EMBO reports

Inhibition of ATM kinase rescues planarian regeneration after lethal radiation

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DOI: 10.15252/embr.202256112

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Review Timeline:

Submission Date:	11th Sep 22
Editorial Decision:	26th Oct 22
Revision Received:	25th Jan 23
Editorial Decision:	15th Feb 23
Revision Received:	20th Feb 23
Accepted:	27th Feb 23

Editor: Esther Schnapp

Transaction Report:

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1st Editorial Decision **26th Oct 2022**

Dear Dr. Adler,

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

As you will see, the referees acknowledge that the main finding is potentially interesting. However, they also point out that the relevant literature is not discussed in an adequate manner, and that the paper needs to be substantially re-written and a number of statements need to be corrected or better explained.
The referees further indicate some controls that should be added, and it would clearly strengthen the paper if the checkpoint regulated by ATM in response to IR in planarians could be identified.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (26th Jan 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
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2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

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4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) At EMBO Press we ask authors to provide source data for the main and EV figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

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Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The paper Inhibition of the ATM kinase rescues planarian regeneration after lethal radiation by Divya A. Shiroor et al (Carolyn Adler as senior author) shows that ATM in planaria triggers apoptosis in response to DNA damage, and that by knocking it down and allowing repair by homologous recombination, the planaria survive levels of irradiation that would normally kill them. The experiments are clearly described, correctly carried out as far as I can tell, and the conclusion is compelling. The question is simply how new or original this finding is. It is somewhat surprising that ATM alone (not p53) controls apoptosis in dNA damaged planaria. This is not the case in - for example - C elegans or most metazoans. Still the data as shown are rather compelling. The paper would benefit from a more extensive discussion about how unique Planaria are, and whether this observation has relevance for other organisms. What are the larger implications of this finding.
For the experimentation and figures, I find them adequate to support the conclusions.

a few points to fix:
1. Clarification of this statement is needed:
This difference may be due to a functional loss of planarian p53.
Despite conservation of the protein, planarian p53 acts primarily to regulate epithelial cell production rather than to respond to genotoxic stress (Pearson and Sánchez Alvarado, 2010; Cheng et al., 2018; Wendt et al., 2022)

Do they really mean "functional loss of p53" or do they mean "loss of a specific function of p53"... ? The p53 is still functional, it just does not regulate DNA damage induced apoptosis. Is it known how ATM regulates apoptosis (p21 ?).

2. What is the checkpoint pathway that loss of ATM compromises ? Some tests or documentation of this pathway would greatly enhance the paper.

Referee #2:

This paper presents evidence that the stem cells of the planarian worm lacking ATM are not sensitive to radiation but rather hyperresistant due to a failure to undergo apoptosis. Indeed exposure of planarian worms lacking ATM are hyperresistant to radiation (ie not just the stem cells). This is a surprising observation and, as such, merits publication. I am also very supportive of papers on distinct model systems, which may differ to mammalian systems. However, much of the paper is not well written, generalisations are made and the comparisons to mammalian cells and analysis are not justified. There appears to be multiple examples where concepts are either not well stated, mis understood or unjustified. I try to summarise my concerns below. I feel the findings have merit but as written this is not acceptable or valid.
1. It is often unclear where the authors are generalising or where they are making statements specific to planarian worms - eg in the abstract line 12 - our results demonstrate that ATM's primary function is to drive apoptosis? - in the worm? Certainly not in other organisms. What is the next line referring to - worms or mammalian cells.
2. Introduction page 3 line 23 - it is stated that cells and patients lackiing ATM are often hypersensitive to IR... but some cell types including thymocytes are radiation resistant. I am not aware of ANY evidence for this. ATM deficient thymocytes are still radiation sensitive compared to control lymphocytes even if they do not die by apoptosis, line 5 - these results suggest ... I think there is no strong evident for this. To my knowledge ALL cells lacking ATM are hypersensitive, although certainly they do not always activate apoptosis.
3. The authors appear woefully lacking in their understanding of the DNA damage response in mammalian cells. Page 4 line 4, they appear to quote a reference that ATM heterozygotes can restore blood faster than homozygotes or WT controls - I have no idea what they mean but ATM deficiency causes stem cell deficiency. The analysis of cell cycle checkpoints in planarians (but quoting human cells) is often incorrect (see details below).
4. Rather as a side point, the authors should perhaps be aware in yeast, the ATM homologue (Tel1) has a much less evident role in the response to IR exposure than in mammalian cells. This in part, is likely to be because yeast readily resect DSBs to give single strand overhands, which can activate ATR. The authors do not seem aware of the difference between ATM and ATR in terms of activation - ATM is activated by DSBs (until extensive resection occurs); ATR is activated by ss regions of DNA. Since replication stalling causes ss regions of DNA, ATR is essential and ATM is not. But if DSBs are avidly resected, then ATR becomes important even after IR exposure. Page 11 line 2 - ATM regulates checkpoints tin response to replciations stress - NO,

ATR is activated after replication stress. The interplay is complex and I will not expand on it here but the major thrust here is not correct. These points all focus on the fact that the way the paper is written - ie the relevance of these findings to human cells does not have validity. They are interesting and information can be learned from them but not as stated.
5. I will now focus on the actual results rather than on the way the paper is written.
6. Figure 1 -the authors nicely show that the piwi-1 stem cells die by apoptosis after IR and are resistant when ATM is lacking. I would like to see a non-stem cell control. Do non-Stem cells die by permanent arrest, senescence? This is important given the focus on stem cells in this paper. It would also be useful to know whether this finding is true of quiescent stem cells or progenitors. It should be appreciated that at least neuronal stem and progenitor cells differ in this context (though this is not true for all stem cell compartments - see PMID: 28489848.
7. Figure 2 examines the checkpoint responses. However this analysis does not appreciate the interplay between checkpoints. ATM regulates G1/S. G2/M and intra S phase checkpoints. The ATM-dependent S phase checkpoint merely prevents new origin firing and is short lived because it is mainly controlled by ATR; the G1/S checkpoint in mammalian cells is significant because it stops cells entering S phase after IR. The replication analysis does not allow a distinction between these. In fact, (page 7 line 3 - the authors write .. the transient progression into S phase after ATM loss phenocopies radioresistant DNA synthesis - firstly progression into S phase is not monitored but additionally this is more likely to be a consequence of G1/S checkpoint loss. Page 7 line 9 - it states that ATM is not required for a metaphase checkpoint? ATM does not have a major role in a metaphase checkpoint? - do the authors mean a G2/M checkpoint.
8. Then it is stated that the worms are reliant on HR? Yet if there are quiescent stem cells in G0, how do they repair DSBs without a sister chromosome. In most organisms, BRCA2 and RAD51 are essential because HR functions when replication stalls. Here, t here is no control showing whether animals survive with siRNA BRCA1 or RAD51 without radiation. Hence the impact of radiation cannot be judged.
9. Page 9 line 21. Our model proposes that planarian stem cells depend on HR for stem cell recovery - HR is essential to deal with stalled/collapsed forms - even without IR, stem cells are reliant on HR (and all cells are reliant on HR when they replicate). The authors do not seem to understand this concept.
10. Page line 19 - a threshold for activation of stem cell apoptosis is proposed - there is NO evidence for this. Most evidence in mammalian cells would say that apoptosis is activated by damage in a linear manner (see eg PMID: 28489848) - here this conclusion of a threshold is reached without ANY dose response analysis. This proposal has no basis at all.
11. Fnally page 11 line 1 - it is stated .. Unlike in other eurkaryotes where ATM regulates checkpoint responses to replication stress ... I mentioned above that this concept is wrong - ATM is non-essential in human cells. ATM responds to DSBs (not ss regions of DNA) - so it is non-essential because DSBs are not common endogenous lesions.
12. The authors should perhaps also look at the situation in yeast, where ATR has a more dominant role - but also in Drosophila, where ATM and ATR have rather distinct functions to mammalian cells. I also recommend collaborating with a group who do understand the damage response mechanisms in mammalian cells. The core finding that ATM deficient worms are hyperresistant to IR is unexpected and interesting - the presentation and some analysis is flawed.

Referee #3:

This manuscript reports the key finding that knocking down ATM kinase in planarians enables their stem cells to survive lethal doses of radiation, which restores regeneration and long-term survival to irradiated animals. The authors show that radiation-induced stem cell apoptosis requires ATM function and that homologous recombination is necessary for the survival of ATM(RNAi) planarians. Based on its implications for the evolution of DNA repair mechanisms and their roles in stem cells, I would expect this work to be of general interest to the molecular biology community. With clarification of some of the following points, the work is robustly documented using independent lines of evidence and is appropriate for publication here.

Points that the authors should consider addressing in a revised manuscript:

1. The authors have done an excellent job showing the specificity of RNAi knockdown of atm via RNAseq analysis of sorted neoblasts under control and atm RNAi conditions, and by using different fragments of the transcript to trigger RNAi. However, the negative results upon which some conclusions are based assume that all genes are being knocked down equivalently. Given that they performed qPCR to measure piwi-1 RNA levels under many of these knockdown conditions, their conclusions would be strengthened by also measuring the levels of targeted transcripts to show that other knockdowns that were important for their conclusions (e.g., dna-pk and all of the atm double RNAi conditions with HR and NHEJ genes, which were performed by bacterial feeding instead of feeding of in vitro-synthesized dsRNA) were also effective.

2. By and large, the paper is well written and the results are clearly presented. However, this reviewer was quite confused by the final paragraph of the Results section (line 2, page 9). It begins, "To confirm that long-term survival after perturbation of NHEJ and HR components was due to aberrant persistence of stem cells..." Looking at Figs 4A and B, which clearly show animals not surviving long-term following perturbation of NHEJ or HR, I don't know how to interpret this. The remainder of the paragraph is quite unclear about which experiments are performed together with atm(RNAi): "Two days after radiation, stem cell abundance was not significantly altered by knockdown of HR or NHEJ components (Fig. 4C)..." Based on the figure, this seems to be referring to simultaneous knockdown of atm and HR or NHEJ components. Similarly, "Seven days after radiation... we found that stem cells were found following knockdown of dna-pk but not brca2 or rad51 (Fig. 4D)..." It seems that the correct wording

should be "stem cells were preserved following double knockdown of atm and dna-pk, but not brca2 or rad51 (Fig. 4D)..."

Other sentences that should be clarified or re-written are:
Page 5, line 5: "This study therefore reveals new mechanisms for promoting stem cell rejuvenation and preventing tumorigenesis in less resilient animals." It is unclear what "less resilient animals" are being referred to and how these results could be used to prevent tumorigenesis in them.
Page 7, line 7, ending: "suggesting that stem cells either fail to atm(RNAi) animals accumulate in S and G2."

3. The authors may wish to consider softening their wording in some of the following places:
Page 6, lines 3-4: "...suggesting that ATM drives stem cell apoptosis primarily after radiation." This is the only DNA damaging agent tested here so it seems difficult to assign it primacy.
Page 8, line 7: "The recovery of regenerative capacity indicates that atm(RNAi) animals have completely restored all stem cell function, including normal differentiation and patterning." Because the authors have only looked at gross morphology and one molecular marker for photoreceptors, "completely" and "all" are vast overstatements, particularly because the regenerates don't look completely normal (the eyes are asymmetric, the head may be indented). Exactly how "normal" these surviving stem cells are will be an interesting avenue for future exploration.

4. Fig. 4E (cited as 4D in the text, page 9, line 20): I may have missed something, but wouldn't the last two panels (depicting NHEJ(RNAi) and HR(RNAi), both of which may lead to some type of repair but do not yield any long-term viable cells) in the context of the experiments reported in this paper, be more useful if they showed the effects of these knockdowns in the context of the atm(RNAi) experiments? It is unclear why the legend says that ATM drives stem cell apoptosis by setting a threshold for cell death at based on levels of DNA damage at 2 dpi, when the effects of atm(RNAi) on apoptosis are already observed 1 dpi.

We are grateful for the overall positive assessment of our work and helpful criticisms offered by all three reviewers. In the revised version we have incorporated these suggestions by primarily improving the clarity of the writing (in particular, substantially revising and extending the discussion as suggested by all three reviewers) and including the proposed experiments.

Referee #1:

Inhibition of the ATM kinase rescues planarian regeneration after lethal radiation by Divya A. Shiroor et al (Carolyn Adler as senior author) shows that ATM in planaria triggers apoptosis in response to DNA damage, and that by knocking it down and allowing repair by homologous recombination, the planaria survive levels of irradiation that would normally kill them. The experiments are clearly described, correctly carried out as far as I can tell, and the conclusion is compelling. The question is simply how new or original this finding is. It is somewhat surprising that ATM alone (not p53) controls apoptosis in dNA damaged planaria. This is not the case in - for example - C elegans or most metazoans. Still the data as shown are rather compelling. The paper would benefit from a more extensive discussion about how unique Planaria are, and whether this observation has relevance for other organisms. What are the larger implications of this finding.
For the experimentation and figures, I find them adequate to support the conclusions.

Thank you for acknowledging the strengths of our work. As suggested, we have substantially revised the discussion to focus on similarities and differences between planarians and other systems with respect to cell cycle checkpoint regulation, DNA repair mechanisms, and stem cell biology.

a few points to fix:
1. Clarification of this statement is needed:
This difference may be due to a functional loss of planarian p53.
Despite conservation of the protein, planarian p53 acts primarily to regulate epithelial cell production rather than to respond to genotoxic stress (Pearson and Sánchez Alvarado, 2010; Cheng et al., 2018; Wendt et al., 2022)

Do they really mean "functional loss of p53" or do they mean "loss of a specific function of p53"... ? The p53 is still functional, it just does not regulate DNA damage induced apoptosis. Is it known how ATM regulates apoptosis (p21 ?).

The reviewer is correct in inferring that our findings support the loss of a specific function-regulation of apoptosis after genotoxic stress. This conclusion is based on existing literature showing that planarian p53 primarily regulates epithelial differentiation and function, and suggests that p53 does not regulate DNA damage induced-apoptosis in these animals. Our work highlights that planarian ATM functions independently of p53. We now more thoroughly reference this important conclusion in the results (lines 139-143) and come back to it in the discussion (lines 276-279, 354-357).

Regulation of apoptosis in planarians is relatively understudied. Our discovery of ATM driving apoptosis after genotoxic stress therefore opens the door to exploring downstream effectors that future studies will target. We have clarified these points in the discussion (lines 271-280).

2. What is the checkpoint pathway that loss of ATM compromises ? Some tests or documentation of this pathway would greatly enhance the paper.

As mentioned above, much like apoptosis, very few regulators of checkpoints have been described in planarians. One hurdle has been a lack of tools in planarians – our only way to perturb gene function is with RNAi, and we still cannot introduce genes via transgenesis. Therefore, we are restricted to pretty

crude tools like EdU, cell cycle analysis, and phosphohistone H3 labeling. Although we feel that a thorough checkpoint analysis is important, we also think that it may be beyond the scope of this current manuscript. Ongoing work in our lab will develop reagents and tools to further aid the dissection of checkpoint mechanisms in greater detail.

Referee #2:

This paper presents evidence that the stem cells of the planarian worm lacking ATM are not sensitive to radiation but rather hyperresistant due to a failure to undergo apoptosis. Indeed exposure of planarian worms lacking ATM are hyperresistant to radiation (ie not just the stem cells). This is a surprising observation and, as such, merits publication. I am also very supportive of papers on distinct model systems, which may differ to mammalian systems. However, much of the paper is not well written, generalisations are made and the comparisons to mammalian cells and analysis are not justified. There appears to be multiple examples where concepts are either not well stated, mis understood or unjustified. I try to summarise my concerns below. I feel the findings have merit but as written this is not acceptable or valid.

We thank the reviewer for appreciating that our findings merit publication. We are grateful for the time and effort they have put in to help clarify our writing and their insight on how to accurately contextualize these findings with mammalian systems. We have rectified our writing errors in accordance with their suggestions, specifics of which are detailed below.

1. It is often unclear where the authors are generalising or where they are making statements specific to planarian worms - eg in the abstract line 12 - our results demonstrate that ATM's primary function is to drive apoptosis? - in the worm? Certainly not in other organisms. What is the next line referring to - worms or mammalian cells.

We have clarified the abstract (line 31) by mentioning that our finding pertains to planarians and have also revised other parts of our manuscript to ensure we make clear species distinctions throughout our text.

2. Introduction page 3 line 23 - it is stated that cells and patients lackiing ATM are often hypersensitive to IR... but some cell types including thymocytes are radiation resistant. I am not aware of ANY evidence for this. ATM deficient thymocytes are still radiation sensitive compared to control lymphocytes even if they do not die by apoptosis, line 5 - these results suggest ... I think there is no strong evident for this. To my knowledge ALL cells lacking ATM are hypersensitive, although certainly they do not always activate apoptosis.

We thank the reviewer for highlighting the important distinction between radiation sensitivity and ultimate apoptosis and explaining the lack of clarity in our writing. We have altered our wording to "*ATM*$^{-/-}$ thymocytes and postmitotic neurons for example are resistant to radiation-induced apoptosis" (line 63-64) to clearly state the pertinent result from the cited publication.

3. The authors appear woefully lacking in their understanding of the DNA damage response in mammalian cells. Page 4 line 4, they appear to quote a reference that ATM heterozygotes can restore blood faster than homozygotes or WT controls - I have no idea what they mean but ATM deficiency causes stem cell deficiency. The analysis of cell cycle checkpoints in planarians (but quoting human cells) is often incorrect (see details below).

We have altered this in our writing as follows "ATM-/- hematopoietic stem cells fail to self-renew long term" (lines 64-65) and thank the reviewer for helping clarify this point.

4. Rather as a side point, the authors should perhaps be aware in yeast, the ATM homologue (Tel1) has a much less evident role in the response to IR exposure than in mammalian cells. This in part, is likely to be because yeast readily resect DSBs to give single strand overhands, which can activate ATR. The authors do not seem aware of the difference between ATM and ATR in terms of activation - ATM is activated by DSBs (until extensive resection occurs); ATR is activated by ss regions of DNA. Since replication stalling causes ss regions of DNA, ATR is essential and ATM is not. But if DSBs are avidly resected, then ATR becomes important even after IR exposure. Page 11 line 2 - ATM regulates checkpoints tin response to replciations stress - NO, ATR is activated after replication stress. The interplay is complex and I will not expand on it here but the major thrust here is not correct. These points all focus on the fact that the way the paper is written - ie the relevance of these findings to human cells does not have validity. They are interesting and information can be learned from them but not as stated.

We thank the reviewer for highlighting this error in our writing and have deleted this statement entirely. As a side note, we have analyzed *atr* knockdown animals and find that they have no phenotypes in unirradiated planarians.

5. I will now focus on the actual results rather than on the way the paper is written.
6. Figure 1 -the authors nicely show that the piwi-1 stem cells die by apoptosis after IR and are resistant when ATM is lacking. I would like to see a non-stem cell control. Do non-Stem cells die by permanent arrest, senescence? This is important given the focus on stem cells in this paper. It would also be useful to know whether this finding is true of quiescent stem cells or progenitors. It should be appreciated that at least neuronal stem and progenitor cells differ in this context (though this is not true for all stem cell compartments - see PMID: 28489848.

Planarian stem cells are thought to divide regularly, and differentiate, in a one-way manner, to produce organ-specific progenitor cells that then mature without dividing, into differentiated cells. It's well established that radiation does not affect the abundance of differentiated cells in planarians (PMID: 16890156), which in part contributes to their ability to survive for weeks after high-dose exposures. Quiescent cells, if they exist, are minor contributors to animal regeneration and survival. We have now included an additional experiment (Fig. EV3, lines 115-128 of the Results) where we examine diverse markers of differentiated neurons and intestinal cells, and early and late stage progenitors of the epithelial lineage. Epithelial cells are the best-characterized lineage in planarians and represent the majority of the progenitors produced by the stem cells. We find no evidence that these cell types are impacted by *atm* knockdown, and therefore conclude that radiation-induced apoptosis likely does not extend to differentiated planarian cells at timepoints relevant to this study.

The reviewer raises interesting points about the dynamics of different cell populations after radiation, and we have added substantial text to the discussion (lines 323-346) to consider the important biological distinctions between the stem cell biology of planarians and mammalian systems. It is unknown whether a senescence-like state exists in planarians, but we also included this in the revised model figure for reference.

7. Figure 2 examines the checkpoint responses. However this analysis does not appreciate the interplay between checkpoints. ATM regulates G1/S. G2/M and intra S phase checkpoints. The ATM-dependent S phase checkpoint merely prevents new origin firing and is short lived because it is mainly controlled by ATR; the G1/S checkpoint in mammalian cells is significant because it stops cells entering S phase after IR. The replication analysis does not allow a distinction between these. In fact, (page 7 line 3 - the authors write .. the transient progression into S phase after ATM loss phenocopies radioresistant DNA synthesis - firstly progression into S phase is not monitored but additionally this is more likely to be a consequence

of G1/S checkpoint loss. Page 7 line 9 - it states that ATM is not required for a metaphase checkpoint? ATM does not have a major role in a metaphase checkpoint? - do the authors mean a G2/M checkpoint.

We thank the reviewer for highlighting this error. We have modified the text to indicate that the G1/S checkpoint is likely to be compromised in *atm*(RNAi) stem cells (lines 151-152), and clarified other text in this paragraph (lines 146-160).

8. Then it is stated that the worms are reliant on HR? Yet if there are quiescent stem cells in G0, how do they repair DSBs without a sister chromosome. In most organisms, BRCA2 and RAD51 are essential because HR functions when replication stalls. Here, t here is no control showing whether animals survive with siRNA BRCA1 or RAD51 without radiation. Hence the impact of radiation cannot be judged.

The reviewer raises an interesting point that highlights a key difference between stem cells in planarians and stem cells in mammalian systems, which we now highlight in the discussion (lines 334-346). The reason we did not consider quiescent stem cells as a major contributor to recovery is that if they exist, they are extremely rare, and would be present in insufficient numbers to drive recovery on the timescale we observe. In planarians, the vast majority of stem cells are thought to be actively proliferating because continued labeling with BrdU results in 99% of neoblasts being labeled (PMID: 10753506). Long-term label retention has only recently been used in planarians (PMID: 33511776) where it was shown that <1% of stem cells retain BrdU label for up to 6 weeks. The simplest explanation for the recovery of stem cell proliferation we observe is that repair occurs in stem cells that have either stalled in G2 or continued replication after IR rather than reactivation of a small number of quiescent stem cells.

Our results are consistent with planarian stem cells dividing regularly - hence the strong reliance on HR. It is documented (PMID: 33890575) that knockdown of *rad51*, but not *brca2*, is required for survival in the absence of radiation. The *rad51* knockdown phenotype takes ~5-6 weeks to appear after the initiation of RNAi, far longer than the duration of our experiments. Therefore, we suspect that the contribution of HR in unirradiated contexts is minimal.

9. Page 9 line 21. Our model proposes that planarian stem cells depend on HR for stem cell recovery - HR is essential to deal with stalled/collapsed forms - even without IR, stem cells are reliant on HR (and all cells are reliant on HR when they replicate). The authors do not seem to understand this concept.

We recognize that HR is necessary for replication even in the absence of radiation – this is also true in planarians, as mentioned above. However, since the focus of our study is radiation-induced genotoxic stress, we have largely restricted our analysis to the context of radiation. Our conclusion that planarian stem cells depend on HR for recovery from radiation comes from two key results in our study. First, blocking the HR pathway by knockdown of *brca2* or *rad51*, either alone or together with *atm*, results in stem cell loss and animal death after radiation. Though components of the NHEJ pathway are intact, they are clearly insufficient to drive sustained repair in planarians. Conversely, blocking the NHEJ pathway by knockdown of *dna-pk* along with *atm* does not impair long-term animal survival after radiation. We have altered the writing throughout to strengthen our conclusion that *atm* acts specifically after ionizing radiation.

10. Page line 19 - a threshold for activation of stem cell apoptosis is proposed - there is NO evidence for this. Most evidence in mammalian cells would say that apoptosis is activated by damage in a linear manner (see eg PMID: 28489848) - here this conclusion of a threshold is reached without ANY dose response analysis. This proposal has no basis at all.

Our previous use of this word was motivated by the fact that we and others observe differences in both stem cell and planarian survival at doses above 1250-1500 rads (PMID: 32386527, PMID: 21566185).

Functional ATM seems to drive radiation-induced apoptosis primarily at higher exposures, but we did not test this rigorously in the previous version. We have completely deleted the word/concept of threshold from the current version of the manuscript, and have revised the model figure accordingly.

11. Fnally page 11 line 1 - it is stated .. Unlike in other eurkaryotes where ATM regulates checkpoint responses to replication stress ... I mentioned above that this concept is wrong - ATM is non-essential in human cells. ATM responds to DSBs (not ss regions of DNA) - so it is non-essential because DSBs are not common endogenous lesions.

Thank you for noticing this error. We have eliminated this statement from this paragraph. Throughout the manuscript, we now emphasize the fact that ATM is strictly activated after radiation, because in every assay we fail to detect any phenotype in unirradiated animals.

12. The authors should perhaps also look at the situation in yeast, where ATR has a more dominant role - but also in Drosophila, where ATM and ATR have rather distinct functions to mammalian cells. I also recommend collaborating with a group who do understand the damage response mechanisms in mammalian cells. The core finding that ATM deficient worms are hyperresistant to IR is unexpected and interesting - the presentation and some analysis is flawed.

We thank the reviewer for appreciating our work and providing important insight into DNA damage responses in vertebrates. As we are newcomers to the DNA repair field, their input on our manuscript has been essential to improving the accuracy of our interpretations.

Referee #3:

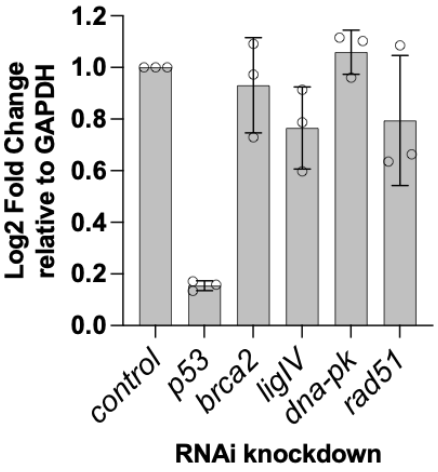
This manuscript reports the key finding that knocking down ATM kinase in planarians enables their stem cells to survive lethal doses of radiation, which restores regeneration and long-term survival to irradiated animals. The authors show that radiation-induced stem cell apoptosis requires ATM function and that homologous recombination is necessary for the survival of ATM(RNAi) planarians. Based on its implications for the evolution of DNA repair mechanisms and their roles in stem cells, I would expect this work to be of general interest to the molecular biology community. With clarification of some of the following points, the work is robustly documented using independent lines of evidence and is appropriate for publication here.

Points that the authors should consider addressing in a revised manuscript:

1. The authors have done an excellent job showing the specificity of RNAi knockdown of atm via RNAseq analysis of sorted neoblasts under control and atm RNAi conditions, and by using different fragments of the transcript to trigger RNAi. However, the negative results upon which some conclusions are based assume that all genes are being knocked down equivalently. Given that they performed qPCR to measure piwi-1 RNA levels under many of these knockdown conditions, their conclusions would be strengthened by also measuring the levels of targeted transcripts to show that other knockdowns that were important for their conclusions (e.g., dna-pk and all of the atm double RNAi conditions with HR and NHEJ genes, which were performed by bacterial feeding instead of feeding of in vitro-synthesized dsRNA) were also effective.

We thank the reviewer for appreciating our efforts to ensure the specificity of RNAi knockdown of *atm*. We recognize the importance of determining the efficacy of knockdown via RNAi for all genes we examined, and have done so using a functional readout. Our reasons for this are as follows:

Based on the reviewer's recommendation, we tried to evaluate knockdown of RNAi genes via qPCR, using either GAPDH (shown below), or UDP as controls. We could consistently detect reliable knockdown for *p53*, while results with other genes were inconclusive (data below). We did this on both single and double-knockdown animals as the reviewer suggested. We suspect that the extremely low expression levels of these individual genes may be skewing their detection via qPCR. A bioinformatic search of RNAseq expression databases for planarians (PlanMine), shows that *rad51*, *brca2*, *dna-pk*, and *ligIV* have very low expression, with FPKMs below 10. We have elected not to include this data in the manuscript. The weak expression of these genes is probably also responsible for our inability to obtain reliable results with *in situ* hybridization for *atm* or these other genes, even though this is a standard technique used in our lab.



qRT-PCR of single gene knockdown of *p53*, *brca2*, *DNA ligase IV, dna-pk,* and *rad51* normalized to GAPDH. Error bars represent standard deviation; each circle represents a biological replicate, which is itself an average of 3-4 technical replicates.

Furthermore, our evaluation of *atm* knockdown with RNAseq suggests that transcript levels might not necessarily correlate with phenotypic outcomes. Despite having only a 50% decrease in transcript abundance in RNAseq, *atm*(RNAi) animals exhibit strong phenotypic effects in terms of stem cell and animal survival after radiation.

Therefore, in lieu of demonstrating knockdown at the molecular level, we have instead strengthened data supporting the conclusions from these experiments. In the previous version, our finding that NHEJ was not necessary for survival rested solely on knockdown of *dna-pk*. Now, we have further verified these findings with *DNA ligase IV* and *artemis* (included in Fig. 4C). Together, these results support the idea that long-term survival after radiation enabled by atm knockdown does not rely on NHEJ.

2. By and large, the paper is well written and the results are clearly presented. However, this reviewer was quite confused by the final paragraph of the Results section (line 2, page 9). It begins, "To confirm that long-term survival after perturbation of NHEJ and HR components was due to aberrant persistence of stem cells..." Looking at Figs 4A and B, which clearly show animals not surviving long-term following perturbation of NHEJ or HR, I don't know how to interpret this. The remainder of the paragraph is quite unclear about which experiments are performed together with atm(RNAi): "Two days after radiation, stem cell abundance was not significantly altered by knockdown of HR or NHEJ components (Fig. 4C)..." Based on the figure, this seems to be referring to simultaneous knockdown of atm and HR or NHEJ components. Similarly, "Seven days after radiation... we found that stem cells were found following knockdown of dna-pk but not brca2 or rad51 (Fig. 4D)..." It seems that the correct wording should be "stem cells were preserved following double knockdown of atm and dna-pk, but not brca2 or rad51 (Fig. 4D)..."

We are grateful to the reviewer for noticing these errors in our writing. We have revised this paragraph (lines 205-220) and hopefully clarified the description of these results.

Other sentences that should be clarified or re-written are:
Page 5, line 5: "This study therefore reveals new mechanisms for promoting stem cell rejuvenation and preventing tumorigenesis in less resilient animals." It is unclear what "less resilient animals" are being referred to and how these results could be used to prevent tumorigenesis in them.

We thank the reviewer for pointing out that this sentence is confusing and we have accordingly deleted this phrase from the revised manuscript (see lines 89-90).

Page 7, line 7, ending: "suggesting that stem cells either fail to atm(RNAi) animals accumulate in S and G2."

We have corrected this typo.

3. The authors may wish to consider softening their wording in some of the following places:
Page 6, lines 3-4: "...suggesting that ATM drives stem cell apoptosis primarily after radiation." This is the only DNA damaging agent tested here so it seems difficult to assign it primacy.
Page 8, line 7: "The recovery of regenerative capacity indicates that atm(RNAi) animals have completely restored all stem cell function, including normal differentiation and patterning." Because the authors have only looked at gross morphology and one molecular marker for photoreceptors, "completely" and "all" are vast overstatements, particularly because the regenerates don't look completely normal (the eyes are asymmetric, the head may be indented). Exactly how "normal" these surviving stem cells are will be an interesting avenue for future exploration.

We have clarified the phrase on page 6 (now line 114), and have toned down our language about animal survival and regeneration accordingly (lines 197-198).

4. Fig. 4E (cited as 4D in the text, page 9, line 20): I may have missed something, but wouldn't the last two panels (depicting NHEJ(RNAi) and HR(RNAi), both of which may lead to some type of repair but do not yield any long-term viable cells) in the context of the experiments reported in this paper, be more useful if they showed the effects of these knockdowns in the context of the atm(RNAi) experiments? It is unclear why the legend says that ATM drives stem cell apoptosis by setting a threshold for cell death at based on levels of DNA damage at 2 dpi, when the effects of atm(RNAi) on apoptosis are already observed 1 dpi.

We appreciate that the illustrated representation of our findings was confusing and have changed our model figure, taking into account suggestions made by the reviewer. We have also eliminated the concept of a threshold from our model figure, taking into account both reviewer 2 and 3's suggestions.

Dear Dr. Adler,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees that were asked to assess it. Referee 2 still has some more minor suggestions that I would like you to address and incorporate before we can proceed with the official acceptance of your manuscript.

A few editorial requests also need to be addressed:

- Your manuscript has 5 main figures but it layed out as a full article. Please either add one more main figure or combine the results and discussion sections to publish it as a short report with a maximum of 28.000 characters. You can find more info about our article types in our guide to authors online.

- Please reduce the number of keywords to 5.

- Please correct the conflict of interest subheading to "Disclosure and Competing Interest Statement"

- Please remove the author credits from the ms file. We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions, if you wish. See also guide to authors https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines.

- Please upload all main and all EV figures as separate files.

- Tables S1 & S2 should be named Dataset EV1 & EV2 and uploaded as excel files using the file type Dataset. Tables S3 & S4 should be named Tables EV1 & EV2 and uploaded using the file type Expanded View.

- The manuscript sections are in the wrong order, please correct.

- I attach to this email a related ms file with comments by our data editors. Please address all comments in the final ms. For example, where n=2 no statistics can be calculated, but instead all data points can be shown.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #2:

This revision is improved and, as previously, the major take home conclusion is valid and interesting. However, I think it is obvious from the reviewers comments (and the massive number of major statements removed during revision), the authors are lax in deriving unwarranted conclusions and in in over-interpreting aspects. This has continued in this revision. Mostly, these issues can be addressed (and do not affect the take home conclusion) by changes to the text. But I do urge the authors to consider whether their discussion points are valid. Before processing to individual points. I make two general comments:
1. The general conclusion is that cells with a DSB replicate and then use HR to repair the DSB. However, replication past a DSB is a major challenge for a cell. HR clearly plays a major role during replication and is required for stem cell expansion. I am not convinced they have shown it is the major driver of DSB repair (they do not actually look at DSB repair at all). As said above, replication past a DSB is challenging - hence the general consensus that NHEJ functions prior to replication and HR in replicated cells (though NHEJ is used there as well). I am not aware of much evidence that cells replicate past a DSB and then use HR - yet that is what is proposed (see eg line 331 - how can they generate a template if the DSB is there during replication?). Please consider carefully how strong you argue that the DSBs are repaired by HR.
(note it is likely that because stem cells are replicating, they will have more late S - G2 cells so HR will have a bigger impact

(which is true in ESCs also) - but this is distinct to what is being said here).

2. The authors admit that their siRNA procedure has limiitations -but still make strong conclusions based on these limitations. See my point about NHEJ proteins below (residual NHEJ function can go a long way). Similarly, in their response letter, they argue that ATR is not required for normal replication. This is not impossible (I have read the ATM replaces ATR in Drosophila) but still highly unlikely. Most likely, there is insufficient knockdown to cause a phenotype.
3. I am concerned about the conclusion that cells replicate past a DSB to create a template to use HR. BIR can do that but they have not shown that. They have not shown that HR repairs the DSB - only that it is required for stem cell propagation.
4. Personally, I think the authors should cut down the discussion and focus on the points they show - abrogation of apoptosis in worms allows the worms to survive and HR is required for this.

Individual points.

5. Abstract: Line 23 - To mitigate potentially deleterious outcomes, cells activate the DDR - which dictates whether cells repair DNA or undergo apoptosis - this sounds like these are alternatives - they are not. Repair can occur even if cells finally undergo apoptosis - and (as written in the response letter), differentiated planarian cells do not seem to die by apoptosis. ATM deficiency leads to only a subtle DSB repair defect. Better to say something like - the DDR network functions to regulate several process affecting cell survival, including apoptosis.

6. Last sentence - ATM is a key regulator of radiation-induced apoptosis. This is not novel - (eg lymphocytes in humans). What is novel is that radiation-induced apoptosis is the major regulator of organismal survival in worms.
(NB in fact in humans, the haematopoeitic system (where apoptosis can arise) is a major sensitive organ and (to some extent) determines organismal survival. However, the difference here is that loss of ATM does not increase organismal survival - probably due to the fact that other stem cells compartments behave differently in humans).
7. My final point in the abstract is that I am not sure its valid to say "without adverse effects". The animal survive indeed as shown in Figure 3 but not all parameters are examined (eg life span or dysfunctional cells in tissues). Perhaps one could say ...without strong or evident adverse effects. Inherently, I believe there must be adverse effects otherwise why activate apoptosis.

8. Introduction
Lines 44 and 55 - again cell death or repair are not alternatives. I understand the point being made but the entire paragraph is not correct - it is not a binary decision - repair or apoptosis.
9. Line 63 - ATM-/- thymocytes and post mitotic neurons are resistant to IR induced apoptosis. Even ATM+ post mitotic neurons do not undergo apoptosis - so although ATM-/- post mitotic neurons are resistant to apoptosis it is not relevant. Thymocyte aspect is OK - and true in the replicating neurons (progenitor cells) and the intestine.
10. 73 - get rid of Rads throughout - its an outdated unit of radiation and not used currently..
11. 83 - you don't show whether it's a choice - you just show that they do not undergo apoptosis (and they require HR for proliferation). Note that the experiment to say that NHEJ does not arise is unreliable since you do not show that you have achieved knock-down of NHEJ proteins (no controls at all) (see below). Lines 85-86 are good and OK

10. 87. - Do they not trigger apoptosis and then undergo HR - or undergo HR and hence do not die by apoptosis - your phrase says the former. It may well be a mix of both depending on where they are at in the cell cycle - indeed if they proceed into S phase, then they will repair by HR. But they need to replicate past a DSB.
11. 89. As per abstract, it is well known that ATM regulates apoptosis after IR - the novel aspect here is that it is apoptosis that causes death of the worms.
12, 94. Just a point of clarification. The definition of stem cells is potentially confusing here - in humans and mice, it is the progenitor stem cells that normally die by apoptosis (see eg. Your reference Barazzuol et al, 2017). Your "stem cells" are effectively replicating stem cells (ie progenitors). So in worms, the difference seem to be that most "stem cells" are actually progenitor cells - ie dividing with few quiescent stem cells. I think this could be stated more clearly in the introduction..
13. 127. The analysis of differentiated cells (Fig EV3) is good. Was apoptosis examined here - I think it is critical to the conclusions to show that the differentiated cells do not undergo apoptosis.
14. 156 - the transient progression into S phase after ATM knockdown is reflective of G1/S arrest in human cells - Radioresistant DNA synthesis if reflective of progression through S phase (ie not stopping replication)
15. 161. The sentence as written here is likely to cause confusion since it is well known that ATM does regulate a G2/M checkpoint. What is known in human cells, is that ATM activates a rapid G2/M checkpoint due to the presence of un-resected DSBs. Then as they get resected, ATR becomes important. I suspect it's the same in worms. Perhaps the best is to add a sentence saying that in human cells also, after IR ATM activates immediate G2/M arrest but is dispensable for longer-term G2/M arrest, which is ATR-dependent (this is work by Mike Kaston PMID: 11809797).

16. 212. As stated above I worry about the conclusions of the experiments involving NHEJ proteins. In human cells, NHEJ proteins are highly abundant (which may not be the case in planaria) and notoriously difficult to knock down. In the response to reviewer 3, it was pointed out that there were not controls to verify the efficacy of knockdown (except for ATM where there was functional examination). There is nothing here to show that knockdown of these proteins has been effective. Just some residual NHEJ proteins can go a long way. The findings suggesting that HR is important post IR in stem cells are good and I do not argue against that (although I am not convinced you have shown that HR repairs the DSBS). However, without some control showing

knockdown has been efficient, it is not valid to conclude that NHEJ is not repairing DSBs. It is bad practice to have no experimental evidence that knockdown has been effective (the fact that NHEJ suppression enhances stem cells is good but this does not negate the need for some control experiments).
17. 363 While its function in regulating checkpoint progression and apoptosis is shared with other systems, its role in promoting downstream DNA repair seems to have diverged - this has not been shown. Indeed, DSB repair has not been examined. And ATM anyway has a very minor role in promoting DSB repair in other cells.

Referee #3:

In this revised manuscript the authors have satisfactorily addressed the concerns raised by the reviewers.

Shiroor et al., Point-by-Point response to Reviewers
Manuscript #56112

Referee #2:

This revision is improved and, as previously, the major take home conclusion is valid and interesting. However, I think it is obvious from the reviewers comments (and the massive number of major statements removed during revision), the authors are lax in deriving unwarranted conclusions and in in over-interpreting aspects. This has continued in this revision. Mostly, these issues can be addressed (and do not affect the take home conclusion) by changes to the text. But I do urge the authors to consider whether their discussion points are valid. Before processing to individual points. I make two general comments:
1. The general conclusion is that cells with a DSB replicate and then use HR to repair the DSB. However, replication past a DSB is a major challenge for a cell. HR clearly plays a major role during replication and is required for stem cell expansion. I am not convinced they have shown it is the major driver of DSB repair (they do not actually look at DSB repair at all). As said above, replication past a DSB is challenging - hence the general consensus that NHEJ functions prior to replication and HR in replicated cells (though NHEJ is used there as well). I am not aware of much evidence that cells replicate past a DSB and then use HR - yet that is what is proposed (see eg line 331 - how can they generate a template if the DSB is there during replication?). Please consider carefully how strong you argue that the DSBs are repaired by HR.
(note it is likely that because stem cells are replicating, they will have more late S - G2 cells so HR will have a bigger impact (which is true in ESCs also) - but this is distinct to what is being said here).

We agree with the reviewer that replication past a DSB is a challenge for cells – this is precisely why we think our manuscript will be of interest to the scientific community! We did, however, tone down our language throughout by adding clauses (e.g. remove the word “strong” in line 351, adding “likely” to line 86 and line 225) to soften our conclusions as suggested.

2. The authors admit that their siRNA procedure has limiitations -but still make strong conclusions based on these limitations. See my point about NHEJ proteins below (residual NHEJ function can go a long way). Similarly, in their response letter, they argue that ATR is not required for normal replication. This is not impossible (I have read the ATM replaces ATR in Drosophila) but still highly unlikely. Most likely, there is insufficient knockdown to cause a phenotype.

We added a clause (lines 223-225) to explicitly state the basis of our conclusion and the possibility of insufficient knockdown.

3. I am concerned about the conclusion that cells replicate past a DSB to create a template to use HR. BIR can do that but they have not shown that. They have not shown that HR repairs the DSB - only that it is required for stem cell propagation.

The reviewer is correct that we do not directly evaluate the extent of repair of the DSB. However, it is a fact that the entire animal has been uniformly exposed to radiation, and yet, cell division resumes. The two possibilities are that 1) cell division can progress despite DSBs remaining repaired, or 2) a selection occurs ~ day 7 that removes severely damaged cells and more mildly damaged cells eventually resume proliferating. We describe these possibilities in the discussion (lines 303-320, 333-343).

4. Personally, I think the authors should cut down the discussion and focus on the points they show - abrogation of apoptosis in worms allows the worms to survive and HR is required for this.

We respectfully disagree with the reviewer on this point. In the revision, we extended the discussion to help explain some of the points raised by all 3 reviewers and are quite happy with its current state.

Individual points.

5. Abstract: Line 23 - To mitigate potentially deleterious outcomes, cells activate the DDR - which dictates whether cells repair DNA or undergo apoptosis - this sounds like these are alternatives - they are not. Repair can occur even if cells finally undergo apoptosis - and (as written in the response letter), differentiated planarian cells do not seem to die by apoptosis. ATM deficiency leads to only a subtle DSB repair defect. Better to say something like - the DDR network functions to regulate several process affecting cell survival, including apoptosis.

We modified this sentence in the abstract (lines 24-25).

6. Last sentence - ATM is a key regulator of radiation-induced apoptosis. This is not novel - (eg lymphocytes in humans). What is novel is that radiation-induced apoptosis is the major regulator of organismal survival in worms.
(NB in fact in humans, the haematopoeitic system (where apoptosis can arise) is a major sensitive organ and (to some extent) determines organismal survival. However, the difference here is that loss of ATM does not increase organismal survival - probably due to the fact that other stem cells compartments behave differently in humans).

We have modified the last sentence of the abstract to highlight the novelty that the reviewer suggested (line 31-32).

7. My final point in the abstract is that I am not sure its valid to say "without adverse effects". The animal survive indeed as shown in Figure 3 but not all parameters are examined (eg life span or dysfunctional cells in tissues). Perhaps one could say ...without strong or evident adverse effects. Inherently, I believe there must be adverse effects otherwise why activate apoptosis.

We toned down this phrase (line 34).

8. Introduction
Lines 44 and 55 - again cell death or repair are not alternatives. I understand the point being made but the entire paragraph is not correct - it is not a binary decision - repair or apoptosis.

We are not necessarily presenting this as a binary decision, but more of a conceptual framework for understanding the outcome of radiation. We disagree that the entire paragraph is incorrect.

9. Line 63 - ATM-/- thymocytes and post mitotic neurons are resistant to IR induced apoptosis. Even ATM+ post mitotic neurons do not undergo apoptosis - so although ATM-/- post mitotic

neurons are resistant to apoptosis it is not relevant. Thymocyte aspect is OK - and true in the replicating neurons (progenitor cells) and the intestine.

It is not clear what change the reviewer would like us to make here.

10. 73 - get rid of Rads throughout - its an outdated unit of radiation and not used currently..

We made this change in the text and in all of the figures.

11. 83 - you don't show whether it's a choice - you just show that they do not undergo apoptosis (and they require HR for proliferation). Note that the experiment to say that NHEJ does not arise is unreliable since you do not show that you have achieved knock-down of NHEJ proteins (no controls at all) (see below). Lines 85-86 are good and OK

We are not sure what the reviewer is suggesting here – line 83 does not have any suggestion of a choice. NHEJ is never mentioned in this paragraph.

10. 87. - Do they not trigger apoptosis and then undergo HR - or undergo HR and hence do not die by apoptosis - your phrase says the former. It may well be a mix of both depending on where they are at in the cell cycle - indeed if they proceed into S phase, then they will repair by HR. But they need to replicate past a DSB.

Once the stem cells stop expressing the stem cell marker *piwi-1*, they disappear, and are therefore unable to undergo HR. We and several other labs have shown this with the available metrics. The biology here is black and white – not a mix of both as the reviewer suggests. However, we tried to clarify the writing of this sentence.

11. 89. As per abstract, it is well known that ATM regulates apoptosis after IR - the novel aspect here is that it is apoptosis that causes death of the worms.

We appreciate this point, and modified the abstract accordingly.

12, 94. Just a point of clarification. The definition of stem cells is potentially confusing here - in humans and mice, it is the progenitor stem cells that normally die by apoptosis (see eg. Your reference Barazzuol et al, 2017). Your "stem cells" are effectively replicating stem cells (ie progenitors). So in worms, the difference seem to be that most "stem cells" are actually progenitor cells - ie dividing with few quiescent stem cells. I think this could be stated more clearly in the introduction..

In the revised version, we have included substantial text (lines 341-353) describing the fact that planarian stem cells are not quiescent to appease this reviewer.

13. 127. The analysis of differentiated cells (Fig EV3) is good. Was apoptosis examined here - I think it is critical to the conclusions to show that the differentiated cells do not undergo apoptosis.

The presence of these retained markers is sufficient to support the conclusion that these cell types are more resistant to apoptosis than the stem cells over the time courses that we analyze here. It is well established that these differentiated cells persist for a long time after radiation.

14. 156 - the transient progression into S phase after ATM knockdown is reflective of G1/S arrest in human cells - Radioresistant DNA synthesis if reflective of progression through S phase (ie not stopping replication)

It is unclear what the reviewer is suggesting here.

15. 161. The sentence as written here is likely to cause confusion since it is well known that ATM does regulate a G2/M checkpoint. What is known in human cells, is that ATM activates a rapid G2/M checkpoint due to the presence of un-resected DSBs. Then as they get resected, ATR becomes important. I suspect it's the same in worms. Perhaps the best is to add a sentence saying that in human cells also, after IR ATM activates immediate G2/M arrest but is dispensable for longer-term G2/M arrest, which is ATR-dependent (this is work by Mike Kaston PMID: 11809797).

We have added a sentence as suggested (lines 160-162) and had already cited that paper in this paragraph.

16. 212. As stated above I worry about the conclusions of the experiments involving NHEJ proteins. In human cells, NHEJ proteins are highly abundant (which may not be the case in planaria) and notoriously difficult to knock down. In the response to reviewer 3, it was pointed out that there were not controls to verify the efficacy of knockdown (except for ATM where there was functional examination). There is nothing here to show that knockdown of these proteins has been effective. Just some residual NHEJ proteins can go a long way. The findings suggesting that HR is important post IR in stem cells are good and I do not argue against that (although I am not convinced you have shown that HR repairs the DSBS). However, without some control showing knockdown has been efficient, it is not valid to conclude that NHEJ is not repairing DSBs. It is bad practice to have no experimental evidence that knockdown has been effective (the fact that NHEJ suppression enhances stem cells is good but this does not negate the need for some control experiments).

We agree with the reviewer on the points raised here and as mentioned above now include a sentence describing our thought process (lines 220-222).

17. 363 While its function in regulating checkpoint progression and apoptosis is shared with other systems, its role in promoting downstream DNA repair seems to have diverged - this has not been shown. Indeed, DSB repair has not been examined. And ATM anyway has a very minor role in promoting DSB repair in other cells.

We changed this sentence (now line 367-368) to read “its role in promoting stem cell exhaustion seems to have diverged.”

Dr. Carolyn Adler
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930 Campus Road, VMC C3-177
Department of Molecular Medicine
Ithaca, NY 14853
United States

Dear Dr. Adler,

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

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Yours sincerely,

Esther Schnapp, PhD
Senior Editor
EMBO reports

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Reporting Checklist for Life Science Articles (updated January 2022)
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 manuscript.
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 replicates.
- 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 Presentation.

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

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	Reagents and Tools Table

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	Materials and Methods, Appendix Table 4

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.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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	Materials 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	Materials 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	Acknowledgements

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.	Not Applicable	

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	Sample sizes are noted in figure captions and in the Materials and Methods section where appropriate.
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?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods, section on Image Quantification
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	Every figure caption mentions the statistical test used.

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	noted in Figure Legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	noted in 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 approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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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	
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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	
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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?	Yes	Data materials availability
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	