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RADX interacts with single-stranded DNA to promote replication fork stability

Lisa Schubert, Teresa Ho, Saskia Hoffmann, Peter Haahr, Claire Guérillon, and Niels Mailand

Corresponding author: Niels Mailand, NNF Center for Protein Research, University of Copenhagen

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Transaction Report:

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

1st Editorial Decision 09 August 2017

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, all three referees highlight the potential interest of the findings. However, all three referees have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn. As the reports are below, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a point-by-point response. Given the time constraints due to the recent publication of a related manuscript, as already indicated in a previous letter to you, we can only offer publication if the revision can be done in a timely manner. We would therefore require that you re-submit your revised manuscript not later than Friday 08.09.2017.

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

REFEREE REPORTS

Referee #1:

In this manuscript, Lisa Schubert and colleagues reported the identification of a novel ssDNA-binding factor, named RADX, that is important to protect against replication fork degradation and

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collapse limiting loading of RPA and excessive RAD51-mediated DNA transactions at fork.

By itself, the study is well-conducted and stands on the track of the several papers describing mechanisms involved in replication fork protection; a hot topic with highly relevant implications for basic biology and for cancer.

The study greatly parallels that recently appeared in Mol. Cell, however, it also contains some novel pieces of science. For instance, it reported the analysis of an OB mutant of RADX and studied in little more detail the effect of OB-fold of RADX on DNA replication, and after DNA damage. In addition, it explored the effect of overexpression and that of RPA-RADX balance.

Clearly, the authors need to acknowledge the publication in Mol. Cell and should consider improving novelty of their work by adding a few experiments to strengthen the section that more differs from the Mol. Cell paper. In particular, the story about competition between RPA and RADX on cellular phenotypes may be interesting to further investigate.

Although I understand that authors wanted to rapidly submit their work after having seen that published in Mol. Cell, and I am sympathetic with authors for that, I would suggest an accurate proofreading of the manuscript that contains several errors and sloppiness in the description of the experiments and to check for consistencies between main text, legends and materials and methods.

Experiments generally support the conclusions, however, I have some comments and suggestions, which are reported below:

1. The behaviour of the OB mutant (*OB) is clear when assessed in vitro, but less clear-cut when assessed in vivo. In particular, it is hard to me to judge if mutation of the OB domain affects DNA binding in the cell. Fig 4F seems to show less PLA spots in cells expressing the *OB mutant. Perhaps, a graph with a count of the number of PLA spot/cell would be helpful. In addition, statistical analysis should be included. Similarly, Fig 4H shows that RADX*OB is found more in the soluble fraction than the wild-type but the amount of the protein in each fraction has not been normalized and quantified.

2. Fig. 2B: It seems to me that RADX is more in chromatin at 8 and 24h of HU, however, it is hard to assess if RADX gets accumulated without any quantification.

3. Fig. 2C: RADX-GFP colocalises with gamma-H2AX in a subset of cells is a little bit vague. Do the authors think that RADX may be also involved during end-resection at DSBs? Indeed, this should be somehow explored as it would represent a novel finding.

4. Fig. 2E: I don't see the added value of this experiment.

5. First of all, are PLA experiments performed to detect interaction between RADX and RPA? I think that the authors would analyse association of RADX and ssDNA (native BrdU assay) instead. Fig. 2F: In the panel authors show pictures from untreated or HU-treated cells, however the quantification includes data from CPT-treated cells (Fig. 2G). The figure legend also does not help as it states that quantification has been performed on data from Fig. 2F. Why did the authors decide to score as positive cells with more than 3 PLA spots when they scored as positive cells with more than 5 PLA spots in Fig. 1? In addition, I still advice authors to report the number of PLA spots/cell and to show wide-filed images. Indeed, quantification of the number of PLA-positive nuclei would be at odd with biochemical assays showing a reduced ability of RADX*OB to associate with chromatin. It is important to clearly define the behaviour of the RADX*OB mutant in vivo as the subsequent replication assays show a phenotype. I tried to understand how the EdU pulse was performed. It should be clearly reported, as it makes no sense what reported generically in materials and methods, where EdU is reported to be added 30min before fixing. Clearly, this would imply that on HU or CPT treatment no incorporation should be detected.

6. In the text, I would not say that PLA experiments have been performed to "further" analyse involvement of RADX in response to replication stress as the sentence is after the experiment with IR, which probably induces DNA damage rather than replication stress.

7. Fig. 4: Why the authors did not analyse replication fork recovery of different OB mutants/overexpression after HU, as the phenotype showed by RADX KD cells is so strong?

8. Fig. 4: WBs showing expression of RPA, RAD51 RADX should be included to assess their amount and to evaluate the ratio between RADX and RPA levels in the different conditions.

9. Fig. 5A, B: In the legend, it is reported a 4h HU treatment while in the scheme it is reported 3h: please clarify.

10. Fig. 5C: Is it the difference between wild-type and RADX*OB statistically significant? There is no statistical analysis of the replication catastrophe data, which should be better explained. (BTW: why is panel D is not labeled instead C, and vice versa?)

11. Fig. 5E: Quantification of "replication catastrophe" does not contain the *OB mutant, why? In general, data from figure 5 could be improved by reporting the effect of different overexpression/depletion as reported in Fig. 4.

12. Fig. 5G, H: Why authors did not analyse sensitivity of cells expressing the OB mutants of RADX, and the effect of RPA overexpression and so on in these mutants?

Referee #2:

This is an interesting manuscript describing a novel ssDNA binding protein called RADX. Overall the experiments describing this protein are well designed and informative. While it appears that the protein does not greatly affect the DNA damage response, despite localizing to sites of DNA damage, it seems to have very clear effects on replication fork progression. Unfortunately, the data presented do not give a clear insight into the role it might play.

While this manuscript was in preparation, a paper from the Cortez lab reported similar studies on RADX. While many of the results appear similar to those described in this manuscript, there appear to be significant differences, particularly with respect to interactions with RAD51. These should be spelled out more clearly.

<u>Specific comments:</u>

Fig. 1F The PLA foci are very difficult to visualize, at least on my screen. If possible clearer images of these foci should be presented.

Fig. 2C The authors state that RADX co-localizes with γH2AX in micro-laser tracts (pg. 8, line 8). However no co-localization of RADX with γH2AX is presented in Fig 2C.

Fig. 3 RADX siRNAs strongly affect replication fork rate in the absence of DNA replication stress, however overall cell cycle distribution is not greatly affected in RADX knockout strains. Do these knockouts show similar effects on DNA replication fork dynamics?

Fig. 5F&G RADX siRNAs weakly affect cellular sensitvity to DNA replication inhibitors. What were the effects of the inhibitors on RADX knockouts? It would be interesting to see more data about the effects of loss of RADX function on overall cellular viability and growth. The authors provide no such data for the RADX knockouts. It is also noteworthy that many tumour cell lines lack detectable RADX and the experiments reported here were done on tumour cell lines having high levels of this protein. Do tumour cell lines having little or no RADX show similar properties? Such data might enhance the reader's understanding of this interesting novel protein.

Referee #3:

In this manuscript, Schubert et al describe the molecular characterization of the RADX (CXorf57) protein and its implication in replication fork progression under normal conditions, or upon

treatment with the DNA damaging agent camptothecin (CPT, a Topoisomerase I inhibitor) or the ribonucleotide reductase inhibitor hydroxyurea (HU). The authors report very similar observation to those of Dungrawala et al., Mol Cell (2017) 67, 1-13. From the technical point of view the work is well executed although it requires a revision as listed below:

1. Introduction and/or discussion, the authors should refer to the other ssDNA binding proteins that have been involved in genome maintenance, such as hssB1 for instance and other RPA interacting proteins. They should also mention published evidence reporting non-canonical pathways leading to checkpoint signaling at stalled forks, including those observed under conditions of limited RPA hyperloading, as they also report in this manuscript. They must also show the mass spectrometry data that allowed them to identify RADX as binding to DNA lesions.

2.Figure 1 and 2F, the PLA foci are barely visible on the provided images. Based on these images it is very difficult to interpret the result.

3. Figure 2B, the binding of RADX to chromatin in the absence of HU is very weak. In order to draw any conclusions the signals need to be carefully quantified. Also, in this experimental set up, an increase of RADX chromatin binding can be observed upon treatment with ATR inhibitor (although the presence of ATR inhibitor does not necessarily strongly increases the binding or RPA to chromatin which I was expected to be more pronounced). The more straightforward conclusion of this experiment to me is that ATR activity inhibits RADX chromatin binding (maybe directly?) while the author's interpretation is different. Curiously, ATR inhibition does not inhibit the phosphorylation of RPA as one may expect. Indeed, a control showing that ATR inhibition was effective is missing (e.g; Chk1 phosphorylation). Direct detection of ssDNA by BrdU detection upon direct labeling of the cells may have provided a better picture and interpretation of this experiment and maybe sustain the authors' claim that "ATR inhibition dramatically increased ssDNA formation" (they only refer to a previous observation described in ref 17).

4.Figure S1C the authors suggest that the punctuate staining observed upon expression of GFPRADX likely represents aggregates, with no supporting evidence. By the way, the delta OB mutant shows pan nuclear staining, suggesting that the punctuate pattern may indeed be specific. Did the authors try to colocalize these foci with replication foci (BrdU or PCNA or RPA) in unstressed cells?

5. Figure S1D, the loading control of this panel is not appropriate and should be actin or tubulin, because expression of RPA and/or MCM2-7 may vary in the indicated cell lines. The authors show that RADX is expressed at very low levels (or not expressed at all) in HeLa cells. Did they analyze mRNA expression of RADX? So, compared to U20S cells, HeLa cells should be more defective in replication fork progression, did the authors checked this? Did the authors analyze the effect of ectopic expression of RADX in HeLa cells?

6. Figure S2B, it would have been interesting and appropriate to check for Pchk2/Chk2 and/or pATM/ATM as signs of double strand breaks formation in this panel.

7. Figure 2E, the authors claim that " GFP-RADX localized to micro-foci corresponding to ssDNA regions embedded within the γ-H2AX" and that "RADX and RPA might occupy adjacent ssDNA areas near the lesions" using laser irradiation of cells. This is an overstatement. To me these can only be considered as double strand breaks, unless again the authors can colocalize RADX with ssDNA under these experimental conditions.

8. Figure 3, the phenotype observed in HCT116 cells upon RADX downregulation by siRNA are not identical to those observed upon RADX deletion by CRISPR/CAS9. This discrepancy cannot apparently be explained by residual amount of RADX observed upon siRNA treatment judged from the blot shown in panel A. Authors have not rescued these phenotypes with wild-type RADX in these cells, but they have done it in U20S cells (Figure 4) although only in siRNA-treated cells.

9. Figure 4, I could not find western blot showing the expression level of cells expressing ectopic version of RPA (super RPA), nor those showing RPA and Rad51 downregulation by siRNA, neither of cells expressing the ectopic versions of RADX (wild-type, and mutants for HCT116). These results are mandatory. This figure is also incomplete as far as the significance tests are concerned in

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several panels. Panels F-H, lanes 3 and 4, are these duplicate or they are supposed to be cells expressing wt (lane3) or RADX siRNA (lane 4) ? This is confusing and as it is the interpretation of the result is difficult.

10. Significance tests must be run for the HU sensitivity experiment shown in Figure 5F-G; S2C-F.

11. Figure S3B, why the x and y axis are expressed in arbitrary units? What do they correspond to? These panels must be quantified. It does not seem to me that the there is a significant difference between wild-type and *OB mutant. I do not see the need to use this assay to assess for gH2AX and RPA binding. A more straightforward chromatin-binding assay would have been more appropriate and easier to interpret.

1st Revision: Authors' response 08 September 2017

Thank you again for giving us the opportunity to revise our manuscript entitled '**RADX interacts with single-stranded DNA to promote replication fork stability**', originally submitted for consideration as a ***Scientific Report*** in ***EMBO Reports***.

We were delighted to see the positive and encouraging reception of our study by the referees, and we found their insightful comments and suggestions to be both helpful and constructive. Prompted by their feedback, we have performed a range of new experiments, which together with appropriate amendments of the text and figures enabled us to address virtually all of the reviewers' concerns.

In the accompanying rebuttal letter, we provide a detailed point-by-point response to each of the issues raised by the referees and explain how the salient new additions to the manuscript strengthen and extend our original conclusion that RADX is a new ssDNAbinding protein with an important role in promoting replication fork stability during replication stress.

We hope that with all of the new additions you will now find our manuscript suitable for publication in ***EMBO Reports***. We look forward to hearing the referees' and your opinion.

[Point-by-point response included in the following pages.]

Point-by-point reply to the reviewers' comments

We would like to thank all the referees for the constructive comments and suggestions they made on our study. In the revised version of our manuscript, we have included the results of a range of new experiments performed on the basis of the referees' helpful comments. We also included additional significance tests for a number of experiments and clarified ambiguities in the text. Together, we believe these revisions address essentially all of the reviewers' concerns.

Below, we provide a detailed point-by-point response to each of the issues raised by the reviewers, explaining how the new additions to the manuscript strengthen and extend our original conclusion that RADX is a new ssDNA-binding protein with an important role in promoting replication fork stability during replication stress.

Referee #1:

In this manuscript, Lisa Schubert and colleagues reported the identification of a novel ssDNA-binding factor, named RADX, that is important to protect against replication fork degradation and collapse limiting loading of RPA and excessive RAD51-mediated DNA transactions at fork.

By itself, the study is well-conducted and stands on the track of the several papers describing mechanisms involved in replication fork protection; a hot topic with highly relevant implications for basic biology and for cancer.

The study greatly parallels that recently appeared in Mol. Cell., however, it also contains some novel pieces of science. For instance, it reported the analysis of an OB mutant of RADX and studied in little more detail the effect of OB-fold of RADX on DNA replication, and after DNA damage. In addition, it explored the effect of overexpression and that of RPA-RADX balance.

Clearly, the authors need to acknowledge the publication in Mol. Cell and should consider improving novelty of their work by adding a few experiments to strengthen the section that more differs from the Mol. Cell paper. In particular, the story about competition between RPA and RADX on cellular phenotypes may be interesting to further investigate.

We thank the referee for the positive and constructive comments and suggestions. We fully agree with the need to mention and acknowledge the recent RADX study by David Cortez and colleagues and its consistency with our work, while at the same time highlighting the novelty aspects of our study. To this end, we added the following sentence to the revised manuscript: *"Similar findings were recently reported by Cortez and colleagues, who demonstrated a role of RADX in antagonizing RAD51 accumulation at replication forks to promote genome integrity [30]. Our study is consistent with this work and additionally suggests that the interplay between RADX and RPA is also important for fork stability."* (page 13). We extended the insights into the interplay between RADX and RPA by showing that an increased abundance of RPA can functionally compensate for the loss of RADX in promoting replication fork stability and survival upon persistent replication stress (new Fig. 5E,H) (please

see our response to point 12 below for details). We also highlighted the observation that RADX expression varies dramatically among different cell lines (and that as a consequence its depletion differentially impacts fork stability in different cell lines), a notion that was not described in the study by Cortez and colleagues, as follows: *"The highly variable RADX expression pattern among different cancer cell lines raises the possibility that alteration of RADX abundance could be a mechanism to mitigate the harmful consequences of chronic replication stress."* (page 13-14).

Although I understand that authors wanted to rapidly submit their work after having seen that published in Mol. Cell, and I am symphatetic with authors for that, I would suggest an accurate proofreading of the manuscript that contains several errors and sloppiness in the description of the experiments and to check for consistencies between main text, legends and materials and methods.

We apologize for any unintentional errors and have carefully amended the manuscript text to ensure full consistency between all sections.

Experiments generally support the conclusions, however, I have some comments and suggestions, which are reported below:

1. The behaviour of the OB mutant (*OB) is clear when assessed in vitro, but less clear-cut when assessed in vivo. In particular, it is hard to me to judge if mutation of the OB domain affects DNA binding in the cell. Fig 4F seems to show less PLA spots in cells expressing the *OB mutant. Perhaps, a graph with a count of the number of PLA spot/cell would be helpful. In addition, statistical analysis should be included. Similarly, Fig 4H shows that RADX*OB is found more in the soluble fraction than the wild-type but the amount of the protein in each fraction has not been normalized and quantified;

We assume the referee is referring to Fig. 1. To clarify this point, as suggested by the reviewer, we included graphs showing average number of PLA foci/cell for both the PLA experiments shown in Fig. 1G and Fig. 2E (new Fig. EV1G and EV2D). We also quantified the ratio between the chromatin-bound and soluble pools of each of the GFP-RADX alleles (data included in Fig. 1I), demonstrating more clearly that the chromatin association of the RADX *OB mutant is reduced compared to RADX WT.

2. Fig. 2B: It seems to me that RADX is more in chromatin at 8 and 24h of HU, however, it is hard to assess if RADX gets accumulated without any quantification;

We clarified this by quantifying the amount of chromatin-bound RADX at each time point relative to the mock control. As can be seen from Fig. 2B, in the absence of ATR inhibition RADX chromatin accumulation is mainly observed upon a 24-h exposure to HU, where extensive replication fork breakage (as determined by H2AX and RPA2 hyperphosphorylation) is apparent. This observation is consistent with RADX being important for mitigating HU-induced replication catastrophe (Fig. 5D).

3. Fig. 2C: RADX-GFP colocalises with gamma-H2AX in a subset of cells is a little bit vague. Do the authors think that RADX may be also involved during end-resection at DSBs? Indeed, this should be somehow explored as it would represent a novel finding;

Whilst we do not wish to rule out that RADX could have a role during end-resection of DSBs, our current observations argue against a key function of RADX in this process: First, RADX depletion does not markedly affect DSB-induced RPA S4/S8 phosphorylation, a marker of DSB end-resection (Fig. EV3B). Second, we found that unlike RPA, RADX accumulation at DSB sites is independent of resection, as knockdown of the key upstream resection factor CtIP does not affect RADX recruitment to DSBs (see Fig. R1 below).

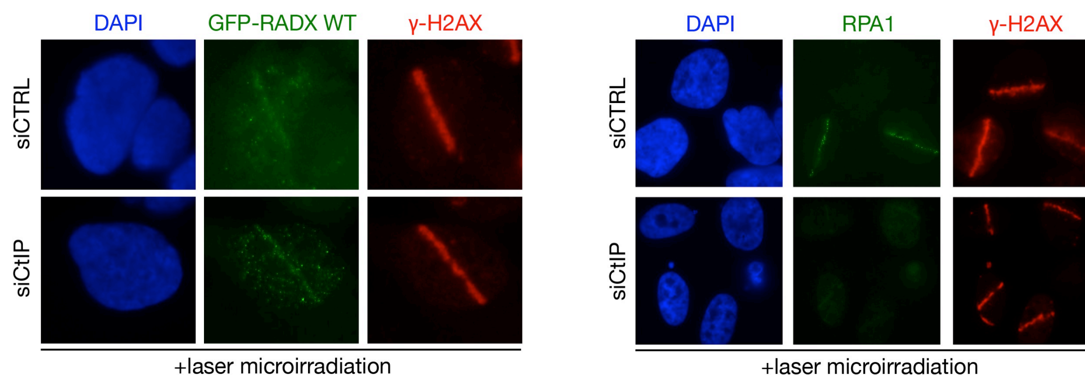

Fig. R1. Inhibition of DSB end-resection by siRNA-mediated knockdown of CtIP impairs accumulation of RPA (right panels) but not RADX (left panels) at DSB sites.

4. Fig. 2E: I don't see the added value of this experiment;

We removed this data from the revised manuscript.

5. First of all, are PLA experiments performed to detect interaction between RADX and RPA? I think that the authors would analyse association of RADX and ssDNA (native BrdU assay) instead. Fig. 2F: In the panel authors show pictures from untreated or HU-treated cells, however the quantification includes data from CPT-treated cells (Fig. 2G). The figure legend also does not help as it states that quantification has been performed on data from Fig. 2F. Why did the authors decide to score as positive cells with more than 3 PLA spots when they scored as positive cells with more than 5 PLA spots in Fig. 1? In addition, I still advice authors to report the number of PLA spots/cell and to show wide-filed images. Indeed, quantification of the number of PLA-positive nuclei would be at odd with biochemical assays showing a reduced ability of RADX*OB to associate with chromatin. It is important to clearly define the behaviour of the RADX*OB mutant in vivo as the subsequent replication assays show a phenotype;
I tried to understand how the EdU pulse was performed. It should be clearly reported as it makes no sense what reported generically in materials and methods, where EdU is reported to be added 30min before fixing. Clearly, this would imply that on HU or CPT treatment no incorporation should be detected;

We used the proximity between GFP-RADX and RPA as a read-out for RADX recruitment to HU- or CPT-induced replication stress sites, which are characterized by the presence of extended RPA-coated ssDNA tracts. We tried to explain this more

clearly in the text, which now reads: *"To probe for recruitment of RADX to ssDNA regions upon replication stress, we used PLAs to assay for GFP-RADX proximity to RPA, which accumulates strongly at these sites."* (page 8-9). For better consistency between the PLA experiments in Fig. 1 and Fig. 2, we now display the quantification of both experiments as the proportion of cells having 3 or more PLA foci (Fig. 1H; Fig. 2F). In Fig. 2E, we included representative images of PLA signals between GFP-RADX and RPA in CPT-treated cells to align this panel better with the quantification of data shown in Fig. 2F. As for Fig. 1G, we also included a graph depicting the average number of PLA spots/cell for the GFP-RADX/RPA PLA experiments (new Fig. EV2D), as well as new and improved images of the PLA foci for all conditions, acquired by confocal microscopy (representative new images shown in Fig. 1G and Fig. 2E). In these experiments, cells were pulse-labeled with EdU prior to treatment with HU or CPT (and hence before replication fork stalling). We apologize for the confusion and have clarified the corresponding figure legend accordingly, so that it now reads: *"Representative images from PLAs with GFP and RPA2 antibodies in U2OS and U2OS/GFP-RADX cell lines labeled with EdU for 30 min and then fixed or exposed to HU or CPT for 4 h before fixation."* (page 26).

While specific point mutations within the OB2 fold (*OB) markedly compromise basic RADX ssDNA binding both *in vitro* and *in vivo* (Fig. 1C,I), this might translate into a more complex pattern of interaction with sites of replication stress, judging from our observation from PLA assays that stably expressed GFP-RADX *OB shows enhanced association with these areas compared to GFP-RADX WT (Fig. 2E,F). Clearly, the reduced ssDNA-binding affinity of the RADX *OB mutant correlates with an impaired ability to support DNA replication integrity in cells (e.g. Fig. 4A-D), and it is possible that such compromised functionality could alter the dynamics of RADX-ssDNA interactions in cells, e.g. by increasing the relative retention time of RADX at ssDNA regions generated in response to replication stress. We now discuss this possibility in the revised manuscript (page 10).

6. In the text, I would not say that PLA experiments have been performed to "further" analyse involvement of RADX in response to replication stress as the sentence is after the experiment with IR, which probably induces DNA damage rather than replication stress;

We concur with this notion and have rephrased the sentence accordingly, so that it now reads: *"To probe for recruitment of RADX to ssDNA regions upon replication stress..."* (page 8-9).

7. Fig. 4: Why the authors did not analyse replication fork recovery of different OB mutants/overexpression after HU, as the phenotype showed by RADX KD cells is so strong?

As suggested, we analyzed replication fork recovery after HU treatment in cell lines stably overexpressing WT and mutant forms of RADX. We found that like RADX depletion, elevated levels of RADX led to a fork recovery defect, however this was seen upon both overexpression of RADX WT and RADX *OB, while the RADX OB mutant had a less pronounced effect (Fig. R2). It is possible that under overexpression conditions, the RADX *OB mutant may adversely affect fork recovery via its residual ssDNA-binding affinity. Additionally, RADX sequences outside the OB fold region

may contribute to interfering with proper replication fork recovery after replication stress. We feel that more detailed studies would be needed to fully understand these observations.

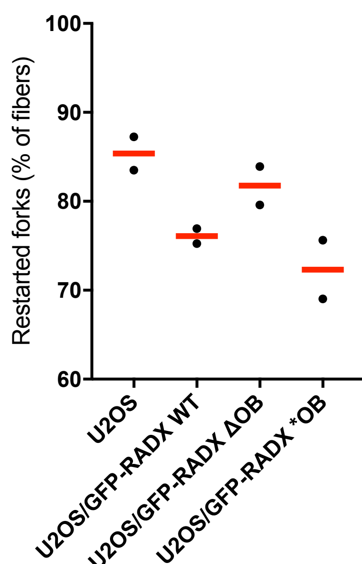

Fig. R2. Proportion of restarted forks (CldU-only tracks, assayed as shown in Fig. 3G) among DNA fibers from U2OS cells or derivative lines stably expressing WT or mutant forms of GFP-RADX (Fig. EV1E,F) (red bars, mean; 200 fibers analyzed per condition; *n*=2 independent experiments).

8. Fig. 4: WBs showing expression of RPA, RAD51 RADX should be included to assess their amount and to evaluate the ratio between RADX and RPA levels in the different conditions;

We apologize for omitting these control immunoblots, which have now been added to the revised manuscript (new Fig. EV4A,B).

9. Fig. 5A, B: In the legend, it is reported a 4h HU treatment while in the scheme it is reported 3h, please clarify;

We think the confusion arises because the reviewer is referring to two different types of analysis (replication fork protection and replication catastrophe assays, respectively), in which different HU exposure times were used: As shown in Fig. 5A, replication fork protection during HU-induced replication stress (Fig. 5B,C) was analyzed under conditions of a 3-h exposure to HU. In the replication catastrophe assays (Fig. 5D,E), cells were exposed to HU treatment for 4 h, as stated in the legend to Fig. 5D. We hope this clarifies the confusion.

10. Fig. 5C: Is it the difference between wild-type and RADX*OB statistically significant? There is no statistical analysis of the replication catastrophe data, which should be better explained. (BTW: why panel D is not labelled insteaed C, and vice versa?);

We included an additional significance test in Fig. 5C, showing that overexpression of RADX *OB significantly compromises replication fork protection, although clearly to

a much milder extent than WT RADX.

We have tried to explain the replication catastrophe data (Fig. 5D,E; Fig. EV5A,B) better, through the addition of more detailed descriptions of the experimental approach and read-out in both the text and the relevant figure legends.

As explained in the preceding point, the data shown in Fig. 5B,C and Fig. 5D,E reflect two distinct experimental set-ups (replication fork protection and replication catastrophe assays, respectively), and we therefore believe it may be confusing to swap panels C and D, as alluded to by the referee.

11. Fig. 5E: Quantification of "replication catastrophe" does not contain the *OB mutant, why? In general, data from figure 5 could be improved by reporting the effect of different overexpression/depletion as reported in Fig. 4;

We have found that for unknown reasons cells expressing the GFP-RADX ΔOB mutant display a high level of HU-induced replication catastrophe even in siCTRL-treated cells. We do not know the precise reason for this, but as we have observed a potential ability of RADX to dimerize via its non-OB fold portion, it is possible that the RADX ΔOB mutant, which unlike RADX WT and *OB does not interact with ssDNA (Fig. 1C), may functionally sequester endogenous RADX away from sites of replication stress, thereby potentially giving rise to the observed increase in replication catastrophe resembling the effect of RADX knockdown (this effect is also partially apparent for the RADX *OB mutant (new Fig. 5D)). Irrespective of the exact mechanism, we believe that the RADX *OB mutant, which contains only two amino acid substitutions in the full-length RADX context (Fig. 1A), is arguably more comparable to RADX WT than RADX ΔOB, and that the observed ability of RADX WT but not RADX *OB to rescue the replication catastrophe phenotype resulting from depletion of endogenous RADX (Fig. 5D) provides strong evidence that the ssDNA-binding ability of RADX via the OB fold region is important for preventing replication fork collapse and breakage.

As suggested by the referee, we performed additional experiments to test the impact of RPA overexpression and RADX depletion on replication catastrophe and cell survival, described in detail in our response to the following point (point 12) below.

12. Fig. 5G, H: Why authors did not analyse sensitivity of cells expressing the OB mutants of RADX, and the effect of RPA overexpression and so on in these mutants?

We have carefully attempted to perform clonogenic survival assays on cell lines stably expressing GFP-RADX constructs using a range of different experimental conditions. However, we invariably found that induction of GFP-RADX WT or mutant alleles strongly impairs long-term cell proliferation and colony formation even in the absence of exogenous stress, thereby precluding us from drawing meaningful conclusions as to the impact of RADX overexpression on clonogenic survival following replication stress.

Instead, as suggested by the referee, we extended the insights into the functional interplay between RADX and RPA by analyzing the impact of mild RPA

overexpression (using the "Super-RPA" cell line (Fig. EV4A)) on the sensitivity of cells to replication stress. Consistent with the previously reported ability of excess RPA complex to mitigate detrimental RPA exhaustion and replication catastrophe (Toledo et al., Cell 2013 (155: 1088-1103)), mild elevation of RPA expression significantly improved long-term survival after CPT treatment (new Fig. 5H). Strikingly, however, we also found that elevated RPA expression fully rescued the cell survival and replication catastrophe defects of RADX-depleted cells exposed to replication stress (new Fig. 5E,H). We can envision at least two non-mutually exclusive explanations for these observations: First, consistent with an antagonistic relationship between RADX and RPA in interacting with ssDNA regions, it is possible that the ssDNA-binding ability of RADX enhances RPA dynamics and mobility at replication forks to increase the effective pool of RPA available to bind and protect extended fork-associated ssDNA stretches generated upon replication stress, thereby rendering cells less prone to replication catastrophe. Second, through its ssDNA-binding affinity RADX may itself have a role in shielding unprotected ssDNA tracts from nuclease-mediated degradation, the loss of which can be functionally compensated for by an increased abundance of RPA. This notion is supported by the observation that RADX preferentially associates with chromatin upon chronic replication stress associated with extensive DNA breakage (Fig. 2B). In the revised manuscript, we now mention and discuss these possibilities (page 12-13).

Referee #2:

This is an interesting manuscript describing a novel ssDNA binding protein called RADX. Overall the experiments describing this protein are well designed and informative. While it appears that the protein does not greatly affect the DNA damage response, despite localizing to sites of DNA damage, it seems to have very clear effects on replication fork progression. Unfortunately the data presented do not give a clear insight into the the role it might play. While this manuscript was in preparation a paper from the Cortez lab reported similar studies on RADX. While many of the results appear similar to those described in this manuscript there appears to be significant differences, particularly with respect to interactions with RAD51. These should be spelled out more clearly.

We thank the referee for the constructive comments and suggestions. In the revised version of our manuscript, we acknowledged the recent RADX study by David Cortez and colleagues and its consistency with our work, while at the same time highlighting the novelty aspects of our study. To this end, we extended the insights into the interplay between RADX and RPA by showing that an increased abundance of RPA can functionally compensate for the loss of RADX in promoting replication fork stability and survival upon persistent replication stress (new Fig. 5E,H), and we added the following sentence to the revised manuscript: *"Similar findings were recently reported by Cortez and colleagues, who demonstrated a role of RADX in antagonizing RAD51 accumulation at replication forks to promote genome integrity [30]. Our study is consistent with this work and additionally suggests that the interplay between RADX and RPA is also important for fork stability."* (page 13). We also further highlighted the observation that RADX expression varies dramatically among different cell lines (Fig. 1F; Fig. EV1B) (and that as a consequence its

depletion differentially impacts fork integrity in different cell lines; see our response below for more detail), a notion that was not described in the study by Cortez and colleagues, as follows: *"The highly variable RADX expression levels among different cancer cell lines raises the possibility that alteration of RADX abundance could be a mechanism to mitigate the harmful consequences of chronic replication stress."* (page 13-14).

Specific comments:

Fig. 1F The PLA foci are very difficult to visualize, at least on my screen. If possible clearer images of these foci should be presented.

In the merged file of the original manuscript, it appears the resolution of the representative images showing PLA foci (Fig. 1F and Fig. 2F) was reduced considerably during conversion of individual figures into the merged and compressed PDF file, making appreciation of the foci difficult. To improve the visualization of the PLA foci, we acquired new and clearer images of cells from each condition in these experiments, using confocal microscopy (new images shown in Fig. 1G and Fig. 2E of the revised manuscript). We hope this improves the visualization of the foci and the interpretation of results.

Fig. 2C The authors state that RADX co-localizes with γH2AX in micro-laser tracts (pg. 8, line 8). However no co-localization of RADX with γH2AX is presented in Fig 2C.

We apologize for unintentionally omitting merged images of GFP-RADX and γH2AX signals in this experiment. These images have now been added to the revised manuscript (Fig. 2C).

Fig. 3 RADX siRNAs strongly affect replication fork rate in the absence of DNA replication stress, however overall cell cycle distribution is not greatly affected in RADX knockout strains. Do these knockouts show similar effects on DNA replication fork dynamics?

We believe the referee might have overlooked the data showing the effect of RADX knockout on replication fork dynamics, which are included in the plots showing the impact of RADX siRNAs (Fig. 3C-F). From these data, it can also be seen that RADX knockdown by siRNAs and CRISPR/Cas9-based RADX knockout affect replication fork dynamics in a highly similar manner (Fig. 3C-F). Likewise, we found that neither RADX knockdown or knockout has a marked impact on overall cell cycle distribution (Fig. EV2A). We consider it likely that the reason no pronounced effect of RADX loss on cell cycle distribution is observed despite the markedly reduced rate of fork elongation under these conditions (Fig. 3C) is that this defect is compensated for by a strong surge in new origin firing (Fig. 3F).

Fig. 5F&G RADX siRNAs weakly affect cellular sensitvity to DNA replication inhibitors. What were the effects of the inhibitors on RADX knockouts? It would be interesting to see more data about the effects of loss of RADX function on overall cellular viability and growth. The authors provide no such data for the RADX knockouts. It is also noteworthy that many tumour cell lines lack detectable RADX

and the experiments reported here were done on tumour cell lines having high levels of this protein. Do tumour cell lines having little or no RADX show similar properties? Such data might enhance the reader's understanding of this interesting novel protein.

We assayed clonogenic survival of RADX knockout cells exposed to replication stress-inducing agents (see Fig. R3 below for an example). As can be seen, while we observed a general trend that RADX knockout mildly sensitized cells to these drugs like RADX siRNAs, the results of four independent repeats were more variable than in the siRNA-based experiments and consequently did not reach statistical significance. In keeping with the notion that many cancer cell lines lack detectable RADX expression (Fig. 1F; Fig. EV1B), it is possible that there are ways for cells to adapt to a chronic absence of RADX, which could at least partially explain why long-term knockout of RADX, as opposed to its acute siRNA-mediated depletion, has an overall milder impact on cell survival following replication stress. In fact, we observed a clear trend that the sensitivity of RADX knockout cells to CPT and HU gradually diminished with increased passaging of these cells (our unpublished observations).

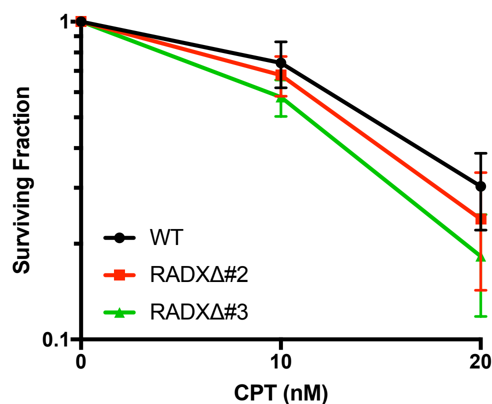

Fig. R3. Clonogenic survival of parental HCT116 cells (WT) and independent RADX knockout cell lines (RADXΔ) subjected to different doses of CPT for 24 h (mean±SEM; *n*=4 independent experiments).

We agree with the referee that the notion many tumour cell lines appear to express RADX at very low levels (or not at all) is intriguing. Prompted by the referees' suggestion, we carried out new experiments to assess the importance of RADX for replication fork stability in such cell lines. For these analyses, we used HeLa cells, in which RADX expression is virtually undetectable (Fig. 1F; Fig. EV1B). First, we found that *RADX* mRNA levels are extremely low in these cells (new Fig. EV1C), suggesting that the paucity of RADX protein primarily reflects a transcriptional effect. In line with its strongly reduced expression, we found that in contrast to HCT116 and U2OS cells, treatment with RADX siRNAs only mildly reduced replication fork elongation rates in HeLa cells (new Fig. EV3F). Thus, it appears that RADX is particularly important for replication fork stability in cell lines such as U2OS and HCT116 where it is expressed at high levels. It seems possible that at least in some cancer cell lines modulation of RADX expression could be a mechanism to

mitigate the harmful consequences of high levels of replication stress, a possibility we now mention in the revised manuscript (page 13-14).

Referee #3:

In this manuscript Schubert et al describe the molecular characterization of the RADX (CXorf57) protein and its implication in replication fork progression under normal conditions, or upon treatment with the DNA damaging agent camptothecin (CPT, a Topoisomerase I inhibitor) or the ribonucleotide reductase inhibitor hydroxyurea (HU). The authors report very similar observation to those of Dungrawala et al., Mol Cell (2017) 67, 1-13. From the technical point of view the work is well executed although it requires a revision as listed below:

1. Introduction and/or discussion, the authors should refer to the other ssDNA binding proteins that have been involved in genome maintenance, such as hssB1 for instance and other RPA interacting proteins. They should also mention published evidence reporting non-canonical pathways leading to checkpoint signaling at stalled forks, including those observed under conditions of limited RPA hyperloading, as they also report in this manuscript. They must also show the mass spectrometry data that allowed them to identify RADX as binding to DNA lesions.

In the Introduction, in addition to BRCA2 and RAD51, we now mention other important ssDNA-binding proteins such as hSSB1 that have been implicated in genome stability maintenance, by addition of the following sentence: *"These include SSB1/NABP2, which facilitates DNA double-strand break signaling and repair, and POT1 and TPP1, components of the telomere-protecting shelterin complex [18]."* (page 5). Due to the strict character limits for Scientific Reports in *EMBO Reports*, it unfortunately has not been feasible to add more elaborate descriptions of canonical and non-canonical mechanisms underlying checkpoint signaling at stalled replication forks.

The systems-wide proteomic screens in which we identified RADX as a potential new genome stability maintenance factor form part of an independent, ongoing study, and we are therefore not able to provide the mass spectrometry data at this point. However, as we had found in parallel through bioinformatic analyses that RADX harbors predicted OB folds, we have rephrased the statement on the identification of RADX in the revised manuscript, so that it now reads: "*In bioinformatic screens for prospective new genome stability maintenance factors, we noted that the uncharacterized human protein CXorf57 contains three potential N-terminal OB folds..."* (page 6).

2.Figure 1 and 2F, the PLA foci are barely visible on the provided images. Based on these images it is very difficult to interpret the result.

In the merged file of the original manuscript, it appears the resolution of the representative images showing PLA foci (Fig. 1F and Fig. 2F) was reduced considerably during conversion of individual figures into the merged and compressed PDF file, making appreciation of the foci difficult. To improve the visualization of the

PLA foci, we acquired new and better images of cells from each condition in these experiments, using confocal microscopy (new images now shown in Fig. 1G and Fig. 2E). We hope this improves the visualization of the foci and the interpretation of results.

3. Figure 2B, the binding of RADX to chromatin in the absence of HU is very weak. In order to draw any conclusions the signals need to be carefully quantified. Also, in this experimental set up, an increase of RADX chromatin binding can be observed upon treatment with ATR inhibitor (although the presence of ATR inhibitor does not necessarily strongly increases the binding or RPA to chromatin which I was expected to be more pronounced). The more straightforward conclusion of this experiment to me is that ATR activity inhibits RADX chromatin binding (maybe directly?) while the author's interpretation is different. Curiously, ATR inhibition does not inhibit the phosphorylation of RPA as one may expect. Indeed, a control showing that ATR inhibition was effective is missing (e.g; Chk1 phosphorylation). Direct detection of ssDNA by BrdU detection upon direct labeling of the cells may have provided a better picture and interpretation of this experiment and maybe sustain the authors' claim that "ATR inhibition dramatically increased ssDNA formation" (they only refer to a previous observation described in ref 17).

These are valid points. To more clearly show that RADX chromatin association is enhanced upon HU-induced ssDNA formation and replication stress, we quantified the RADX signal in the chromatin fraction at each time point relative to the mock condition (quantification now shown in Fig. 2B). While it may seem counterintuitive at first glance, the strong induction of RPA2 hyperphosphorylation seen in cells treated with both HU and ATR inhibitor (ATRi) is in fact expected and reflects its ATM-mediated (and ATR-independent) phosphorylation resulting from extensive DNA breakage under these conditions (as described in Toledo et al., Cell 2013 (155: 1088-1103); Haahr et al., Nature Cell Biol. 2016 (18:1196-1207)). We now explain this better in the text, and we have added a γ-H2AX blot to indicate the relative amount of DNA double-strand break formation at the different time points. In addition, as suggested by the reviewer, to show that ATR signaling is indeed efficiently inhibited in ATRi-treated cells, we included a control blot for Chk1 pS317, an ATR-specific phosphorylation site, from which it can be clearly appreciated that ATR activity is strongly suppressed under these conditions (new blots added to Fig. 2B). We consider it unlikely that ATR directly inhibits RADX chromatin loading, as ATR is highly active upon a 24-h exposure to HU (as judged from Chk1 S317 phosphorylation), yet RADX clearly accumulates at chromatin under these conditions (Fig. 2B). However, we agree with the reviewer that direct detection of ssDNA by native BrdU staining would more directly show that combined HU and ATRi treatment exacerbates ssDNA production. Accordingly, we included in the revised manuscript representative images visualizing the extent of ssDNA formation at each time point, supporting the notion that HU-induced ssDNA generation is accelerated in the presence of ATRi (new Fig. EV2A).

4. Figure S1C the authors suggest that the punctuate staining observed upon expression of GFPRADX likely represents aggregates, with no supporting evidence. By the way, the delta OB mutant shows pan nuclear staining, suggesting that the punctuate pattern may indeed be specific. Did the authors try to colocalize these foci with replication foci (BrdU or PCNA or RPA) in unstressed cells?

The reviewer is correct that the GFP-RADX foci seen in unperturbed cells are lost by deletion of the entire OB fold region. However, we found by immunostaining of unstressed cells that these foci show little colocalization with PCNA or RPA (representative images are now shown in new Fig. EV2B,C), suggesting that they most likely do not correspond to replication foci. The GFP-RADX foci in unperturbed cells also did not show pronounced co-localization with other nuclear structures, incl. transcription and pre-mRNA splicing sites, PML and Cajal bodies (data not shown), leading us to speculate that the RADX foci seen in unperturbed cells, the formation of which appears to directly correlate with the relative expression levels of RADX, might represent aggregates. However, as we do not have formal proof these structures indeed correspond to RADX aggregates, we have amended the manuscript text describing this observation, so that it now reads: *"Stably expressed GFP-RADX predominantly localized to the nucleus, displaying a pronounced puncta-like pattern in unstressed cells that required the OB region but did not co-localize with replication foci or a range of other nuclear compartments..."* (page 8).

5. Figure S1D, the loading control of this panel is not appropriate and should be actin or tubulin, because expression of RPA and/or MCM2-7 may vary in the indicated cell lines. The authors show that RADX is expressed at very low levels (or not expressed at all) in HeLa cells. Did they analyze mRNA expression of RADX? So, compared to U20S cells, HeLa cells should be more defective in replication fork progression, did the authors checked this? Did the authors analyze the effect of ectopic expression of RADX in HeLa cells?

We agree that expression of RPA and MCMs could vary between individual cell lines, and we therefore included Vinculin blots as a more suitable control for equal loading (new blots added to Fig. 1F and Fig. EV1B (previously Fig. 1D)). As requested by the referee, we analyzed *RADX* mRNA levels in HeLa cells, where RADX is virtually undetectable, and HCT116 and U2OS cells, in which RADX abundance is much higher. We found that *RADX* mRNA levels in these cells closely mirrored the expression levels of RADX protein (new Fig. EV1C; compare with Fig. 1F), suggesting that the difference in RADX expression among different cell lines is largely a transcriptional effect.

We also tested how RADX depletion affects replication fork speeds in HeLa cells. As might be expected from the very low levels of RADX expression in this cell line, we found that in contrast to HCT116 and U2OS cells, treatment with RADX siRNAs only mildly reduced replication fork elongation rates in HeLa cells (new Fig. EV3F). Average replication fork speeds were overall comparable in U2OS and HeLa cells (compare Fig. EV3C and Fig. EV3F), suggesting that the chronic downregulation of RADX in HeLa cells can likely be functionally compensated for by other mechanisms and/or differences in the genetic makeup of these cell lines, the nature of which will require more dedicated studies to understand. It seems possible that at least in some cancer cell lines modulation of RADX expression levels could be a mechanism to mitigate the harmful consequences of a high burden of replication stress, a possibility we now mention in the revised manuscript (page 13-14).

6. Figure S2B, it would have been interesting and appropriate to check for Pchk2/Chk2 and/or pATM/ATM as signs of double strand breaks formation in this

panel.

We have now included blots for pCHK2, CHK2, pATM and ATM to this experiment (new blots added to Fig. EV3B (previously Fig. S2B)).

7. Figure 2E, the authors claim that " GFP-RADX localized to micro-foci corresponding to ssDNA regions embedded within the γ-H2AX" and that "RADX and RPA might occupy adjacent ssDNA areas near the lesions" using laser irradiation of cells. This is an overstatement. To me these can only be considered as double strand breaks, unless again the authors can colocalize RADX with ssDNA under these experimental conditions.

We concur with this notion and have removed these statements from the revised manuscript, so that it now simply describes RADX recruitment to laser-induced DNA damage sites as follows: *"…WT RADX rapidly accumulated at sites of microlaser irradiation-inflicted DNA damage sites in a subset of cells, co-localizing with γ-H2AX and PCNA, while the ΔOB mutant showed no detectable recruitment (Fig 2C,D)."* (page 8).

8. Figure 3, the phenotype observed in HCT116 cells upon RADX downregulation by siRNA are not identical to those observed upon RADX deletion by CRISPR/CAS9. This discrepancy cannot apparently be explained by residual amount of RADX observed upon siRNA treatment judged from the blot shown in panel A. Authors have not rescued these phenotypes with wild-type RADX in these cells, but they have done it in U20S cells (Figure 4) although only in siRNA-treated cells.

We disagree with the referee on this point. We believe the impacts of RADX knockdown (by different siRNAs) and RADX knockout (in cell lines generated by CRISPR/Cas9, using independent sgRNAs) on replication fork dynamics are in fact highly similar, thus strongly supporting the notion it does not represent an off-target effect, although as pointed out by the referee RADX knockout consistently gives rise to a slightly more pronounced effect in these assays. We consider it likely that this may be due to very low levels of residual RADX expression in siRNA-treated cells, although this cannot be clearly appreciated from our RADX immunoblots, as long exposures of the blots give rise to a high background signal that might potentially obscure any remaining RADX signal.

Although we tried extensively, we have so far failed to generate HCT116 cell lines stably expressing WT and mutant forms of GFP-RADX in a homogenous fashion, hence precluding us from performing rescue experiments in a *RADX* knockout background. However, as we have demonstrated that siRNA-mediated RADX depletion has very similar impacts on replication fork dynamics in U2OS and HCT116 cells (Fig. 3 and Fig. EV3), which additionally recapitulate the effects of RADX knockout in HCT116 cells (Fig. 3), and we show that these defects can be fully corrected by stable complementation of U2OS cells with GFP-RADX WT but not the OB fold mutants (Fig. 4), we believe we have proven beyond reasonable doubt that the observed impact of RADX depletion on replication fork parameters do not represent off-target effects.

9. Figure 4, I could not find western blot showing the expression level of cells expressing ectopic version of RPA (super RPA), nor those showing RPA and Rad51 downregulation by siRNA, neither of cells expressing the ectopic versions of RADX (wild-type, and mutants for HCT116). These results are mandatory. This figure is also incomplete as far as the significance tests are concerned in several panels. Panels F-H, lanes 3 and 4, are these duplicate or they are supposed to be cells expressing wt (lane3) or RADX siRNA (lane 4) ? This is confusing and as it is the interpretation of the result is difficult.

We apologize for omitting control immunoblots showing RPA and RAD51 knockdown efficiency and expression of the ectopic RPA alleles in the Super-RPA cell line and in the pratental U2OS cells. These have now been added to the revised manuscript (new Fig. EV4A,B). Expression levels of the various GFP-RADX alleles in stable U2OS cell lines are shown in Fig. EV1E (we did not generate and use HCT116 cell lines stably expressing GFP-RADX alleles). Following discussions with the editor, we have removed statistical significance tests from all DNA fiber experiments (including those shown in Fig. 4), as these were repeated only twice (yet in all cases yielding highly similar results in independent repeats).

We apologize if the labeling of Fig. 4F-H, lanes 3 and 4 was not straightforward to interpret: As indicated in these panels, lane 3 represents HCT116 WT cells treated with RPA1 siRNAs, while lane 4 corresponds to HCT116 RADX-null cells treated with RPA1 siRNAs, therefore these are clearly different conditions. To avoid ambiguity, we amended the figure legend for Fig. 4F so that it now reads: *"Replication fork speeds in HCT116 cells with indicated genotypes…"* (page 28).

10. Significance tests must be run for the HU sensitivity experiment shown in Figure 5F-G; S2C-F.

We added significance tests for the clonogenic survival assays (Fig. 5F,G). As explained above, following the editor's instructions, we have removed the significance tests from all DNA fiber experiments.

11. Figure S3B, why the x and y axis are expressed in arbitrary units? What do they correspond to? These panels must be quantified. It does not seem to me that the there is a significant difference between wild-type and *OB mutant. I do not see the need to use this assay to assess for gH2AX and RPA binding. A more straightforward chromatin binding assay would have been more appropriate and easier to interpret.

The data presented in Fig. EV5A and Fig. EV5B (previously Fig. S3B and Fig. S3C) show total detergent-resistant (chromatin-bound) RPA and mean γH2AX fluorescence intensities in individual cells (each dot representing a single cell, signal intensities depicted as arbitrary units), quantified using automated microscopy analysis. We believe that such unbiased high-content quantification of chromatin-bound levels of RPA and γH2AX across thousands of individual cells offers a degree of resolution that is superior to a traditional ensemble chromatin-binding assay. The validity of this imaging-based approach for monitoring replication catastrophe (characterized by γH2AX hyperphosphorylation accompanied by maximal RPA chromatin loading) in individual cells, which could not be gleaned from a conventional chromatin-binding

assay, has been clearly established previously (Toledo et al., Cell 2013 (155:1088-1103); see also Toledo et al., Mol Cell 2017 (66:735-749)). The quantification of cells undergoing replication catastrophe (cells in red in Fig. EV5A,B) is shown in Fig. 5D,E. To facilitate the readers' interpretation of these data, we have tried to more carefully explain this experimental approach and the read-out in the revised manuscript, through the addition of more detailed descriptions in both the text and the relevant figure legends. In addition, instead of showing only results from representative experiments in Fig. 5D,E, we now include the data from a series of independent repeats, allowing us to more firmly conclude that ectopically expressed RADX WT, but not RADX *OB, alleviates the replication catastrophe defect caused by knockdown of endogenous RADX (Fig. 5D).

3rd Editorial Decision: Acceptance 18 September 2017

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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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship 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.

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

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical method was used to predetermine sample size (page 20 (Materials and methods)).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were included from the analysis (page 20 (Materials and methods)).
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No - samples were not randomized (page 20 (Materials and methods))
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No - the investigators were not blinded to the group allocation during experiments and outcome assessment (page 20 (Materials and methods)).
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes.
Is there an estimate of variation within each group of data?	No.
Is the variance similar between the groups that are being statistically compared?	No.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Catalog numbers/source and working dilutions of all antibodies used in this study have been reported (page 17 (Materials and methods)).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All parental cell lines were obtained from ATCC. The cell lines were not authenticated, but were regularly tested for mycoplasma infection (page 15 (Materials and methods)).

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Not applicable.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not applicable.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Not applicable.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable.
12. 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.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
16. 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.
17. 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.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Not applicable for this study.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	We will positively consider providing raw data such as microscopy images for a public repository such as Figshare.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable.

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Not relevant for this study.