have a "characteristic pattern." The bottom band looks like a full length RAD51. The next up, some kind of degradation product and the third band up, maybe a full length hybrid. When they show that this mutant forms foci, are they sure that they are looking at the full length mutant? Is the N-terminally flag tagged hybrid able to form foci? If they overexpress the WT in the presence of the SMRAD51 can they compete off the SMRAD51?

Minor points:
1. Methods are incomplete. For example, it is unclear if the survival assays are done under conditions of daily treatment or just one treatment followed by incubation for the indicated number of days. How were MEFs immortalized?
2. Multiple spelling mistakes.
3. Figure 7A is impossible to see
4. What is the quantification of Figure appendix Figure S2D? Annotation of the micrographs is necessary.
5. In Appendix Table S1- is n/a =not assessed or not applicable?
6. The authors overuse the word "remarkably". The fact that they did not see what they expected means that they were incorrect.

Referee #2:

- general summary and opinion about the principle significance of the study, its questions and findings

This is a mouse study to investigate the activity of RAD51, which mediates DNA double-strand break repair through the homologous recombination (HR) pathway, and also protects newly-synthesized DNA at replication forks. Whereas other HR factors (notably BRCA1 and BRCA2) are known as tumor suppressors, RAD51 is not well known as a tumor supressor, and this is something of a paradox. The authors set out to engineer a mouse model expressing a RAD51 mutant that disrupts HR without stimulating other forms of error-prone DNA repair. This approach allows the authors to test whether RAD51-mediated HR has a specific tumor suppressor function. The study therefore does not represent the first use of a targeted mouse allele for RAD51, but nonetheless tests an important question.

The authors draw on their experience of dominant-negative RAD51 mutants, which dates back more than 20 years (e.g. Lambert & Lopez, EMBO J, 2000). They use the "SMRAD51" allele, and present supplemental data to indicate that it behaves similarly to WT RAD51 but suppresses HR strand invasion. Adapting this allele to a Dox-inducible gene-targeted mouse model avoids issues with RAD51-/- embryonic lethality. Strikingly, induced expression of SMRAD51 caused dramatically-reduced lifespan of mice in the test group, together with symptoms (curved spine, reduced weight, altered progentor pools) that could reasonably be taken as indicative of premature aging. The premise of the study is good, the mouse work appears to have been done to a high standard, and the overall finding is very striking and interesting. However, there are some potential areas that could be improved.

- specific major concerns essential to be addressed to support the conclusions

1. I would debate whether the data shown give information about tumor susceptibility. It's fair to say that tumorigenesis is not a

primary phenotype of these mice, but it is possible that the mice are tumor prone, and this phenotype is masked because the mice die of aging-related / inflammation pathology first. The tumor susceptibility question could be better tested by expressing SMRAD51 in some kind of tumor model with short latency.

2. In the case of the RAD51 paralog, RAD51c, tumorigenesis was seen after crossing to a p53-deficient background (Kuznetsov et al, Cancer Research, 2009). Is it possible that mice expressing SMRAD51 would also show tumor susceptibility on a p53-deficient background? (This is possibly beyond the scope of the current report.)

3. Discuss more the mechanism leading to inflammation. How does the cellular phenotype (reduced replication rate) contribute to the organism phenotype (inflammation, progeria)?

4. Expansion of the hematopoietic stem cell pool is an aging-related adaptation to reduced stem cell function (Morrison SJ et al, Nature Medicine, 1996). Did the authors observe this in mice expressing SMRAD51? If the answer is 'no', then that warrants comment.
5. The range of phenotypes described does not include issues with the intestinal epithelium, a rapidly-dividing tissue that might be expected to be especially sensitive to disrupted HR. Did the authors observe any effects on the gut? If not, do they consider that surprising?

6. Mice expressing SMRAD51 protein do not appear to get tumors, according to the data in this paper. There is also some evidence that tumor growth is reduced in mice treated with RAD51 inhibitors (see for example, Huang & Mazin, Plos One, 2014). Can the authors please comment on this in the discussion? To what extent does existing literature support the concept of RAD51 inhibition as an anti-cancer strategy?

- minor concerns that should be addressed

1. I am surprised that SMRAD51 dissociates from ssDNA with equivalent kinetics to wtRAD51. I would have expected that if it forms a complex that is non-productive, it would remain bound to the ssDNA?

2. In the discussion, consider citing (Murga M, Nat Genet, 2009) along with (Ruzinakina et al, 2007) as an example of progeria associated with loss of ATR.

3. The authors may want to note that mouse FANC-knockout models do not really show the tumor susceptibility of human FA patients. Therefore, it is possible that gene-targeting studies in mice may underestimate the importance of certain DNA repair factors as tumor suppressors in humans.

4. "Indeed other dominant negative forms of RAD51, either mutated in Fanconi anemia group R (RAD51-T131P) or mutated in the ATP binding site (RAD51-K133R or RAD51-K133A) do not bind damaged DNA..." I would appreciate the authors to comment a little more about this. My understanding is that RAD51-T131P does bind to DNA, albeit with reduced affinity compared to wtRAD51. (This may not affect the broader point.)

- any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

1. Consider breaking the 'Discussion' into a more appropriate number of paragraphs, to improve readability.

Referee #3:

In vivo inactivation of RAD51-mediated homologous recombination leads to premature aging but not to tumorigenesis

The Manuscript by Matos-Rodrigues et al. reports the phenotypes of engineered mouse models with inducible expression of either wild type RAD51, or a dominant negative form of the same protein. At cellular level, the dominant negative form of RAD51 supressed homologous recombination and led to replication stress and DNA damage. In vivo, it caused inflammation, premature ageing and a reduced lifespan in mice, but interestingly it did not promote tumorigenesis. Overall, the findings reported in this paper contribute to our understanding of RAD51 roles in vivo which hitherto have been impossible to establish due to the embryonic lethality of Rad51 KO mice (which couldn't even be rescued in a Trp53-deficient background). The current manuscript complements with in vivo phenotypes the cellular functions of mutant RAD51 reported in their recently published

paper (So et al. NAR 2022). However, several issues need to be addressed before publication.

Major points:

Page 5 - 'Dox-induced SMRad51 expression decreased homology-directed gene targeting efficacy, decreased viability and increased sensitivity to mitomycin C and olaparib (a PARP inhibitor) compared with the absence of Dox (Appendix, Fig. S1A-D).' The method used for assessing viability upon SMRad51 expression is not indicated here, or in any other figure.

Page 5 and Discussion - 'It should be noted that ssDNA occupancy by SMRAD51 also accounted for its capacity to prevent accessibility to nonconservative repair, as proposed (Ayeong et al, 2019; So et al, 2022).' No assays were performed in the current manuscript to show non-conservative repair in the mouse cells used here. Instead, two papers (Ayeong et al, 2019; So et al, 2022) are used as reference for such assays throughout the manuscript, although these were performed in human cells. One additional problem is that, on closer inspection, the two papers turn out to be one and the same paper! In itself, the carelessness in preparing this manuscript precludes its publication at this stage, in addition to the lack of rigorous controls.

Authors must perform assays that show A-EJ and SSA are present in MEFs with exMmRad51, but not SMRAD51 expression otherwise cannot claim that they have supressed HR activity, whilst preventing nonconservative repair mechanisms. Need also to make sure this isn't overstated in the discussion.

Fig. 1E: It is not stated what the error bars represent. Also, not stated is how many mice for each genotype were used in each experiments. According to this figure, there are half as many exMmRad51 mice compared to SMRad51 mice (n= 5 vs n=10) at 3 months but in Fig. 1F there were no deaths of exMmRad51 mice reported. Is this technically impossible, in other words, are exMmRad51 mice harder to establish and, if so, why is this the case?

Fig. 1F: The authors need to provide more details about how the survival curves were generated and the number of mice included in each genotype.

Fig. 1H: The authors must define how many samples are in each condition and what the line is showing.

Fig. 1I: Is there an example of the follicular bulb (red arrow in control) and hypodermis (blue arrow in control) structures in SMRad51 cells? Please indicate in representative image.

Fig. 2: It must be indicated how many of the 29 upregulated factors are proinflammatory.

Fig. 3C-E: The authors must specify how many cells were analysed, the number of independent biological replicates and what the dots and lines represent. Also, do the red and black samples in C and D correspond to the legend in E?

Fig. 5: 'SMRad51 mutants.' was written in figure legend, should this be 'SMRad51-expressing mice'? Also, indicate the exact number of biological replicates in all of the panels.

Fig. 5A: please use red and blue arrows to indicate the structures referred to in SMRad51 cells.

Fig. 5C is not referred to in text. Also please explain what has been stained and quantitate the different between control and SMRad51 cells.

Fig. 6A, C, D: what do the lines in the graph represent and how many images were analysed per biological replicate?

Page 7 - '(ii) the fact that growing mice, in which many tissues are proliferating, were more sensitive than adult mice;' please clarify this statement, what are the younger mice more sensitive to than adult mice and how would this indicate that replication may be perturbed?

Fig 7: It is not indicated how many nuclei or fibres were counted per biological replicate (and how many biological replicated were quantified) and what the dots and lines represent (not specified for all panels in the figure)

Fig. 8: check all text, some symbols have been converted to boxes and the figure title appears incomplete. Also, none of the panel labels match the figure legend. Specify number biological replicates, individual images/nuclei quantitated and what the lines represent. It does not appear that all panels have been cited in the text, is there Fig. 8C mentioned?

Page 8: The last 4 paragraphs must be reformatted and full stops must be inserted. Interpretation of the H2AX signal in BrdU+ cells is incorrect: H2AX indicates DNA damage, and not necessarily replication stress, although it can be speculated that the damage stems from replication stress. RPA or pRPA Ser33 would be needed to show that there is replication stress.

Fig. 9 is not referred to in text; presumably Fig. 8 mentioned in Discussion should be Fig. 9?

Appendix S1B: Is the first plasmid combination only lacking the gRNA, or gRNA and Cas9 activity as stated in the figure legend? Also, are there 'No SMRad51' -DOX cells. Has the same experiment been performed in exMmRad51 cells and do they show a similar response to SMRad51 cells?

Appendix S4: the scale bar is not defined.

Supplementary Table S3 and 4: 'values' appear in many of the cells, probably because there is an error in the formula used.

Supplementary Table S5 is missing.

Minor points:

Both 'SMRad51' and 'SMRAD51' are used; please check consistency in text and figures.

Fig. 1F: the legend for 1G has also accidently been put in this section.

Fig 1I: No statistical analyses are shown in this panel; P values should be removed from the figure legend.

Page 6 - 'These data show that disruption of RAD51 function in HR leads to a systemic inflammatory response in adult mice.' In PDF 'in adult mice' has been crossed out.

Fig. 4C legend: 'Student's t test. * $p<0.05$.' No statistical analyses are shown in panel C; this needs to be corrected.

Appendix S1: this figure doesn't have very good resolution.

Appendix S1A: The units for DOX treatment are not listed properly: there is a symbol missing replaced by a box.

Appendix S2A, legend: should 'Electrophorese' be 'Electrohporesis'?

Appendix S2D legend: '&00%' instead of '100%'. Also scale bar size in this figure doesn't match what is stated in the legend.

Appendix S2E,F legend: what 'specific antibody' is used?

Appendix S3A: replace 'Testicules' with 'testicles'

Appendix S6: in the title 'change' is missing a 'h'.

Appendix Table S1: ERCC1-/- doesn't appear properly, there is a box instead of -/-.

Appendix items are sometimes referred to as 'SI Appendix'.

Supplementary Methods D-loop assay: 'final concentration of 2,5' need to change comma.

Supplementary Methods TEM analysis of RAD51 filaments: 'overhang (the construction of which has been described in ((Tavares et al, 2019))) using' - need to remove unnecessary brackets.

Supplementary Methods Whole-exome sequencing: 'whole-exome sequence ng ArrayExpress accession number is E-MTAB-8625.' - 'sequencing' is misspelled.

Dear Hartmut,

We are grateful that you are willing to consider our revised manuscript now entitled " *In vivo reduction of RAD51-mediated homologous recombination triggers aging but impairs oncogenesis*".

We also thank the reviewers for their comments. Addressing the reviewers' comments substantially improved the manuscript. Therefore, we hope that the revised manuscript will now be suitable for publication in the *EMBO Journal*.

On behalf of the coauthors,
Yours sincerely,

Please find answers to the issues you specifically underlined in our responses below.

"- Strengthening the evidence for absence of tumorigenesis upon SMRAD51 expression, e.g. by utilizing mouse models with decreased tumor latency or p53 deficiency [refs 1 & 2]".

We crossed SMRad51 mice with PyMT breast cancer model mice (PMID: 1312220), which show 100% penetrance of tumor development. This choice was driven by the fact that most genes mutated in familial breast cancer are involved in homologous recombination (HR). We observed that SMRAD51 expression in PyMT mice reduced the formation and progression of mammary tumors. These results strongly suggests that SMRAD51 suppresses tumorigenesis. This important statement was not included in the first version of the manuscript, in which we stated only that ablation of RAD51-dependent HR did not lead to tumorigenesis; in the revision, we added that, in fact, SMRAD51 expression counteracts oncogenesis. This statement is provocative because it challenges current dogma linking HR defects to cancer development. These newly added results bring *in vivo* data accounting for the "RAD51 paradox" (doi.org/10.1093/narcan/zcab016).
We also found that SMRAD51 expression leads to an enhanced activation of the DNA damage response (DDR) in PyMT tumors, further supporting the notion that decreasing RAD51 function via *SMRad51* expression induces DNA damage in cancer cells.
In summary, the explanation we propose for this tumor-suppressing effect is that SMRAD51 suppresses HR, generating replication stress in proliferating cells such as tumor cells (and progenitor cells, as shown in the first version of the manuscript), inhibiting tumor initiation and progression.

We also crossed the SMRad51 model mice with p53-knockout mice. Interestingly, the double mutant mice did not develop tumors, reinforcing the idea that *SMRad51* expression suppresses tumorigenesis. However, this observation was complicated by the unexpected and rapid death of the double-mutant mice. Understanding the cause(s) of this accelerated death requires in-depth investigations and extends beyond the scope of the current report (as pointed out by Referee #2).

We believe that the results with the PyMT model mice address the referees' comments, and we have therefore added them to the manuscript. We believe that adding the p53 data would overload the manuscript (which already contains many complex data) and confuse readers. These data will be completed for publication in a future report.

"- Deeper investigation of the aging/inflammation-related phenotypes (and how they may originate from HR deficiency) on the hematopoietic system and on rapidly-dividing tissues [ref 2] "

Referee#2's comment:" Discuss more the mechanism leading to inflammation. How does the cellular phenotype (reduced replication rate) contribute to the organism phenotype (inflammation, progeria)? »

On the one hand, an increasing number of publications is showing that replication stress induces the production of inflammatory cytokines and triggers innate immunity through the production of cytosolic DNA and induction of the STING pathway, which induces the expression of inflammatory cytokine genes. These mechanisms have been described in several reviews (e.g., Ragu et al. Genes, 2020 PMID: 32283785; Lin et al. Curr Opin Genet Dev, 2021 PMID: 34455237). Moreover, we recently showed that replication stress can more directly induce the expression of inflammatory cytokine gene expression through the activation of NFκB mediated by PARP1 (Ragu et al. 2023 PMID: 36869180; DOI: 10.1038/s41418-023-01141-0).

On the other hand, HR defects have been convincingly shown to cause replication stress (as we also observed in our model mice). Therefore, HR defects would induce inflammation through replication stress, an observation confirmed with BRCA2-defective cells (Reisländer et al. Nat Commun, 2019 PMID: 31316060).

Proliferative progenitor cells, which divide, thereby replicating the genome, are particularly affected by replication stress, and inflammation exacerbates their disappearance. The exhaustion of stem cell pools alters the renewal of tissues, leading to aging phenotypes.

Obviously, we were not clear enough in our explanations in the first version of the manuscript, and we have tried to improve our argument in the revised version.

Referee#2's comment: ". Expansion of the hematopoietic stem cell pool is an aging-related adaptation to reduced stem cell function (Morrison SJ et al, Nature Medicine, 1996). Did the authors observe this in mice expressing SMRAD51? If the answer is 'no', then that warrants comment. »

This point indeed deserves attention. First, splenomegaly (which we reported in the first version of the manuscript) is a marker of hematopoietic stem cell pool expansion.

In the revised version, we added new data to show that expressing *SMRad51* for 3 months via Dox treatment does not induce changes in hematopoietic stem cells, but after 6 months of Dox treatment, some mice showed an increase in hematopoietic stem cells (Lin (-), Sca-1 (+), c-Kit (+) and FLK2(-), rendered as LSK/FLK2– cells). The density and percentage of LSK/FLK2- cells are shown in Figure S4B in the revised version). The data suggest that hematopoietic stem cell accumulation is not related to *SMRad51* expression *per se* but to the aging phenotypes related to the expression of *SMRad51*. We added these data in the revised

version (see Figure S4B), and the text has been amended as follows: *"We observed hematopoietic cells in the spleens of SMRad51 mice after 6 months of Dox exposure, revealing extramedullary hematopoiesis (Appendix, Fig. S4A). Splenomegaly associated with extramedullary hematopoiesis is a common feature of aged and prematurely aging mice and is a compensatory mechanism triggered by bone marrow progenitor cell exhaustion (Pettan-Brewer & M. Treuting, 2011; French et al, 2002; Mann et al, 2018). Then, we investigated whether these changes in spleen histology were associated with alterations in hematopoiesis. First, we analyzed the blood composition of mice with and without SMRad51 expression. The number of red blood cells (RBCs) and platelets (PLTs), but not that of white blood cells (WBCs), was decreased in the blood of SMRad51 mice compared with control mice after three months of Dox treatment (Fig. 4A). Expansion of the hematopoietic stem cell pool is an aging-related adaptation to reduced stem cell function (Morrison et al, 1996). After three months of Dox treatment, SMRad51 expression did not lead to a reduction in the overall number of bone marrow stem cells (Lineage [Lin]$^-$Sca-1$^+$-c-Kit$^+$-Fkl2$^-$ or LSK, Flk2$^-$ cells), but after 6 months of Dox treatment, the SMRad51-expressing mice showed an tendency to present with an increased stem cell pool (Appendix, Fig. S4B). Next, we evaluated whether these changes correlated with hematopoietic changes in the bone marrow and found a reduction in the amount of common lymphocyte progenitors (CLPs; Lin$^-$Sca-1$^-$c-Kit$^+$IL7R$^+$ cells) and B cells (B220$^+$ B cells) after SMRad51 was expressed for 3 months (Fig. 4B). These data indicate that SMRAD51 altered hematopoiesis by reducing the progenitor cell pool (Appendix, Fig. S4. Altogether, our data show that the expression of SMRad51 disrupted blood cell production, leading to thrombocytopenia (a reduction in the number of PLTs) and anemia (a reduction in the number of RBCs), which are associated with compromised hematopoiesis.".*

"- Conclusively confirming that SMRAD51 expression abolishes HR (and A-EJ, SSA) in the murine system (MEFs) and better defining its role in fork protection [refs 1 & 3]"

Evaluating HR efficiency in our mouse model is indeed essential. Because our mouse model already carries GFP in the rtTA transgene construct, we could not use classical HR and SSA reporter systems based on GFP expression. For this reason, we performed gene-targeting assays to evaluate HR efficiency, which was decreased after *SMRad51* was expressed. We also observed a defect in replication fork restart as well as an increase in sensitivity to MMC and PARP inhibitors. Together, these data are consistent with previous reports of HR defects after *SMRad51* was expressed in hamster and human cells (So et al. NAR, 2022 PMID: 35137208; Lambert & Lopez EMBO J, 2000 PMID: 10856252; Wilhelm et al. Plos Genet, 2016 PMID: 27135742).
Notably, we had presented indirect arguments suggesting the repression of SSA by SMRAD51: the binding of SMRAD51 is shown *in vitro* in Appendix S2C and in the MEFs in Appendix S2E. Moreover, we have previously shown that *in vitro* the SMRAD51 protein prevents the annealing of complementary ssDNA, an essential step for both SSA and A-EJ (So et al. NAR, 2022 PMID: 35137208; we used the same protein in the present study).

Nevertheless, to support these observations and more directly address the reviewer's comments concerning SSA and A-AJ, as requested by Referee #3, we have mutagenized MEFs obtained from SMRad51 model mice using a CRISPR–Cas9 strategy. Then, we introduced the SSA and EJ reporter substrates that we had previously used in human cells (So et al. NAR, 2022 PMID: 35137208) into *gfp*-defective MEFs. Although SMRad51 expression inhibited HR (see above), it stimulated neither SSA nor A-EJ. Notably, the absence of *Rad51*

expression (knocked down by *Rad51* siRNA) led to the stimulation of SSA in MEFs, as previously shown with human cells (So et al. NAR, 2022 PMID: 35137208). These data have been added in the revised version.

" In addition, all referees note that experimental descriptions and presentations are presently insufficient to properly assess the results, and that a revised manuscript would require significantly more careful preparation in various aspects."

We apologize for missing points that were necessary to properly relate our experimental descriptions and results. We carefully updated the revised manuscript in many ways to clarify and support our results.

Answers to specific referees' comments:

Referee #1:

The Manuscript by Matos-Rodrigues et al. describes a mouse that overexpresses a hybrid form of RAD51, which contains the N terminus of the yeast RAD51. The allele is dox inducible and its induction in the three-month-old mice resulted in phenotypes associated with aging, including increased serum levels of pro-inflammatory cytokines, suppression of bone marrow function accompanied by extramedullary hematopoiesis, and premature death. Expression of the allele in younger mice (P12-14) resulted in loss of progenitor cells in multiple tissues, high inflammatory response, and death in less than three weeks. The cellular phenotypes associated with this allele in MEFs included increased DNA damage, decreased replication speed, fork asymmetry, but apparent presence of fork protection. The experimental work is fine, but I have major issues with the authors conclusions that lack of HR in their model leads to lack of tumorigenesis. The text needs to be re-written to de-emphasize absence of tumorigenesis and less emphasis on the lack of HR in the mutant.

To address this point raised by Reviewer #1, in the revised version, we have written "decreased HR," or "affected HR" or “downregulated HR", which corresponds to the actual situation in the mouse model.
In the first version, we did not observe tumorigenesis. In the revised version, we crossed SMRad51 mice with PyMT breast cancer model mice (PMID: 1312220), which show 100% penetrance of tumor development. This choice was driven by the fact that most genes mutated in familial breast cancer are involved in homologous recombination (HR). We observed that SMRAD51 expression in the PyMT mice impeded the formation and progression of mammary tumors. These results strongly suggested that SMRAD51 suppresses tumorigenesis. This important statement was not included in the first version of the manuscript, in which we stated only that the ablation of RAD51-dependent HR did not lead to tumorigenesis; in the revision, we show that, in fact, SMRAD51 expression counteracts oncogenesis. This statement is provocative because it challenges current dogma linking HR defects to cancer development, but *in vivo* data support the “RAD51 paradox” (doi.org/10.1093/narcan/zcab016)**.**
We also found that *SMRad51* expression leads to the activation of the DNA damage response (DDR) in PyMT tumors, further supporting the notion that decreasing RAD51 function via *SMRad51* expression induces DNA damage in cancer cells.
In summary, the explanation we propose for this tumor-suppressing effect is that SMRAD51 suppresses HR, generating replication stress in proliferating cells such as tumor cells (and progenitor cells, as shown in the first manuscript submitted), inhibiting tumor initiation and progression.

1. *" The authors never show absence of HR. The assays shown in Appendix Figure 1 shows partial HR deficiency."*

We have modified the text with “decrease in HR” instead “absence of HR” (similar to what is generally observed with siRNA). Note that the KO is lethal.
Nevertheless, the expression of *SMRad51* leads to not only impaired HR but also substantial replication stress, inflammation (phenotypes that are consistent with HR defects), and strong phenotypes (premature ageing, reduced lifespan) *in vivo* (in contrast with those of control mice or mice expressing WT *Rad51*).

" The sensitivities to olaparib and MMC are also very mild. Without proper controls of RAD51 s and BRCA2 KD, which I suspect would lead to a much greater sensitivity, they cannot say how much HR is missing. They should also test HU sensitivity. Cells should be very HU sensitive if they lack HR."

This comment is based on drug sensitivities generally measured in human cancer cells. Here, we used rodent cells, embryonic cells and noncancer cells (this is the first description of drug sensitivity in these kinds of cells); therefore, comparisons cannot be made. Moreover, because RAD51 and BRCA2 knockout causes lethality, we cannot construct such controls in with our MEFs. Note that designing clones that are knocked-out for a target gene leads to selecting cells that have adapted to the lack of the targeted genes, and therefore, this can interfere with the primary pathway analyzed (here HR). This is not the case with the use of an inducible dominant negative form, where cells are not selected before inhibition of the targeted process (here, HR). This strategy allowed us to specifically analyze the primary consequences of HR alteration. Using siRNA to knock down genes also only partially and transiently downregulated *Rad51* and *Brca2*. Therefore, these approaches are not suitable for clonogenic assays, such as those we performed. Therefore, a quantitative comparison could not be made.
It is important to stress that SMRAD51 inhibits HR in a unique way: since SMRAD51 protein still binds damaged DNA, it represses HR but also prevents SSA and A-EJ (So et al. NAR 2022, PMID: 35137208); in contrast, BRCA2 or RAD51 KD makes damaged DNA accessible to alternative repair pathways and stimulates SSA and A-EJ (So et al. NAR 2022, PMID: 35137208). Therefore, comparisons would not be relevant. To our knowledge, there is no proper way to compare the sensitivity of the MEFs we derived from our mouse model to that of other cells with completely abrogated HR (which is an essential process).
HR-deficient cells generally show mild sensitivity to HU, less than that to MMC or olaparib, likely because NHEJ can also process HU-induced DSBs (PMID: 11447127), in contrast to interstrand crosslinks generated by MMC, SSBs generated by olaparib, or other multiple consequences of PARP inhibition. For example, in the following publication, HR-deficient cells were less but not more sensitive to HU than to olaparib (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3261725/or https://elifesciences.org/articles/30523).
Nevertheless, our results clearly show that SMRAD51 expression significantly decreased HR-dependent gene targeting and increased sensitivity to both MMC and PARP inhibition,

all of which are phenotypes consistent with HR inhibition. The consequences (replication stress and expression of inflammatory cytokine genes) are also consistent with defective HR.

2. *"The authors are surprised that they do not see tumorigenesis. However, previous work with conditional BRCA2-/- mice indicates that only after p53 is deleted, the phenotype of tumorigenesis is revealed EMBO J. 2007 Jun 6; 26(11): 2732-42. Without p53, the phenotype of BRCA2 deficiency is degenerative in nature. Their studies in young mice are still postnatal. If they wanted to seriously test that mutant as a tumor suppressor, they would have to delete it specifically in tissue of interest during embryogenesis or just after birth when they are still proliferative, and they would have to do it in p53-/- background. "*

A large number of articles, reviews, and textbooks have connected HR defects to tumorigenesis predisposition. This is the reason why our data might be surprising to some, as they contradict general dogma.
In the first version of the manuscript, we did not claim that the mutant is a tumor suppressor. However, we agree that the point raised by the reviewer deserves to be considered. First, the results of postnatal analyses are highly informative to decipher the impact of *SMRad51* expression itself. Indeed, generally, most tumors develop during postnatal period, and tumors developing during embryogenesis are special cases. For example, in familial breast or ovary cancer, tumors develop after the loss of the WT allele (frequently encoding an HR gene) in adults.
The embryogenesis program can indeed interfere with tumorigenesis. However, here, the induction of SMRAD51, at a postnatal step, more directly addressed the impact of HR alteration on tumorigenesis, eliminating potential interference of the embryogenesis/fetal program (which is indeed an interesting question but far beyond the scope of the present manuscript).
There are numerous articles showing that inactivation of HR genes leads to cancer formation in the presence of WT p53 (for a review, see PMID: **33923105)**. This means that, in an HR-defective background, deletion of p53 is not absolutely necessary to induce tumor development.
Surprisingly, the consequences of Rad51 defect have not been analyzed with elaborate strategies, and because of the specificities of RAD51, which differ from its partners (supporting "the RAD51 paradox" PMID 34316706), the consequences of alterations of RAD51 cannot be deduced from the one of its mediator/accessory partners.
Nevertheless, because we agree with the reviewer that this question is important, we addressed it. We crossed SMRad51 mice with p53-KO mice and induced *SMRad51* expression in embryos at mid-gestation (embryonic Day 10.5) in WT or p53 knocked out mice. Wild type p53 mice expressing *SMRad51* during embryogenesis were born but died shortly after birth. In agreement with our analysis with adult mice (see above), embryonic expression of *SMrad51* in p53-KO mice exacerbated the lethality phenotype, being embryonic lethal in p53-KO mice. As mentioned above, understanding the cause(s) of this

accelerated death in p53-KO mice requires an in-depth investigation that extends beyond the scope of the current report (as pointed out by Referee #2). We believe that adding the p53 data would overload the manuscript (which already contains many complex data) and may confuse readers.

To address the impact of the mutant in a cancer-prone background, we crossed our model mice with PyMT model mice. This process is explained above in response to your comment on this point.
The results showed that *SMRad51* expression reduced the number and size of mammary tumors in the PyMT model mice. Therefore, in addition to failing to stimulate cancerogenesis (as shown in the first manuscript version), SMRAD51 suppressed tumor growth.

"Appendix Figure S3 shows that one out of the six mice developed SCC. SCCs are characteristic of FA patients so maybe RAD51is a tumor suppressor after all?"

The text "1 out of 6 with SCC" means that 5 of 6 mice did not present with SCC.
More generally, none of the 15 mice analyzed in this study developed tumors. Therefore, we cannot conclude that RAD51 is a tumor suppressor.
Finally, expressing SMRAD51 in the PyMT model mice confirmed that SMRAD51 does not favor tumorigenesis but revealed, in contrast, that it hinders it.

3. *"They use the lack of nascent strand degradation phenotype as indicating that the aging phenotypes are due to HR deficiency ("Therefore, these data confirmed that the phenotypes generated by SMRAD51 expression resulted from HR deficiency rather than the HR-independent role of RAD51.") There is a distinct possibility that the reason they do not see nascent fork degradation is because the mutant does not support fork reversal. This has to be tested because the phenotypes might be secondary to lack of reversal and not lack of HR. "*

We have removed the sentence "These data also showed that SMRad51 induced replication stress even when the arrested replication forks were efficiently protected."

We did not propose that aging stems from the lack of nascent strand degradation; we stated that aging is caused by defective HR, reduced velocity and defects in the resumption of arrested replication forks. Using SMRAD51 allowed us to focus on the HR function of RAD51, which binds to damaged DNA, preventing DNA from undergoing degradation and annealing (which would trigger SSA and A-EJ). Nevertheless, we agree with the referee that it might affect fork reversal, as suggested by the defect in fork resumption. We have added a commentary on this interesting point in the discussion of the revised version. " *We cannot*

exclude the possibility that SMRAD51 may mediate the reversal of arrested replication forks. Indeed, SMRAD51 may retain the capacity to induce fork reversal since this function of RAD51 might not require strand exchange activity (Berti et al, 2020; Thomas et al, 2023; Mason et al, 2019). Addressing these questions is an exciting challenge for future research. Nevertheless, the important point is that, in fine, SMRAD51 generates replication stress, thereby preferentially affecting proliferating cells.".

4. *" Another issue (related to above) is poor explanation of what the hybrid RAD51 is. There are three bands upon expression of the mutant allele. The authors only indicate that they have a "characteristic pattern." The bottom band looks like a full length RAD51. The next up, some kind of degradation product and the third band up, maybe a full length hybrid. When they show that this mutant forms foci, are they sure that they are looking at the full length mutant? Is the N-terminally flag tagged hybrid able to form foci? If they overexpress the WT in the presence of the SMRAD51 can they compete off the SMRAD51?"*

These three bands have been observed since SMRAD51 was first discovered in mammalian cells more than 20 years ago (see Lambert & Lopez, EMBO J, 2000 PMID: 10856252 and in all the other papers published since the original report: Saintigny et al. EMBO J, 2001 PMID: 11350949; Lambert et al. Oncogene, 2001 PMID: 11641788, 2002; Bertrand et al. Oncogene, 2003 PMID: 14576820; Guirouilh-Barbat Mol Cell, 2004 PMID: 15175156; Daboussi et al. Oncogene, 2005 PMID: 15782136; Daboussi et al. J Cell Sci., 2008 PMID: 18089650; Wilhelm et al. PNAS, 2014 PMID: 24347643; PLoS Genet 2016 PMID: 27135742; So et al. NAR, 2022 PMID: 35137208). Therefore, the 3 bands are the "diagnosis" of the expression of SMRAD51 in living cells. The full length of the hybrid protein corresponds to the upper band, as confirmed by the analysis of the purified proteins (Appendix appendix S1A and S2A in this manuscript). The other forms correspond to degradation products and/or internal translation starts. The foci analyzed in Appendix S2E were detected using an antibody specifically against the N-terminus part of yeast RAD51, which showed that the hybrid protein indeed formed foci. These results were confirmed by SO et al. (NAR 2022), as we also identified foci with N-terminus-flagged SMRAD51. Collectively, these *in vitro* and *in cellulo* experiments indicate binding of the full-length SMRAD51 protein to DNA. Taken together, the *in vitro* and *in cellulo* experiments all indicated binding of the full-length SMRAD51 protein to DNA. Importantly, the ultimate phenotype acquired exhibits decreased HR efficiency (without increasing SSA and A-EJ activity). Moreover, using purified full-length SMRAD51, we showed (i) inhibition of D-loop formation by purified SMRAD51 and (ii) DNA binding by SMRAD51 and formation of nucleofilaments via transmission electron microscopy in *in vitro* assays. The biochemical data shown in Appendix S2 demonstrate that the full-length hybrid protein (SMRAD51) prevented D-loop formation even in the presence of wtRAD51, demonstrating that only a few molecules of SMRAD51 incorporated into the nucleofilament were sufficient to abrogate

the global activity of this filament. These data support the findings obtained with cultured cells and mice.

Minor points:

1. *Methods are incomplete. For example, it is unclear if the survival assays are done under conditions of daily treatment or just one treatment followed by incubation for the indicated number of days. How were MEFs immortalized?*

We apologize for the lack of clarity in the description of our methods. We reviewed this section of our manuscript and added a full description of our experimental procedures to the figure legends.

2. *Multiple spelling mistakes.*

We apologize for the spelling mistakes. We conducted a careful grammar and review of our manuscript before resubmission.

3. *Figure 7A is impossible to see*

We changed Figure 7A, which is now Figure 8A, to improve its clarity.

4. *What is the quantification of Figure appendix Figure S2D? Annotation of the micrographs is necessary.*

One hundred percent of the DNA molecules were coated by MmRAD51 or SMRAD51 or both (this information is indicated in the text and in the legend).

5. *In Appendix Table S1- is n/a =not assessed or not applicable?*

This has been corrected in the revised version.

6. *The authors overuse the word "remarkably". The fact that they did not see what they expected means that they were incorrect.*

We apologize for the overuse of the term remarkably and have removed all but one instance of the term.
We did not expect to “see” something, and therefore, the results do not indicate that we were incorrect. However, our results do contradict current dogma, which suggests that HR defects are associated with cancer predisposition. That is why our results are remarkable. Our manuscript revises this incorrect point of view coming from the current dogma.

Referee #2:

- general summary and opinion about the principle significance of the study, its questions and findings

This is a mouse study to investigate the activity of RAD51, which mediates DNA double-strand break repair through the homologous recombination (HR) pathway, and also protects newly-synthesized DNA at replication forks. Whereas other HR factors (notably BRCA1 and BRCA2) are known as tumor suppressors, RAD51 is not well known as a tumor supressor, and this is something of a paradox. The authors set out to engineer a mouse model expressing a RAD51 mutant that disrupts HR without stimulating other forms of error-prone DNA repair. This approach allows the authors to test whether RAD51-mediated HR has a specific tumor suppressor function. The study therefore does not represent the first use of a targeted mouse allele for RAD51, but nonetheless tests an important question.

The authors draw on their experience of dominant-negative RAD51 mutants, which dates back more than 20 years (e.g. Lambert & Lopez, EMBO J, 2000). They use the "SMRAD51" allele, and present supplemental data to indicate that it behaves similarly to WT RAD51 but suppresses HR strand invasion. Adapting this allele to a Dox-inducible gene-targeted mouse model avoids issues with RAD51-/- embryonic lethality. Strikingly, induced expression of SMRAD51 caused dramatically-reduced lifespan of mice in the test group, together with symptoms (curved spine, reduced weight, altered progenitor pools) that could reasonably be taken as indicative of premature aging. The premise of the study is good, the mouse work appears to have been done to a high standard, and the overall finding is very striking and interesting. However, there are some potential areas that could be improved.

- specific major concerns essential to be addressed to support the conclusions

1. *« I would debate whether the data shown give information about tumor susceptibility. It's fair to say that tumorigenesis is not a primary phenotype of these mice, but it is possible that the mice are tumor prone, and this phenotype is masked because the mice die of aging-related / inflammation pathology first. The tumor susceptibility question could be better tested by expressing SMRAD51 in some kind of tumor model with short latency. »*

Although not the main point of the first manuscript version, we fully agree with the Referee's comment/question. We crossed SMRad51 mice with the PyMT model mice with breast cancer (PMID: 1312220), which show 100% penetrance of tumor development. This choice was driven by the fact that most genes mutated in familial breast cancer are involved in homologous recombination (HR). We observed that SMRAD51 expression in PyMT mice impeded the formation and progression of mammary tumors (the results are presented in revised Figure 3). These results strongly suggest that SMRAD51 not only fails to promote tumorigenesis but hinders it. This important statement was not included in the first version of the manuscript, in which we stated only that ablation of RAD51-dependent HR did not lead to tumorigenesis.
We also found that SMRAD51 enhanced the activation of the DNA damage response (DDR) in PyMT tumors, further supporting the notion that decreasing RAD51 function via SMRAD51 induced DNA damage in cancer cells (New Figure 3G and H).

In summary, the explanation we propose for this tumor suppressor effect is that SMRAD51 suppresses HR, generating replication stress in proliferating cells such as tumor cells (and progenitor cells, as shown in the first manuscript submitted) inhibiting tumor initiation and progression.
We have added these data (revised Figure 3) and related comments in the results and discussion sections of the revised version.

2. *« In the case of the RAD51 paralog, RAD51c, tumorigenesis was seen after crossing to a p53-deficient background (Kuznetsov et al, Cancer Research, 2009). Is it possible that mice expressing SMRAD51 would also show tumor susceptibility on a p53-deficient background? (This is possibly beyond the scope of the current report.) »*

We crossed our model mice with p53-KO mice. Interestingly, the double-mutant mice did not develop tumors, reinforcing the idea that SMRAD51 expression suppresses tumorigenesis. However, our observation was complicated by the unexpected, rapid death of the double mutant mice. Therefore, we believe that adding the p53 data would overload the manuscript (which already contains many complex data) and may confuse readers. Understanding the cause(s) of this accelerated death requires an in-depth investigation and extends beyond the scope of the current report, as mentioned by the reviewer.

3. *"Discuss more the mechanism leading to inflammation. How does the cellular phenotype (reduced replication rate) contribute to the organism phenotype (inflammation, progeria)?"*

On the one hand, an increasing number of publications are showing that replication stress induces the production of inflammatory cytokines and innate immunity through the production of cytosolic DNA and induction of the STING pathway, which induces the expression of inflammatory cytokine genes. These mechanisms were described in several reviews (e.g., Ragu et al. Genes, 2020 PMID: 32283785; Lin et al. Curr Opin Genet Dev, 2021 PMID: 34455237). Moreover, we recently showed that replication stress can more directly induce the expression of inflammatory cytokine genes through the activation of NFκB mediated by PARP1 (Ragu et al. 2023 PMID: 36869180; DOI: 10.1038/s41418-023-01141-0).

On the other hand, HR defects have been shown to lead to replication stress (as we also observed in our model). Therefore, HR defects induce inflammation through replication stress, an observation confirmed with BRCA2-defective cells (Reisländer et al. Nat Commun, 2019 PMID: 31316060).

Proliferative progenitor cells, which are dividing and replicating their genome, are particularly affected by replication stress, and inflammation exacerbates their disappearance.

The exhaustion of stem cell pools should alter the renewal of tissues, leading to the acquisition of aging phenotypes.

Obviously, we were not clear enough in our explanations in the first version of the manuscript, and we have tried to improve our argument in the revised version. *" An increasing number of publications show that replication stress induces the production of inflammatory cytokines and innate immunity, notably through the production of cytosolic DNA and activation of the STING pathway (for reviews, see Ragu et al, 2020; Lin & Pasero, 2021). Moreover, replication stress can directly induce the expression of proinflammatory cytokine genes through the activation of NFκB mediated by PARP1 (Ragu et al, 2023). Since HR defects lead to replication stress, they are also expected to induce inflammation, as observed when cells express dysfunctional BRCA2 mutants (Reisländer et al, 2019). Given that SMRad51 expression leads to replication stress, (Daboussi et al, 2008; Wilhelm et al, 2016, 2014), including in MEFs (present data), it is therefore consistent that it also leads to the expression of inflammatory cytokine genes and to systemic inflammation."*

4. *« Expansion of the hematopoietic stem cell pool is an aging-related adaptation to reduced stem cell function (Morrison SJ et al, Nature Medicine, 1996). Did the authors observe this in mice expressing SMRAD51? If the answer is 'no', then that warrants comment. »*

We agree with the reviewer that this point deserves attention. First, splenomegaly (which we reported in the first version of the manuscript) is a marker of hematopoietic stem cell pool expansion.

In addition, we provide new data in the revised version that support the assumption of Reviewer 2 (Figure S4B). We show that expressing *SMRad51* for 3 months via dox treatment did not induce changes in hematopoietic stem cells, but after 6 months of Dox treatment, some mice showed an increase in the number of hematopoietic stem cells (Lin (-), Sca-1 (+), c-Kit (+) and FLK2(-), rendered as LSK/FLK2-) (Figure 4 and Figure S4B). These results suggest that hematopoietic stem cell accumulation is not related to *SMRad51* expression *per se* but to the aging phenotypes that are related to the expression of *SMRad51*. We have added these data to the results section of the revised version and amended the text as follows: *" We observed hematopoietic cells in the spleens of SMRad51 mice after 6 months of Dox exposure, revealing extramedullary hematopoiesis (Appendix, Fig. S4A). Splenomegaly associated with extramedullary hematopoiesis is a common feature of aged and prematurely aging mice and is a compensatory mechanism triggered by bone marrow progenitor cell exhaustion (Pettan-Brewer & M. Treuting, 2011; French et al, 2002; Mann et al, 2018). Then, we investigated whether these changes in spleen histology were associated with alterations in hematopoiesis. First, we analyzed the blood composition of mice with and without SMRad51 expression. The number of red blood cells (RBCs) and platelets (PLTs), but not that of white blood cells (WBCs), was decreased in the blood of SMRad51 mice compared with control mice after three months of Dox treatment (Fig. 4A). Expansion of the hematopoietic stem cell pool is an aging-related adaptation to reduced stem cell function (Morrison et al, 1996). After three months of Dox treatment, SMRad51 expression did not lead to a reduction in the overall number of bone marrow stem cells (Lineage [Lin]$^-$Sca-1$^+$-c-Kit$^+$-Fkl2$^-$ or LSK, Flk2$^-$ cells), but after 6 months of Dox treatment, the SMRad51-expressing*

mice showed an tendency to present with an increased stem cell pool (Appendix, Fig. S4B). Next, we evaluated whether these changes correlated with hematopoietic changes in the bone marrow and found a reduction in the amount of common lymphocyte progenitors (CLPs; Lin$^-$ Sca-1$^-$c-Kit$^+$IL7R$^+$ cells) and B cells (B220$^+$ B cells) after SMRad51 was expressed for 3 months (Fig. 4B). These data indicate that SMRAD51 altered hematopoiesis by reducing the progenitor cell pool (Appendix, Fig. S4. Altogether, our data show that the expression of SMRad51 disrupted blood cell production, leading to thrombocytopenia (a reduction in the number of PLTs) and anemia (a reduction in the number of RBCs), which are associated with compromised hematopoiesis".

5. *« The range of phenotypes described does not include issues with the intestinal epithelium, a rapidly-dividing tissue that might be expected to be especially sensitive to disrupted HR. Did the authors observe any effects on the gut? If not, do they consider that surprising? »*

We agree with the reviewer that the intestine should be affected. As described in Appendix Figure S7 of our manuscript, we analyzed the intestinal epithelium in young growing mice. We showed the induction of the DNA damage response (DDR) in this tissue. We added a description of the analysis in adult mice to the revised version of the manuscript (Figure S4C).

6. *«Mice expressing SMRAD51 protein do not appear to get tumors, according to the data in this paper. There is also some evidence that tumor growth is reduced in mice treated with RAD51 inhibitors (see for example, Huang & Mazin, Plos One, 2014). Can the authors please comment on this in the discussion? To what extent does existing literature support the concept of RAD51 inhibition as an anti-cancer strategy? »*

As pointed out by Referee #2, several publications have identified the RAD51 pathway as a pharmacological target for anticancer therapy associated with treatments of genotoxicity induced by agents. However, to our knowledge, no report describes the effects of hypomorphic forms of RAD51 on de novo tumor formation *in vivo*, placing our work as the first proof of concept. Moreover, in the new manuscript, we added new results obtained with PyMT model mice that support the finding that expressing *SMRad51* inhibits tumor formation and progression. In addition, we have published a review describing what we called the "RAD51 paradox" (Matos-Rodrigues et al. NAR Cancer, 2021 PMID: 34316706): although it plays a central role in HR, RAD51 is not a gene in which mutation predisposes the carrier to familial breast or ovarian cancer, in contrast with most of RAD51 partners and accessory HR factors.

We have discussed all these points in the revised manuscript: *" RAD51 is a pharmacological target, and research is being performed to develop RAD51 inhibitors (Huang & Mazin, 2014; Grundy et al, 2020). However, taking into account the impact of these inhibitors on alternative nonconservative repair processes such as SSA and A-EJ might be important: molecules that selectively inhibit the HR activity of RAD51 without stimulating nonconservative repair processes may limit the risks of increased genetic instability and its*

associated consequences. Our data at a genetic level support this suggestion and show that hypomorphic forms of Rad51 can affect de novo tumor formation in vivo, establishing a novel proof of concept.."

- minor concerns

1. I am surprised that SMRAD51 dissociates from ssDNA with equivalent kinetics to wtRAD51. I would have expected that if it forms a complex that is non-productive, it would remain bound to the ssDNA?

These are factual data. Notably, similar results were also found with human cells (So et al. NAR, 2022 PMID: 35137208), supporting the data from the present study.

2. In the discussion, consider citing (Murga M, Nat Genet, 2009) along with (Ruzinakina et al, 2007) as an example of progeria associated with loss of ATR.

We have discussed ATR in the following paragraph: "*According to the intrauterine programming model, developmental issues generated by replication stress during embryogenesis are the underlying cause of tissue degeneration and malfunction that result in a premature aging-like phenotype in adulthood (Fernandez-Capetillo, 2010; Murga et al, 2009). Here, functional inactivation of RAD51 was performed in adults but still resulted in premature aging phenotypes. Therefore, the intrauterine programming model cannot account for the phenotypes observed in the current study. Instead, our results are consistent with data showing that inactivation of the replicative stress response factor Atr leads to premature aging in adult mice (Ruzankina et al, 2007)*.".

We have added the reference (Ruzinakina et al., 2007) to the paragraph and Table S1.

3. The authors may want to note that mouse FANC-knockout models do not really show the tumor susceptibility of human FA patients. Therefore, it is possible that gene-targeting studies in mice may underestimate the importance of certain DNA repair factors as tumor suppressors in humans.

We thank Referee 2 for highlighting this concern.
We agree with the reviewer, and we are aware of the limitations of using model mice to study human diseases. More specifically, several aspects of DNA repair are different between humans and rodents. In addition, cells obtained from clones with genes knocked out have adapted to the lack of the targeted genes, and therefore, the results can be affected. However, this drawback was not a concern with the inducible dominant negative form because cells were not selected before the targeted process was altered (here HR). This allowed us to specifically analyze the primary consequences of HR alteration. In addition, RAD51 mutations are not classified as tumor-prone mutants ("The RAD51 paradox") in human tumors, suggesting that our data obtained with mice recapitulate the situation in humans.
In the revised version, we have added a sentence in the discussion to discuss this point: *" DNA repair sometimes differs between rodents and humans. However, we can point out that mutated RAD51 is not classified as a tumor-prone ("the RAD51 paradox") in humans,*

suggesting that our present data obtained with mice truly recapitulated the situation in humans and the "RAD51 paradox".

4. "Indeed other dominant negative forms of RAD51, either mutated in Fanconi anemia group R (RAD51-T131P) or mutated in the ATP binding site (RAD51-K133R or RAD51-K133A) do not bind damaged DNA..." I would appreciate the authors to comment a little more about this. My understanding is that RAD51-T131P does bind to DNA, albeit with reduced affinity compared to wtRAD51. (This may not affect the broader point.)

In vitro assays showed that these mutant forms of RAD51 can bind DNA, but we observed that in living cultured cells, these mutants did not form foci (or formed very few) and impaired focus formation of endogenous WT-RAD51. In addition, the expression of these mutant forms stimulated alternative nonconservative repair (SSA and A-EJ), similar to the depletion of RAD51 mediated by siRNA or the inactivation of BRCA2, which resulted in the failure of RAD51 to be loaded onto damaged DNA (So et al. NAR, 2022 PMID: 35137208). Collectively, these data support the conclusion suggesting low DNA-binding abilities of such mutant forms of RAD51 in living cells (in contrast to the ability of SMRAD51 to bind DNA). In the revised version of the manuscript, we replaced the sentence with *"Indeed, the expression of other dominant-negative forms of RAD51, such as that in Fanconi anemia Group R (RAD51-T131P) or that encoding for a dysfunctional ATP-binding site (RAD51-K133R and RAD51-K133A), impair foci formation (including that of endogenous WT-RAD51) after the induction of DNA damage (So et al, 2022)"*

- any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

We added many additional data and revised the related text.

1. Consider breaking the 'Discussion' into a more appropriate number of paragraphs, to improve readability.

We thank Referee 2 for this suggestion. We changed the structure of our paragraphs to improve their readability as requested.

Referee #3

In vivo inactivation of RAD51-mediated homologous recombination leads to premature aging but not to tumorigenesis

The Manuscript by Matos-Rodrigues et al. reports the phenotypes of engineered mouse models with inducible expression of either wild type RAD51, or a dominant negative form of the same protein. At cellular level, the dominant negative form of RAD51 supressed homologous recombination and led to replication stress and DNA damage. In vivo, it caused inflammation, premature ageing and a reduced lifespan in mice, but interestingly it did not promote tumorigenesis. Overall, the findings reported in this paper contribute to our understanding of RAD51 roles in vivo which hitherto have been impossible to establish due to the embryonic lethality of Rad51 KO mice (which couldn't even be rescued in a Trp53-deficient background). The current manuscript complements with in vivo phenotypes the cellular functions of mutant RAD51 reported in their recently published paper (So et al. NAR 2022). However, several issues need to be addressed before publication.

Major points:

« Page 5 - 'Dox-induced SMRad51 expression decreased homology-directed gene targeting efficacy, decreased viability and increased sensitivity to mitomycin C and olaparib (a PARP inhibitor) compared with the absence of Dox (Appendix, Fig. S1A-D).' The method used for assessing viability upon SMRad51 expression is not indicated here, or in any other figure. »

As highlighted in the figure legend, we used a "colony formation assay" to assess viability. To clarify the description, we changed the sentence to "*SMRad51 expression also increased the sensitivity of the MEFs to mitomycin C and olaparib (a PARP inhibitor) compared with the absence of Dox (Appendix, Fig. S1C), as indicated by a colony formation assay.*".

« Page 5 and Discussion - 'It should be noted that ssDNA occupancy by SMRAD51 also accounted for its capacity to prevent accessibility to nonconservative repair, as proposed (Ayeong et al, 2019; So et al, 2022).' No assays were performed in the current manuscript to show non-conservative repair in the mouse cells used here. Instead, two papers (Ayeong et al, 2019; So et al, 2022) are used as reference for such assays throughout the manuscript, although these were performed in human cells. »

"Authors must perform assays that show A-EJ and SSA are present in MEFs with exMmRad51, but not SMRAD51 expression otherwise cannot claim that they have suppressed HR activity, whilst preventing nonconservative repair mechanisms. Need also to make sure this isn't overstated in the discussion."

Evaluating HR efficiency in our model mice is indeed essential. Because our model mice already carry GFP in the rtTA transgene construct, we could not use the classical HR and SSA reporter systems based on GFP expression. For this reason, we performed gene-targeting assays to evaluate HR efficiency, which was decreased after SMRad51 was expressed. We also observed a defect in replication fork restarting as well as increased sensitivity to MMC and PARP inhibitors. Together, these data are consistent with a previous report of HR defects induced after SMRAD51 expression in hamster and human cells (So et al. NAR, 2022 PMID: 35137208; Lambert & Lopez EMBO J, 2000 PMID: 10856252; Wilhelm et al. Plos Genet, 2016 PMID: 27135742).

To complete these observations and score alternative pathways as requested by Reviewer 3 in the revised version, we first selected *gfp*-negative MEFs after performing CRISPR–Cas9 mutagenesis. Then, we introduced SSA or end-joining substrates and selected clones (as described in So et al. NAR, 2022 PMID: 35137208). We then compared the impact of expressing SMRAD51 with silencing endogenous RAD51, which makes broken DNA ends accessible to alternative nonconservative repair processes. Indeed, we compared the effect of SMRAD51, which bind DNAs (see Appendix Figure S2. and So et al. NAR, 2022 PMID: 35137208) to that caused by the abrogation of RAD51 (note that overexpressing MmRAD51, which does not alter the in vivo phenotypes, did not answer this question because it did not impair RAD51 binding to damaged DNA, which was still protected from alternative nonconservative pathways; therefore, no conclusion could be drawn). In the revised version of the manuscript, we show that, similar to the effect in human cells, silencing RAD51 (via siRNA) stimulated alternative nonconservative repair processes, and in contrast, SMRAD51 stimulated neither SSA nor A-EJ (which is the critical point here). These new results are presented in Figure S3, and the text has been amended according to the results.

These data are consistent with the DNA-binding capacity of SMRAD51 (shown *in vitro* in Appendix S2). Moreover, we had previously shown that, *in vitro*, the SMRAD51 protein prevents the annealing of complementary ssDNA, an essential step for both SSA and A-EJ (So et al. NAR, 2022 PMID: 35137208). Importantly, the protein used in this study was the same as that used in the previous study.

« One additional problem is that, on closer inspection, the two papers turn out to be one and the same paper! »

This is true; one is the BioRxiv version, and the other the published version (NAR, 2022). The paper had not been published in NAR but was in press at the time of submission. Therefore, we added the two refs to ensure access to the full paper by the referees. In the revised version, only the publication in NAR is quoted.

Fig. 1E: It is not stated what the error bars represent. Also, not stated is how many mice for each genotype were used in each experiments. According to this figure, there are half as many exMmRad51 mice compared to SMRad51 mice (n= 5 vs n=10) at 3 months but in Fig.

1F there were no deaths of exMmRad51 mice reported. Is this technically impossible, in other words, are exMmRad51 mice harder to establish and, if so, why is this the case?

All animal handling procedures and experiments were performed according to French government regulations. We followed the recommendations to minimize the use of the mice. Since none of the exMmRad51 mice exposed to doxycycline expressed an altered phenotype, fewer exMmRad51 than SMRad51 mice were treated. Among the 6 exMmRad51 mice described in Figure 1F, none died during the course of the experiment. Error bars represent SEM, and this information has been added to the figure legend.

Fig. 1F: The authors need to provide more details about how the survival curves were generated and the number of mice included in each genotype.

We reviewed the "mice" section of the Materials and Methods and added the following sentence: "*Survival curves were generated comparing littermates of at least three different litters.*". We also added the number of mice per genotype group in the legends.

Fig. 1H: The authors must define how many samples are in each condition and what the line is showing.

We apologize for the lack of clarity in the description of our methods. We reviewed this section of our manuscript and added a full description of our experimental procedures to the legends.

Fig. 1I: Is there an example of the follicular bulb (red arrow in control) and hypodermis (blue arrow in control) structures in SMRad51 cells? Please indicate in representative image.

There are no examples of follicular bulbs in the figure and the hypoderms is not well defined in the histological sections of SMRad51 mice. However, in the revised version we added hatched arrows in the figure at the place where the structures are missing.

Fig. 2: It must be indicated how many of the 29 upregulated factors are proinflammatory.

Proinflammatory cytokines are now highlighted with an asterisk (*) in Figure 2, and a classification is presented in Appendix Table 2.

Fig. 3C-E: The authors must specify how many cells were analysed, the number of independent biological replicates and what the dots and lines represent. Also, do the red and black samples in C and D correspond to the legend in E?

Fig. 3C-E is now Fig. 4C-E. The figure legend has been changed according to the reviewer's suggestion.

Fig. 5: 'SMRad51 mutants.' was written in figure legend, should this be 'SMRad51-expressing mice'? Also, indicate the exact number of biological replicates in all of the panels.

Fig. 5 is now Fig. 6. The figure legend has been changed according to the reviewer's suggestion.

Fig. 5A: please use red and blue arrows to indicate the structures referred to in SMRad51 cells.

There are no examples of follicular bulbs in the figure and the hypoderms is not well defined in the histological sections of SMRad51 mice. However, in the revised version we added hatched arrows in the figure at the place where the structures are missing.

Fig. 5C is not referred to in text. Also please explain what has been stained and quantitate the different between control and SMRad51 cells.

Fig. 5C is now Fig. 6C, the text has been updated, and now Fig. 6C is cited as follows: *"More specifically, SMRad51 expression led to decreases in progenitor cell populations in tissues with high cell proliferation: $SOX9^+$ cells in the skin (Fig. 6B), $PLZF^+$ cells in the testis (Fig. 6C and 6D), and $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) stem cells/progenitor cells in the bone marrow (Fig. 6E).."*

Fig. 6A, C, D: what do the lines in the graph represent and how many images were analysed per biological replicate?

Fig. 6 is Fig. 7 in the revised manuscript. We reviewed the figure and updated the description of the graph with the following sentence whenever needed: "*Each point represents a biological replicate, and the horizontal line represents the mean"*. The following sentence was added to the "Statistical analysis" in the Materials and methods section: "*The number of biological replicates per experiment is presented in each graph or legend. Quantifications of the histological sections were based on at least three biological sections in each biological sample.* ".

Page 7 - '(ii) the fact that growing mice, in which many tissues are proliferating, were more sensitive than adult mice;' please clarify this statement, what are the younger mice more sensitive to than adult mice and how would this indicate that replication may be perturbed?

We changed the sentence to "*Our data suggested that replication dynamics might be affected in SMRad51-expressing cells as indicated by (i) the pronounced effects of in vivo SMRad51 expression on tissues with proliferating cells; (ii) the fact that growing mice, in which many tissues are proliferating, were more sensitive to the expression of SMRad51 than adult mice; and (iii) the role of HR in arrested replication fork protection and resumption.* ".

Fig 7: It is not indicated how many nuclei or fibres were counted per biological replicate (and how many biological replicated were quantified) and what the dots and lines represent (not specified for all panels in the figure)

The figure legend has been changed according to the reviewer's suggestion.

Fig. 8: check all text, some symbols have been converted to boxes and the figure title appears incomplete. Also, none of the panel labels match the figure legend. Specify number biological replicates, individual images/nuclei quantitated and what the lines represent. It does not appear that all panels have been cited in the text, is there Fig. 8C mentioned?

We apologize for the issues with the figure. The figure was updated, and the legend was changed according to the request.
Fig. 9C is now cited as follows: "After 5 days of *SMRad51* expression in young mice, the number of γH2AX foci in $BrdU^+/Sox9^+$ (proliferating) cells (Fig. 9*C*) increased, confirming that *SMRad51* expression induced DNA damage, likely arising from replicative stress *in vivo*.."

"Page 8: The last 4 paragraphs must be reformatted and full stops must be inserted. Interpretation of the γH2AX signal in BrdU+ cells is incorrect: γH2AX indicates DNA damage, and not necessarily replication stress, although it can be speculated that the damage stems from replication stress. RPA or pRPA Ser33 would be needed to show that there is replication stress. "

We apologize for the poor formatting of this part of the paper. We reformatted the paragraphs.

The text has been amended as follows to correct the relevance of γH2AX staining: *". After 5 days of SMRad51 expression in young mice, the number of γH2AX foci in $BrdU^+/Sox9^+$ (proliferating) cells (Fig. 9C) increased, confirming that SMRad51 expression induced DNA damage, likely arising from replicative stress in vivo."*
We would like to stress that although RPA or pRPA Ser33 are markers of replication stress, they are also found in cells undergoing DSB repair after resection. Therefore, to our knowledge, no clear replication stress marker exists. RPA focus formation can be observed during meiosis or after treatment with genotoxic agents, but it is challenging to observe RPA foci *in vivo* without the use of genotoxic agents. Nevertheless, as suggested by reviewer 2, we attempted to perform RPA and pRPA2 immunostaining to analyze replication stress *in vivo;* unfortunately, although multiple assays were performed, we failed to obtain suitable staining results.

Fig. 9 is not referred to in text; presumably Fig. 8 mentioned in Discussion should be Fig. 9?

We apologize for this mistake; Fig. 9 is now Fig. 10 in the revised version.

Appendix S1B: Is the first plasmid combination only lacking the gRNA, or gRNA and Cas9 activity as stated in the figure legend? Also, are there 'No SMRad51' -DOX cells. Has the

same experiment been performed in exMmRad51 cells and do they show a similar response to SMRad51 cells?

The first group represents plasmidic DNA (donor) + CAS9 lacking gRNA1 or gRNA2, as stated in the figure legend "three combinations of plasmids: 1) Cas9+Donor (no Cas9 activity) and 2) Cas9+Donor+gRNA1 (Cas9 active) or 3) Cas9+Donor+gRNA2 (Cas9 active)". The experiment was performed with exMmRad51 cells (Manuscript Appendix Figure S1B). exMmRAD51 did not inhibit homologous recombination, although SMRAD51 does.

Appendix S4: the scale bar is not defined.

The scale bar represents 100 µm. This is now included in the figure legend.

Supplementary Table S3 and 4: 'values' appear in many of the cells, probably because there is an error in the formula used.

The formula was correct, but we modified the values because the comma and dot in different versions of Excel led to conflicting data. The tables have been updated accordingly in the revised version.

Supplementary Table S5 is missing.

Table S5 is not part of this manuscript, and the section was erased from the text.

Minor points:

Both 'SMRad51' and 'SMRAD51' are used; please check consistency in text and figures.

We checked the text and used the standard notation for the mice:
SMRad51 = refers to a transgene or to the expression of a transgene
SMRad51 = refers to the mice
SMRAD51 = refers to the protein

Fig. 1F: the legend for 1G has also accidently been put in this section.

We apologize for this mistake, which has been corrected in the revised version.

Fig 1I: No statistical analyses are shown in this panel; P values should be removed from the figure legend.

This has been corrected in the revised version.

Page 6 - 'These data show that disruption of RAD51 function in HR leads to a systemic inflammatory response in adult mice.' In PDF 'in adult mice' has been crossed out.

This has been corrected in the revised version.

Fig. 4C legend: 'Student's t test. * $p<0.05$.' No statistical analyses are shown in panel C; this needs to be corrected.

This has been corrected in the revised version.

Appendix S1: this figure doesn't have very good resolution.

This has been corrected in the revised version.

Appendix S1A: The units for DOX treatment are not listed properly: there is a symbol missing replaced by a box.

This has been corrected in the revised version.

Appendix S2A, legend: should 'Electrophorese' be 'Electrohporesis'?

This has been corrected in the revised version.

Appendix S2D legend: '&00%' instead of '100%'. Also scale bar size in this figure doesn't match what is stated in the legend.

This has been corrected in the revised version.

Appendix S2E,F legend: what 'specific antibody' is used?

This has been described in the legend of the revised version.

Appendix S3A: replace 'Testicules' with 'testicles'

This has been corrected in the revised version (now Appendix S4A).

Appendix S6: in the title 'change' is missing a 'h'.

This has been corrected in the revised version (now Appendix S7).

Appendix Table S1: ERCC1-/- doesn't appear properly, there is a box instead of -/-.

This has been corrected in the revised version.

Appendix items are sometimes referred to as 'SI Appendix'.

This has been corrected in the revised version.

Supplementary Methods D-loop assay: 'final concentration of 2,5' need to change comma.

This has been corrected in the revised version.

Supplementary Methods TEM analysis of RAD51 filaments: 'overhang (the construction of which has been described in ((Tavares et al, 2019))) using' - need to remove unnecessary brackets.

This has been corrected in the revised version.

Supplementary Methods Whole-exome sequencing: 'whole-exome sequence ng ArrayExpress accession number is E-MTAB-8625.' - 'sequencing' is misspelled.

This has been removed from the revised version because we do not show these data in the revised version.

Dr. Bernard S Lopez
Institut Cochin
Reproduction and Cancer
24 rue St Jacques
Paris 75014
France

21st Aug 2023

Re: EMBOJ-2022-110844R
In vivo reduction of RAD51-mediated homologous recombination triggers aging but impairs oncogenesis

Dear Bernard,

Thank you for submitting your final revised manuscript for our consideration. Given the positive re-reviews (below) by the three original referees, I am happy to inform you that we have now accepted it for publication in The EMBO Journal!

Your article will be processed for publication in The EMBO Journal by EMBO Press and Wiley, who will contact you with further information regarding production/publication procedures and license requirements. You will also be provided with page proofs after copy-editing and typesetting of main manuscript and expanded view figure files.

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

With best regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
h.vodermaier@embojournal.org

Referee #1:

The authors responded to many of my comments and the manuscript is improved. I believe that the data that are presented are solid and the mouse phenotypes, both on the side of functional decline and tumorigenesis, will be of interest to the readers.

Referee #2:

I reviewed an earlier version of this manuscript in February 2022 and I was impressed by the study at that time. I made a number of comments, which I thought would improve the manuscript, but which I did not consider essential for publication. In the revised manuscript, the authors have done an excellent job of addressing the suggestions that I made. I find the revised manuscript to be substantially stronger than the earlier version, and although I defer to the judgment of the editor, it is my opinion that this report should be published expeditiously in its current form.

In particular, I would draw attention to the extensive work that the team have done with mouse genetics, introducing a tumor model into their RAD51 mutant line. The results from this work, which must have been a really big job, strongly substantiate the argument that this RAD51 mutant suppresses tumor formation. That is a very important result, and it is a credit to the team that they took on this substantial experiment.

I am also impressed by the new data on hematopoietic stem cells. I note that the team chose not to include data on a cross to a p53 knockout background in this revision. I think that is absolutely fine. The manuscript already contains enough data. I made clear in my earlier coments that I saw a cross to p53 as a bonus, which was not necessary for this paper. However, I look forward to reading about the results of the p53 knockout cross in a future manuscript!

I look forward to seeing this report in print, and I am confident that I will be citing it myself soon, because I think it is important and relevant work.

Spacific major concerns to be addressed: none
Minor concers to adress: none
Additional non-essential suggestions: none.

Referee #3:

The authors have substantially improved their manuscript and addressed all of the issues that I raised and in my opinion this work is now suitable for publication in The EMBO Journal. There were a couple of minor comments:
- 'shown/see above' is used several times on page 9, it would be clearer to specify the figure/s this refers to.
- The Discussion has a few typos and confusing language such as ' in fine' page 10 and 'concourse' page 11.
- Page 20: figure legend Figure 1E. 'Each point represents a biological replicate.'. The appears to show means + error bars rather than dots to represent each biological replicate.

EMBO Press Author Checklist

Corresponding Author Name: Bernard S. Lopez, Emmanuelle Martini
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2022-110844R

USEFUL LINKS FOR COMPLETING THIS FORM
The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures
1. Data
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Material and methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Material and methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Material and methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Material and methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Material and methods

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Material and methods
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Material and methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Material and methods, expending data

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure legends, data
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Material and methods, data
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Material and methods, data, figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Material and methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	