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RNase H2 degrades toxic RNA:DNA hybrids behind stalled forks to promote replication restart

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Dr. Philippe Pasero
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20th Dec 2022

Re: EMBOJ-2022-113104
RNase H2 degrades toxic RNA:DNA hybrids behind stalled forks to promote replication restart

Dear Philippe,

Thank you for submitting your study on postreplicative RNA:DNA hybrids to The EMBO Journal. I have now heard back from three expert referees, whose reports you will find copied below. As you will see, all referees consider your findings interesting and potentially important, but at the same time raise a number of major and minor concerns that would have to be addressed prior to publication. These issues include various specific points (such as quantifications and statistics, and conclusiveness of some of the data), but especially referee 2 also mentions some more conceptual caveats with potential impact on the study's conclusions. Should you be able to adequately respond to these criticisms, we would be happy to pursue a revised version of the study further.

Since it is our policy to consider only a single round of major revision and therefore important to fully answer to all comments at the time of resubmission, I would invite you to consider the reports together with your coworkers, and to get back to me with a tentative response letter/revision plan early next month. On the basis of this response, we could then further discuss the revision requirements and how to best address the key concerns e.g. via a follow-up video call. I should add that we could also offer extension of the default three-months revision period if needed, with our 'scooping protection' (meaning that competing work appearing elsewhere in the meantime will not affect our considerations of your study) remaining of course valid also throughout this extension.

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

Yours sincerely,

Hartmut Vodermaier

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

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

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In the interest of ensuring the conceptual advance provided by the work, we recommend submitting a revision within 3 months (20th Mar 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

Referee #1:

In the manuscript entitled "RNase H2 degrades toxic RNA:DNA hybrids behind stalled forks to promote replication restart" the authors explore the mechanism how co-transcriptional R-loop structures interfere with replication resulting in DNA replication stress. As RNAseH enzymes are the main catalytic enzymes to degrade RNA:DNA hybrids in cells, the authors focused on mutant or knockdown cells of RNAseH1 and RNAseH2 in both yeast and human cells and monitored fork progression, arrest and restart under normal as well as replication stress conditions (by HU or MMS treatment). In yeast, the results show that RNaseH enzymes are required to recover stressed replication forks and this is mediated by the failure to load RPA at stressed forks (Figure 1). This is not caused by single ribonucleotide incorporation (Figure 2), nor by the activity of translesion polymerases and can can be rescued by overexpression of Sen1, a master regulator of RNA:DNA hybrid levels in yeast (Figure 3). Mechanistically, a nascent RNA-Seq approach indicates that the RNA:DNA hybrid preexists and is not nascently formed after passage of the replication fork (Figure 4), indicating that the helicase may translocate across the hybrid forming region. Finally, the authors show similar functions of RNAseH2 in human cells by siRNA knockdown experiments (Figure 5-7), where the authors show that the cells have fork resection defects in the absence of RNaseH2 that can be rescued by triptolide as a specific inhibitor that degrades RNAPII, but not DRB that physically leaves RNAPII on chromatin and can therefore continue to interfere with fork resection.
This is overall a very nice manuscript addressing an important question in the field. The data are convincing and support all main conclusion and therefore I'm generally supportive of this manuscript to be published in EMBO Journal.

Major comment:
The authors use a nascent RNA-Seq approach (HT-Seq) in combination with a copy number analysis to detect whether a region has been replicated or not in order to address the question whether the RNA that is forming the RNA:DNA hybrid is formed before or after replication fork passage (Figure 4). This is a very nice approach but I'm a bit unsure about whether the authors have really the required resolution in genomic space as well as time to draw this conclusion. The authors went down to 500bp genomic bin size in the supplement but would the results change if 100bp or 200bp windows are considered? Regarding the timing this may be technically challenging but the authors should at least discuss this limitation in the manuscript.

Minor comments
1) Figure 1F: Can the authors also provide RPA-ChIP data for the RNaseH1 or RNAseH2 single mutants?
2) Figure EV3: The FACS profiles of 240min after release without overexpression in the double mutant (second panels from the left) look different in the number of G2/M cells between -HU and -MMS treatment even though this is technically an identical experiment in the absence of drugs. The authors should comment on this discrepancy.

Referee #2:

This manuscript examines the role(s) of RNase H enzymes in the processing of so-called toxic RNA:DNA hybrids forming at or near stalled replication forks induced in both yeast in human cells. This is a relevant topic given the importance of transcription replication conflicts (TRCs) in setting up genome destabilization events and the potential role(s) or R-loops or RNA:DNA hybrids in potentiating these encounters. The manuscript reports interesting findings that have the potential to push the field further, but it also suffers from significant weaknesses including lack of statistical rigor, over-interpretation of findings, inconsistent experimental settings, and missing experiments.

A major conclusion reached by the authors is that RNase H enzymes, particularly, RNase H2, work to resolve toxic RNA:DNA hybrids behind stalled replication forks. The presence of toxic hybrids is inferred indirectly from the importance of RNase H1/2 activity in supporting the viability of yeast strains treated with fork stalling agents HU and MMS. The presence of these hybrids behind the fork is again inferred indirectly from the fact that lack of RNase H activity affects fork resection which is a postreplicative process. However, the manuscript doesn't contain any direct evidence that these "toxic" hybrids form behind the forks as stated throughout the text and in the manuscript title. Thus, some of the key conclusions of the manuscript are not supported by direct evidence (see below for detailed comments).

General comments:

Figure 1: When discussing panel E, the authors state that RPA binding at both origins of replication are "strongly reduced" in the rnh1Δ, rnh2Δ, and rnh1Δ rnh2Δ mutants compared to wildtype cells however no statistically analysis is performed to measure the degree of reduction of RPA binding. The authors should consider indicating the fold change and relevant tests of significance within panel E. Similar consideration should be given to comparisons made from the data presented in panel F. Likewise, while the differences shown in figure 1B are fairly apparent by eye, the cell cycle profiles should be accompanied by quantifications of the 1C/2C distributions from the 180 and 240 min timepoints over multiple replicates and statistical significance should be indicated. In terms of interpretation, the conclusion that "RNase H activity is required for the timely restart of MMS-arrested forks" is too specific. Authors have not assayed the kinetics of fork restart and therefore can't comment on the timeliness of that process.

Figure 2 : In panel B, the authors ask if RER activity of rnh201 is required for RPA loading under conditions of replicative stress induced by addition of HU. Similar to figure 1E, there are no symbols to indicate if a statistical test was performed for the analysis of RPA accumulation among the different yeast strains (WT, rnh1△, rnh1△rnh201△, and rnh1△rnh201-RED). The authors should perform and apply the appropriate statistical tests to these figures.

Figure 3: Based on the results from Figures 3A, 3B, and figures EV3B and EV3C, the authors conclude that "high levels of Sen1 efficiently suppressed the G2/M accumulation of RNase H-deficient cells". To be frank, the data in figures EV3B and EV3C are not convincing of an "efficient suppression". As suggested in figure 1, the FACS data needs to be quantified for the two latest timepoints over multiple replicates and shown with proper statistics to buttress that claim.

The data in figures 3C and 3D is used to claim that in the absence of RNase H, toxic RNA:DNA hybrids are present at stalled replication forks. However, the slot blot assay provides no information whatsoever regarding the location of RNA:DNA hybrids. It is essential for the authors to add data using S9.6 ChIP-qPCR to support that RNA:DNA hybrids exist at stalled forks, preferably behind the fork. Given the authors claim that HU treatment represses transcription, I'd expect that co-transcriptional R-loops should be proportionally decreased under HU. The observation of a significant hybrid increase in HU-treated double mutants (3C, 3D), therefore suggests that there should be a rather dramatic hybrid / R-loop increase around stalled forks that more than compensates for the genome-wide reduction of co-transcriptional R-loops. Adding S9.6 ChIP-qPCR data is essential to support the conclusion regarding fork stalling resulting from hybrid / R-loop formation behind the replication forks.

As an important side note, there seems to be a disconnect between the quantification (3F) and the raw Western blot (3E). The most obvious difference is seen in the Rpb1-S5P S-Phase sample. The quantification (3F) indicates an approximately 25% reduction in Rpb1-S5P antibody signal when Senataxin is overexpressed in S-phase. When looking at the corresponding samples in the Western Blot, the reduction in signal seems to be much higher, perhaps as high as 80-90%. It is advised to repeat/review the quantification results, or if necessary, the blot itself. Additionally, the authors should consider graphing the quantification results as a jitter plot (not bar graph) and providing statistical significance calculations for figure 3F. Although the authors conclude that Senataxin overexpression leads to the reduction of chromatin-bound RNAPII, there is no data provided to indicate the location on RNAPII in relation to stalled replication forks. This issue could be remedied by performing RNAPII ChIP-qPCR. Finally, it seems that figure 3E,F were performed without HU treatment, when the effect of Senataxin is only detected when cells are treated. This should be remedied.

Figure 4: In this figure the authors aim to address whether increased R-loop formation under HU arrest is due to transcription occurring prior to fork arrest or to de novo transcription at arrested forks. To do this they use CRAC to measure nascent transcription in WT HU-treated yeast cells and compare replicated and unreplicated regions for both co-directional and head-on transcription units. Using this technique they observe no change in nascent transcription between replicated and unreplicated regions in the HT-seq count and conclude "that toxic RNA:DNA hybrids accumulating at stressed forks upon HU addition are not caused by de novo transcription but may result from pre-existing R-loops". There is a significant disconnect with this figure and the others in the paper. Most of the paper focuses on the RNaseH KO mutants, however here the authors chose to analyze WT cells treated with HU. Based on Fig.3C/D the RNase H KO line has the most dramatic increase in S9.6 signal and would seem the best model to investigate changes in nascent transcription relative to fork arrest that could result in changes to R-loop metabolism. Additionally if the authors want to measure whether transcription occurs before HU addition or from RNA synthesis during HU arrest they should show data in WT cells not treated with HU. These experiments support that under replication stress in WT cells, nascent transcription does not increase at stalled forks. However, it is a vast overstatement to say that this is a readout for de novo RNA:DNA hybrid formation. If authors aim to report on hybrids, then they need to use S9.6 ChIP approaches. Of course, doing this in RNase H-deficient cells is a must. Overall, the title of this section, "Post-replicative RNA:DNA hybrids do not form de novo after HU-mediated fork arrest" is not supported by the data and should be significantly improved or deleted.

Side note Fig.4B/C: The axis of the graphs, HTseq count, is not explained in the methods section of the referenced papers that previously performed the protocols. Figure EV4A/B: Bars for statistics in this figure do not include a statistical call (ns).

In figures 5 and 6, the authors switch to human cells and focus on DNA combing assays to evaluate the role of RNases H on resection of nascent DNA at stressed forks. The switch to a human system is jarring after four figures dedicated to yeast. The evidence that resection is reduced upon depletion of RNase H2 subunits indicates that the removal of RNA:DNA hybrids may be necessary for resection. The observation that triptolide treatment can suppress this resection defects suggests that RNAPII contributes to blocking resection when RNase H2 is depleted. Based on this and some rather suggestive effect of HU versus Aph treatment, the authors propose a model in which previous R-loops are bypassed and converted to RNA:DNA hybrids that then need to be resolved to allow resection.

Some elements of the model are interesting and offer hints that may shake up the usual dogma involving R-loops at sites of TRCs. However, quite a few aspects remain unclear or untested. For instance, the authors imply that an HO R-loop is bypassed to become a hybrid on the lagging strand. Why does an R-loop need to be involved here? Couldn't this happen to an elongating RNAPII complex without an R-loop? From figure 7B, the authors imply that a second R-loop is necessary to cause fork stalling. There is no evidence for this. Likewise, the authors' data indicate that RNAPII itself is also an obstacle to resection but the model doesn't show this. The authors also do not indicate where they believe Senataxin plays a role. While it is clear from the model that hybrid resolution might be needed for RPA loading, how would the presence of a possibly long hybrid on the lagging strand cause defects in fork restart and/or fork elongation? Finally, the authors suggest that such toxic hybrids may result from R-loops formed at the end of genes based on arguments about head-on collision frequencies but there is no mapping of these events whatsoever. Therefore, while the work offer some interesting insights, the model serves to highlight just how speculative things are at this junction.

Other comments

- Throughout the manuscript, inconsistent concentrations of MMS are used ranging from a 0.015% treatment in figure 3A to a 0.1% treatment in figure 2C. This more than 6-fold difference in concentrations between experiments reduces one's ability to compare and synthesize results into a cohesive conclusion. The authors should elaborate on the experimental considerations for the use of different concentrations of MMS.

- Personal communication should not be cited as evidence towards a claim (Discussion, Paragraph 2, last sentence).

- Top of page 11 authors refer to the "9.6 antibody", this should be corrected to "S9.6 antibody"

Referee #3:

It has long been known that the loss of RNase H activity renders cells sensitive to replication stress, however the nature of this sensitivity has yet to be understood. In this manuscript, Kemiha et al, use a combination of yeast genetics and human cell culture combined with molecular combing to demonstrate that the persistent presence of RNA-DNA hybrids prevents the generation of ssDNA at stalled replication forks, which in turn prevents replication fork recovery. The authors convincingly demonstrate that it is ongoing, and not de novo, transcription, that is responsible for the R-loops at stalled forks using nascent RNA seq in the presence and absence of replication stress. They also demonstrate that it is likely RNAPII itself, and not the hybrid per se, that is responsible for the replication stress. In yeast, the overexpression of Sen1 is able to rescue many of the phenotypes of the rnh1 rnh201 mutants and it also promotes removal of RNAPII. In agreement, in human cells only the addition of triptolide, and not DRB, can rescue the fork recovery phenotypes and this is likely due to the degradation of RNAPII. Based on the results, the authors propose a model consistent with their data which suggests that in the case of head on TRCs a hybrid gets bypassed by the replisome and is left behind on the lagging strand. The hybrids would then need to be removed in order to allow recovery following resection. Importantly, the authors demonstrated in both systems that the accumulation of RNA-DNA hybrids does not affect checkpoint activation.
The manuscript is clearly written from start to finish and all figures are clear and easy to interpret. I feel that this study adds important and relevant findings to understanding how replication fidelity is upheld in the presence of ongoing transcription. I would like to suggest 3 or 4 experiments that I feel would improve the study even further.

1. It would be interesting to see if the hybrids that are detected in figure 3C are also localized at replication origins and if these are the hybrids that are being removed by Sen1 overexpression (i.e. through DRIP of R-ChIP as done with RPA in Figure 1)
2. Figure 3E &F, the effect of Sen1 overexpression on RNAPII removal is minor, would this be more prevalent in a rnh1 rnh201 background
3. Sen1 OE can help in hybrid removal, but does it? How is recovery in sen1-1 mutants or with shSenataxin?
4. In the discussion the authors speculate that, like at DSBs, RNA:DNA hybrids may have both positive and negative roles during replication recovery. Was the overexpression of RNase H1 ever attempted, especially in human cells this would be interesting as Rnh1 OE in yeast has very few phenotypes.
5. Finally, can the model be expanded a bit to illustrate what happens in the presence and absence of RNase H enzymes. I think it would come across more clearly.

Referee #1:

In the manuscript entitled "RNase H2 degrades toxic RNA:DNA hybrids behind stalled forks to promote replication restart" the authors explore the mechanism how co-transcriptional R-loop structures interfere with replication resulting in DNA replication stress. As RNAseH enzymes are the main catalytic enzymes to degrade RNA:DNA hybrids in cells, the authors focused on mutant or knockdown cells of RNAseH1 and RNAseH2 in both yeast and human cells and monitored fork progression, arrest and restart under normal as well as replication stress conditions (by HU or MMS treatment). In yeast, the results show that RNaseH enzymes are required to recover stressed replication forks and this is mediated by the failure to load RPA at stressed forks (Figure 1). This is not caused by single ribonucleotide incorporation (Figure 2), nor by the activity of translesion polymerases and can can be rescued by overexpression of Sen1, a master regulator of RNA:DNA hybrid levels in yeast (Figure 3). Mechanistically, a nascent RNA-Seq approach indicates that the RNA:DNA hybrid preexists and is not nascently formed after passage of the replication fork (Figure 4), indicating that the helicase may translocate across the hybrid forming region. Finally, the authors show similar functions of RNAseH2 in human cells by siRNA knockdown experiments (Figure 5-7), where the authors show that the cells have fork resection defects in the absence of RNaseH2 that can be rescued by triptolide as a specific inhibitor that degrades RNAPII, but not DRB that physically leaves RNAPII on chromatin and can therefore continue to interfere with fork resection. This is overall a very nice manuscript addressing an important question in the field. The data are convincing and support all main conclusion and therefore I'm generally supportive of this manuscript to be published in EMBO Journal.

We are grateful to this reviewer for his/her very positive comments.

Major comment:

The authors use a nascent RNA-Seq approach (HT-Seq) in combination with a copy number analysis to detect whether a region has been replicated or not in order to address the question whether the RNA that is forming the RNA:DNA hybrid is formed before or after replication fork passage (Figure 4). This is a very nice approach but I'm a bit unsure about whether the authors have really the required resolution in genomic space as well as time to draw this conclusion. The authors went down to 500bp genomic bin size in the supplement but would the results change if 100bp or 200bp windows are considered? Regarding the timing this may be technically challenging but the authors should at least discuss this limitation in the manuscript.

In principle, CRAC has near-nucleotide resolution. Here, the resolution is limited by the average length of the reads, which is around 30 nt. This resolution is close to the RNAPII occupancy on DNA and is sufficient for the scope of our study, but it could be further increased by mapping only the 3'-end of the reads. Actually, the main limitation comes from the time resolution of the experiment, especially regarding the position of replication forks. Indeed, fork position was derived from changes in DNA copy number in the same samples at the population level and may vary in individual cells. These limitations are now discussed in the manuscript. The window size was originally chosen to include a sufficiently large number of genes for statistical analysis. As requested by this reviewer, we now display analyses with bin sizes ranging from 0.1 to 1 kb, showing no difference in CRAC signals between replicated and unreplicated regions (**Appendix Figure 1B**).

To address this timing issue with an independent approach, we also analyzed RNAPII occupancy on both sides of *ARS305* by ChIP-qPCR by collecting samples every 3 minutes after releasing G_1-arrested cells into S phase in the presence of HU (**EV Fig. 4E** and **4F**). Although this experiment was performed only once, we decided to include it in the manuscript as it is consistent with CRAC data and is supported

with biological replicates performed at two other early origins (*ARS306* and *ARS607*), showing also no RNAPII enrichment in HU-arrested cells (**EV Fig. 4D**).

Minor comments

1) Figure 1F: Can the authors also provide RPA-ChIP data for the RNaseH1 or RNAseH2 single mutants?

Figure 1E now shows a new series of experiments including single *rnh1Δ* and *rnh201Δ* mutants.

2) Figure EV3: The FACS profiles of 240min after release without overexpression in the double mutant (second panels from the left) look different in the number of G2/M cells between -HU and -MMS treatment even though this is technically an identical experiment in the absence of drugs. The authors should comment on this discrepancy.

We agree that there were more G_2 cells in the MMS-free control experiment (**Fig. EV3C**) compared to the HU-free control experiment (**Fig. EV3B**), although both samples should behave similarly. We have therefore performed three additional biological replicates using the same protocol and found a comparable number of G_2 cells at t=240 min after release from G_1 in both wild type and *rnh1Δ rnh201Δ* cells (see figure below). The correct control samples are now shown in **Fig. EV3C and EV3D**.

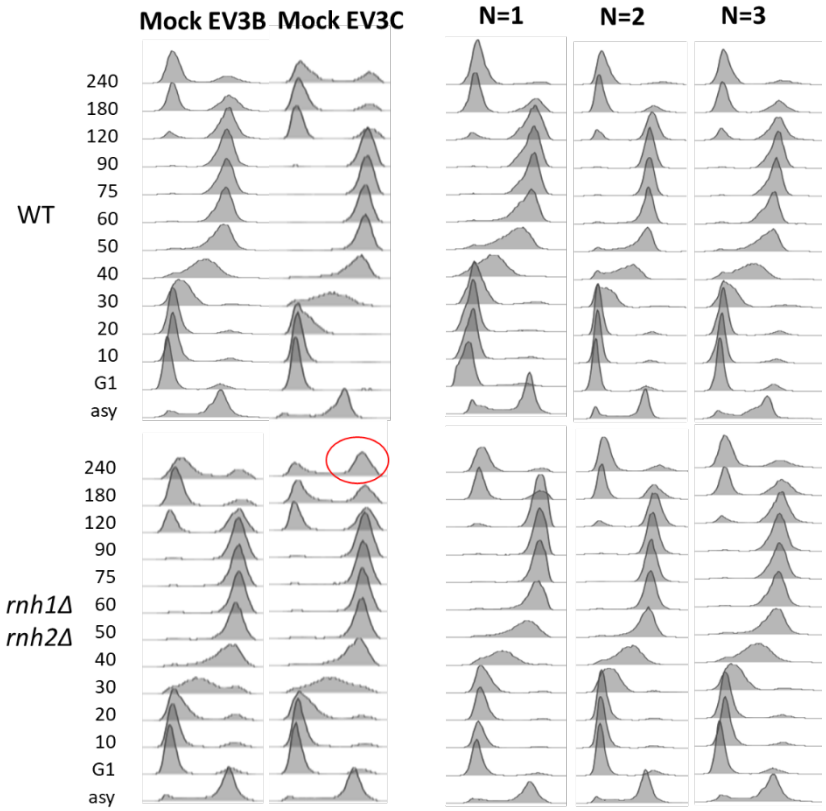

Figure legend: Cells were synchronized in G_1 with alpha-factor and released into S phase by the addition of pronase. Cell cycle kinetics was measured by cytometry. Mock EV3B and EV3C correspond to the results presented in the first version of the manuscript.

Referee #2:

This manuscript examines the role(s) of RNase H enzymes in the processing of so-called toxic RNA:DNA hybrids forming at or near stalled replication forks induced in both yeast in human cells. This is a relevant topic given the importance of transcription replication conflicts (TRCs) in setting up genome destabilization events and the potential role(s) or R-loops or RNA:DNA hybrids in potentiating these encounters. The manuscript reports interesting findings that have the potential to push the field further, but it also suffers from significant weaknesses including lack of statistical rigor, over-interpretation of findings, inconsistent experimental settings, and missing experiments.

A major conclusion reached by the authors is that RNase H enzymes, particularly, RNase H2, work to resolve toxic RNA:DNA hybrids behind stalled replication forks. The presence of toxic hybrids is inferred indirectly from the importance of RNase H1/2 activity in supporting the viability of yeast strains treated with fork stalling agents HU and MMS. The presence of these hybrids behind the fork is again inferred indirectly from the fact that lack of RNase H activity affects fork resection which is a postreplicative process. However, the manuscript doesn't contain any direct evidence that these "toxic" hybrids form behind the forks as stated throughout the text and in the manuscript title. Thus, some of the key conclusions of the manuscript are not supported by direct evidence (see below for detailed comments).

We thank this Reviewer for his/her positive comments and for constructive criticisms that helped us improve our manuscript.

General comments:

Figure 1: When discussing panel E, the authors state that RPA binding at both origins of replication are "strongly reduced" in the rnh1Δ, rnh2Δ, and rnh1Δ rnh2Δ mutants compared to wildtype cells however no statistically analysis is performed to measure the degree of reduction of RPA binding.
The authors should consider indicating the fold change and relevant tests of significance within panel E. Similar consideration should be given to comparisons made from the data presented in panel F.

As suggested by this Reviewer, we now also present results from **Fig. 1E** and **Fig. EV1C** as fold changes (**Fig EV1D**) and show statistical tests.

Likewise, while the differences shown in figure 1B are fairly apparent by eye, the cell cycle profiles should be accompanied by quantifications of the 1C/2C distributions from the 180 and 240 min timepoints over multiple replicates and statistical significance should be indicated.

Figure EV3B now shows the average percentage of cells in G_2 phase for three biological replicates for the 180 and 240 min timepoints, as well as the statistical significance of differences with or without *SEN1* overexpression.

In terms of interpretation, the conclusion that "RNase H activity is required for the timely restart of MMS-arrested forks" is too specific. Authors have not assayed the kinetics of fork restart and therefore can't comment on the timeliness of that process.

We have removed references to the speed of fork restart. For example, we have replaced "RNase H activity is required for the timely restart of MMS-arrested forks" with “RNase H activity is required for the efficient restart of MMS-arrested forks”.

Figure 2 : In panel B, the authors ask if RER activity of rnh201 is required for RPA loading under conditions of replicative stress induced by addition of HU. Similar to figure 1E, there are no symbols to

indicate if a statistical test was performed for the analysis of RPA accumulation among the different yeast strains (WT, rnh1△, rnh1△rnh201△, and rnh1△rnh201-RED). The authors should perform and apply the appropriate statistical tests to these figures.

As above, results are now presented as fold changes in **Figure EV1H** and statistical tests are provided for **Figure 2B**.

Figure 3: Based on the results from Figures 3A, 3B, and figures EV3B and EV3C, the authors conclude that "high levels of Sen1 efficiently suppressed the G2/M accumulation of RNase H-deficient cells". To be frank, the data in figures EV3B and EV3C are not convincing of an "efficient suppression". As suggested in figure 1, the FACS data needs to be quantified for the two latest timepoints over multiple replicates and shown with proper statistics to buttress that claim.

To address this issue, we quantified the proportion of G_2/M cells in three biological replicates and conducted statistical tests (**Fig. EV3B**). The data indicate that *SEN1* overexpression did not significantly prevent the accumulation of G_2/M cells at 180 minutes, but did so at 240 minutes. We therefore rephrased the sentence "high levels of Sen1 efficiently suppressed the G_2/M accumulation of RNase H-deficient cells… " to "high levels of Sen1 also efficiently reduced the late G_2/M accumulation of RNase H-deficient cells … (t=240 min, Figure EV3B-D)".

The data in figures 3C and 3D is used to claim that in the absence of RNase H, toxic RNA:DNA hybrids are present at stalled replication forks. However, the slot blot assay provides no information whatsoever regarding the location of RNA:DNA hybrids. It is essential for the authors to add data using S9.6 ChIP-qPCR to support that RNA:DNA hybrids exist at stalled forks, preferably behind the fork. Given the authors claim that HU treatment represses transcription, I'd expect that co-transcriptional R-loops should be proportionally decreased under HU. The observation of a significant hybrid increase in HU-treated double mutants (3C, 3D), therefore suggests that there should be a rather dramatic hybrid / R-loop increase around stalled forks that more than compensates for the genome-wide reduction of co-transcriptional R-loops. Adding S9.6 ChIP-qPCR data is essential to support the conclusion regarding fork stalling resulting from hybrid / R-loop formation behind the replication forks.

This is indeed a very important point that was also raised by Reviewer #3. The DNA-RNA immunoprecipitation (DRIP) assay with the S9.6 antibody was never applied before to HU-arrested cells and it took us much time and efforts to improve existing protocols. We now show in a new **figure 4A** the DRIP-qPCR analysis of RNA:DNA hybrid levels around three early origins (*ARS305*, *ARS306* and *ARS607*) as well as control loci in wild type and *rnh1Δrnh201Δ* cells exposed or not to HU. RNA:DNA hybrid levels did not change at newly replicated and unreplicated loci in both wild type and *rnh1Δ rnh201Δ* cells. However, we detected a 2- to 3-fold increase in RNA:DNA hybrids in front of HU-arrested forks in RNase H-deficient cells relative to wild type cells, at regions encompassing at least 7 kb per fork. Although the spatial and temporal resolution of the assay was not sufficient to formally demonstrate the presence of hybrids behind the forks, these data explain the overall increase in S9.6 signal observed on slot blots in RNase H-deficient cells exposed to HU. Indeed, considering that there are approximately 200 active origins in HU-arrested cells, these regions enriched in R-loops would account for nearly 3 Mb of genomic DNA, equivalent to approximately one-fourth of the yeast genome.

As an important side note, there seems to be a disconnect between the quantification (3F) and the raw Western blot (3E). The most obvious difference is seen in the Rpb1-S5P S-Phase sample. The quantification (3F) indicates an approximately 25% reduction in Rpb1-S5P antibody signal when Senataxin is overexpressed in S-phase. When looking at the corresponding samples in the Western Blot, the reduction in signal seems to be much higher, perhaps as high as 80-90%. It is advised to repeat/review the quantification results, or if necessary, the blot itself. Additionally, the authors should consider graphing the quantification results as a jitter plot (not bar graph) and providing statistical

significance calculations for figure 3F. Although the authors conclude that Senataxin overexpression leads to the reduction of chromatin-bound RNAPII, there is no data provided to indicate the location on RNAPII in relation to stalled replication forks. This issue could be remedied by performing RNAPII ChIP-qPCR. Finally, it seems that figure 3E,F were performed without HU treatment, when the effect of Senataxin is only detected when cells are treated. This should be remedied.

We thank Reviewer #2 for pointing that out. As requested by this Reviewer, we have repeated the experiments with and without HU. The data are now presented as a jitter plot with statistics comparing the different conditions (**Fig. EV4A** and **EV4B**). We also performed ChIP-qPCR of Rpb1, the large subunit of RNAPII, at genes known to generate transcription-replication conflicts (TRCs) to quantify the amount of chromatin-bound RNAPII relative to replication forks. As previously described (see Poli *et al.*, 2016 and Hurst *et al.* 2021), we observed a strong reduction of RNAPII on chromatin at these loci (see **Fig. EV4C** and **EV4D**). These data indicate that *rnh1Δ rnh201Δ* cells are proficient to remove RNAPII from chromatin at TRC sites in response to HU and that *SEN1* overexpression does not affect overall levels of chromatin-bound RNAPII, even though it significantly reduced RNA:DNA hybrid levels in the presence of HU.

Figure 4: In this figure the authors aim to address whether increased R-loop formation under HU arrest is due to transcription occurring prior to fork arrest or to de novo transcription at arrested forks. To do this they use CRAC to measure nascent transcription in WT HU-treated yeast cells and compare replicated and unreplicated regions for both co-directional and head-on transcription units. Using this technique they observe no change in nascent transcription between replicated and unreplicated regions in the HT-seq count and conclude "that toxic RNA:DNA hybrids accumulating at stressed forks upon HU addition are not caused by de novo transcription but may result from pre-existing R-loops". There is a significant disconnect with this figure and the others in the paper. Most of the paper focuses on the RNaseH KO mutants, however here the authors chose to analyze WT cells treated with HU. Based on Fig.3C/D the RNase H KO line has the most dramatic increase in S9.6 signal and would seem the best model to investigate changes in nascent transcription relative to fork arrest that could result in changes to R-loop metabolism.
Additionally if the authors want to measure whether transcription occurs before HU addition or from RNA synthesis during HU arrest they should show data in WT cells not treated with HU. These experiments support that under replication stress in WT cells, nascent transcription does not increase at stalled forks. However, it is a vast overstatement to say that this is a readout for de novo RNA:DNA hybrid formation. If authors aim to report on hybrids, then they need to use S9.6 ChIP approaches. Of course, doing this in RNase H-deficient cells is a must. Overall, the title of this section, "Post-replicative RNA:DNA hybrids do not form de novo after HU-mediated fork arrest" is not supported by the data and should be significantly improved or deleted.

The aim of the CRAC experiment was to address the possibility that the remodeling of nascent chromatin behind HU-arrested forks could promote pervasive transcription and potentially induce the formation of post-replicative RNA:DNA hybrids. The original event sought was therefore a possible increase in RNAPII occupancy in regions where replication is blocked, much like what we showed at the replication fork barrier in a recent report (Delamarre, Mol Cell 2020). With the caveats mentioned in our response to Reviewer #1, we observed comparably low levels of CRAC signals at replicated and unreplicated regions. We also performed ChIP-qPCR analyses of RNAPII levels at HU-arrested forks in both wild type and *rnh1Δ rnh201Δ* cells and found no sign of increased RNAPII occupancy behind HU-arrested forks. We therefore decided to focus our efforts on other aspects such as DRIP experiments and we did not pursue in analyzing CRAC signals in HU-arrested *rnh1Δ rnh201Δ* mutants.

Side note Fig.4B/C: The axis of the graphs, HTseq count, is not explained in the methods section of the referenced papers that previously performed the protocols. Figure EV4A/B: Bars for statistics in this figure do not include a statistical call (ns).

We thank this Reviewer for pointing this out. The revised version of the manuscript includes a full description of Log2 Rpb1-HTP CRAC count as well as information about the statistics in the figure legends.

In figures 5 and 6, the authors switch to human cells and focus on DNA combing assays to evaluate the role of RNases H on resection of nascent DNA at stressed forks. The switch to a human system is jarring after four figures dedicated to yeast. The evidence that resection is reduced upon depletion of RNase H2 subunits indicates that the removal of RNA:DNA hybrids may be necessary for resection. The observation that triptolide treatment can suppress this resection defects suggests that RNAPII contributes to blocking resection when RNase H2 is depleted. Based on this and some rather suggestive effect of HU versus Aph treatment, the authors propose a model in which previous R-loops are bypassed and converted to RNA:DNA hybrids that then need to be resolved to allow resection.

The switch to human cells allowed us to directly assess the effect of RNA:DNA hybrids on the resection of nascent DNA. Indeed, the ChIP-qPCR experiments performed in yeast show differences in RPA-coated ssDNA, which could potentially result from other mechanisms such as hyper-unwinding or uncoupling of leading and lagging strand synthesis in wild type cells, but not in RNase H mutants. Moreover, experiments in human cells also allowed us to investigate the effect of transcription inhibitors, which is not possible in budding yeast because active transcription is critical to enter S phase. We have tried to present the data from yeast alongside the data from human cells, however, the outcome of this combination hindered the clarity of message. We therefore maintained the order of presenting yeast and human cells data separately.

Some elements of the model are interesting and offer hints that may shake up the usual dogma involving R-loops at sites of TRCs. However, quite a few aspects remain unclear or untested. For instance, the authors imply that an HO R-loop is bypassed to become a hybrid on the lagging strand. Why does an R-loop need to be involved here? Couldn't this happen to an elongating RNAPII complex without an R-loop?

We agree with Reviewer #2 that many aspects of our model remain to be tested. In principle, an elongating RNAPII complex could indeed be bypassed to generate a post-replicative RNA:DNA hybrid, but this would require a MUS81-dependent cleavage of the fork, as reported by the Janscak lab in cells exposed to CPT (Chappidi, Mol Cell 2020). Moreover, recent *in vivo* and *in vitro* studies indicate that head-on TRCs do not block fork progression in budding yeast (Tsirkas, NAR 2022; Kumar, eLife 2021). This is consistent with the fact that the $Mec1^{ATR}$ kinase is able to disengage RNAPII from chromatin during S phase (Poli, Gene Dev 2016; Hurst, EMBO J 2021) and that ATR is activated when replication and transcription converge at TTS in human cells (Promonet, 2020). Together, these data indicate that cells can easily deal with TRCs, unless if Top1 is limiting (Promonet, Nat Commun 2020) or if fork progression is slowed down by HU or ROS (Andrs, Nat Commun 2023). Our model integrates this information and proposes that the combined removal of R-loops and RNAPII at TRCs avoids deleterious consequences on forks. In contrast, in the absence of RNase H, the persistence of RNA:DNA hybrids on the lagging strand could interfere with the processing of nascent DNA and with fork restart, especially when DNA replication is challenged with HU. We have expanded the presentation of the model in the discussion and figure legend to better reflect this complexity.

From figure 7B, the authors imply that a second R-loop is necessary to cause fork stalling. There is no evidence for this. Likewise, the authors' data indicate that RNAPII itself is also an obstacle to resection

but the model doesn't show this. The authors also do not indicate where they believe Senataxin plays a role.

Our recent analysis of the distribution of R-loops at TRC sites in human cells (Promonet, Nat Commun 2020) indicates that these structures expand over a large region downstream of the TTS, which contrasts with the narrower distribution of R-loops at TSS. This finding, along with the results from others (Skourti-Stathaki, Mol Cell 2011) argue for the view that replication forks encounter multiple R-loops at TTS. Our DRIP-qPCR analyses also indicate that R-loops accumulate over several kb in front of the forks, which is consistent with our model. When overexpressed, Senataxin compensates for the lack of RNase H and restores fork restart in budding yeast, but endogenous levels are not sufficient to fulfill this role.

While it is clear from the model that hybrid resolution might be needed for RPA loading, how would the presence of a possibly long hybrid on the lagging strand cause defects in fork restart and/or fork elongation?

As discussed in the manuscript, the mechanism by which RNA:DNA hybrids interfere with fork restart is currently unclear. Work in fission yeast has shown that fork resection is required to promote the HR-mediated restart of forks arrested at the *RTS1* replication barrier (Teixeira-Silva, Nat Commun 2017). Since fork resection depends on fork reversal, one possibility could be that postreplicative RNA:DNA hybrids interfere with fork reversal, but further work involving EM studies is required to address this possibility, which goes beyond the scope of our work.

Finally, the authors suggest that such toxic hybrids may result from R-loops formed at the end of genes based on arguments about head-on collision frequencies but there is no mapping of these events whatsoever. Therefore, while the work offers some interesting insights, the model serves to highlight just how speculative things are at this junction.

We have now mapped RNA:DNA hybrids by DRIP-qPCR in HU-arrested cells and found that hybrids accumulate in front of replication forks in *rnh1Δ rnh201Δ* cells. These new data support our model that toxic hybrids do not form *de novo* behind the forks but rather result from replication bypass. These aspects are now discussed in the manuscript.

Other comments

● Throughout the manuscript, inconsistent concentrations of MMS are used ranging from a 0.015% treatment in figure 3A to a 0.1% treatment in figure 2C. This more than 6-fold difference in concentrations between experiments reduces one's ability to compare and synthesize results into a cohesive conclusion. The authors should elaborate on the experimental considerations for the use of different concentrations of MMS.

We use different doses of MMS depending on the type of experiments. Low doses (0.015%) are used for chronic exposures (**Fig. 3A**) and higher doses (0.1% or 0.033%) are used for fork arrest and restart (**Fig. 2C**). This is now better explained in the revised version of the manuscript.

● Personal communication should not be cited as evidence towards a claim (Discussion, Paragraph 2, last sentence).

This manuscript is now published and the reference (Stoy, Nat Struct Mol Biol 2023) is cited in the manuscript.

● Top of page 11 authors refer to the "9.6 antibody", this should be corrected to "S9.6 antibody"

Corrected, thanks.

Referee #3:

It has long been known that the loss of RNase H activity renders cells sensitive to replication stress, however the nature of this sensitivity has yet to be understood. In this manuscript, Kemiha et al, use a combination of yeast genetics and human cell culture combined with molecular combing to demonstrate that the persistent presence of RNA-DNA hybrids prevents the generation of ssDNA at stalled replication forks, which in turn prevents replication fork recovery. The authors convincingly demonstrate that it is ongoing, and not de novo, transcription, that is responsible for the R-loops at stalled forks using nascent RNA seq in the presence and absence of replication stress. They also demonstrate that it is likely RNAPII itself, and not the hybrid per se, that is responsible for the replication stress. In yeast, the overexpression of Sen1 is able to rescue many of the phenotypes of the rnh1 rnh201 mutants and it also promotes removal of RNAPII. In agreement, in human cells only the addition of triptolide, and not DRB, can rescue the fork recovery phenotypes and this is likely due to the degradation of RNAPII. Based on the results, the authors propose a model consistent with their data which suggests that in the case of head on TRCs a hybrid gets bypassed by the replisome and is left behind on the lagging strand. The hybrids would then need to be removed in order to allow recovery following resection. Importantly, the authors demonstrated in both systems that the accumulation of RNA-DNA hybrids does not affect checkpoint activation.
The manuscript is clearly written from start to finish and all figures are clear and easy to interpret. I feel that this study adds important and relevant findings to understanding how replication fidelity is upheld in the presence of ongoing transcription. I would like to suggest 3 or 4 experiments that I feel would improve the study even further.

We are grateful to this Reviewer for his/her interest in our work and for constructive comments.

1. It would be interesting to see if the hybrids that are detected in figure 3C are also localized at replication origins and if these are the hybrids that are being removed by Sen1 overexpression (i.e. through DRIP of R-ChIP as done with RPA in Figure 1)

This is an important point that was also raised by Reviewer #2. As discussed above, we performed DRIP-qPCR +/- HU treatment and quantified the level of RNA:DNA hybrids in the vicinity of replication forks (**Fig. 4**). We found that RNA:DNA hybrids accumulate over several kb in front of the forks. Even though the resolution of our assay is not sufficient to formally demonstrate that part of these hybrids is transferred behind forks, these data support our model that postreplicative RNA:DNA hybrids originate from pre-existing cotranscriptional R-loops.

2. Figure 3E &F, the effect of Sen1 overexpression on RNAPII removal is minor, would this be more prevalent in a rnh1 rnh201 background

We have performed chromatin fractionation in wild type and *rnh1Δ rnh201Δ* cells overexpressing or not *SEN1* +/- HU (**Fig. EV4A** and **EV4B**) and found that *SEN1* overexpression has a limited effect on overall levels of RNAPII on chromatin, regardless of the condition tested.

3. Sen1 OE can help in hybrid removal, but does it? How is recovery in sen1-1 mutants or with shSenataxin?

We have analyzed fork resection by DNA fiber spreading in human cells depleted for Senataxin using shRNAs (n=3) and did not detect any significant differences relative to control cells (**Appendix Fig. 2E-**

F). These data indicate that Senataxin is dispensable for the resection of nascent DNA at stalled forks when RNase H is present.

4. In the discussion the authors speculate that, like at DSBs, RNA:DNA hybrids may have both positive and negative roles during replication recovery. Was the overexpression of RNase H1 ever attempted, especially in human cells this would be interesting as Rnh1 OE in yeast has very few phenotypes.

We have shown that the overexpression of RNase H1 in human cells does not significantly affect the progression of replication forks in HeLa cells (Promonet, Nat Commun 2020). It would be interesting to test whether it affects the kinetics of fork restart, but this goes beyond the scope of this study.

5. Finally, can the model be expanded a bit to illustrate what happens in the presence and absence of RNase H enzymes. I think it would come across more clearly.

We have revised the model to provide a more comprehensive explanation how replication forks are affected by R-loops in the RNase H-proficient and deficient cells.

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20th Sep 2023

Re: EMBOJ-2022-113104R
RNase H2 degrades toxic RNA:DNA hybrids behind stalled forks to promote replication restart

Dear Philippe,

Thank you again for submitting your revised manuscript to The EMBO Journal. We have now received re-reviews from all three original referees, and I am happy to say that all were satisfied with the revisions. After addressing of the following, important editorial issues, we should therefore be able to proceed with acceptance and publication of the study:

- Pre-acceptance checks by our data editors have raised several queries with the data descriptors in the figure legends, which you will find as comments in the attached edited/commented Word document with activated "Track changes" option. I would appreciate if you incorporated the requested final text modifications and answered the Figure legend queries directly in this version (and modified figures where necessary), uploading the edited main text document upon resubmission with changes/additions still highlighted via the "Track changes" option, to facilitate our final checking.

- As we are switching from a free-text author contribution statement towards a more formal statement based on Contributor Role Taxonomy (CRediT) terms, please remove the present Author Contribution section and instead specify each author's contribution(s) directly in the Author Information page of our submission system during upload of the final manuscript. See https://casrai.org/credit/ for more information.

- Since we mandate addition of ORCID identifiers for all (co-)corresponding authors, please encourage Armelle Lengronne to obtain and add an ORCID to her author profile in our submission system. This needs to be added by her personally and can unfortunately not be done by others on her behalf.

- Related to the two previous points: I wanted to double-check the authorship listing on the manuscript with you. Although we often see broad-based collaborations between different laboratories leading to shared corresponding authorship on many research papers in The EMBO Journal, it is unusual to include a full four co-corresponding authors all sharing the same affiliation. What is even more exceptional is that the main author (PP) we corresponded with during the whole course of the submission process is not at all listed as corresponding author on the manuscript itself. We certainly understand that authorship position is relevant for research assessment and academic credit, but please remember that an excessively distributive authorship also dilutes and diminishes this credit; therefore, we would consider a detailed description of each author's specific contributions (using CRediT taxonomy as well as the free-text boxes in the submission system) clearly more valuable. I would therefore appreciate your further explanation and possible reconsideration of the authorship status assignments on this paper.

- In the "Data Availability" section, please include a direct URL to the deposited dataset in the GEO, and make sure that the data are becoming publicly released at this point.

- Please rename the "Supplementary information" PDF as "Appendix" on its front page (and indicate also the paper title there for ease of identification. Rename all the included figures, both in the Appendix Table of Contents, in the respective legends, as well as when referencing them in the main text (!), into "Appendix Figure S1/2/3.." or "Appendix Table S1/2/3...", both when referencing them in the text, and within the single Appendix PDF, in which they should all be combined. Make sure to also move the Appendix Figure legends from the main text into the Appendix PDF. The Appendix should have page numbers, and start with a brief table of contents listing the included figures, legends and tables and their respective page numbers.

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- Please double-check to make sure to all relevant funding information in the manuscript is congruent with the info entered into

our submission system. Centre National de la Recherche Scientifique (CNRS), the Fondation pour la Recherche Médicale (FRM, programme Equipes 2019, (ANR-16-CE12-0022-01 , ANR-21-CE12-0040-01, ANR-19-CE12-0016-01, the Institut Universitaire de France, the French Ministère de la Recherche et de l'Enseignement Superieur (MRES), ANR-20-CE12-0016-01, ANR-19-CE12-0023-03, Université Paris Cité IdEx #ANR-18-IDEX-0001, French Government -listed in the text- are currently all missing in the submission system.

- Finally, our routine pre-acceptance image checks found that certain panels showing yeast colony serial dilutions appear to have been duplicated between Figure panels 1A, 2A, and EV2A. I realize that these figures may originate from the same initial experiment and certain panels therefore be repeated for comparison, but if so, this would need to be explicitly state and explained in all respective figure legends. Please clarify.

I am therefore returning the manuscript to you for a final round of minor revision, to allow you to make these adjustments and upload all modified files. Once we will have received them, we should hopefully be ready to proceed with formal acceptance and production of the manuscript.

With kind regards,

Hartmut

Hartmut Vodermaier, PhD
Senior Editor, The EMBO Journal
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In the interest of ensuring the conceptual advance provided by the work, we recommend submitting a revision within 3 months (19th Dec 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

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Referee #1:

The authors have sufficiently addressed my previous concerns and now provide sufficiently strong evidence that RNaseH2 degrades toxic RNA:DNA hybrids behind stalled forks to promote replication restart.

Referee #2:

I have carefully read the revised manuscript, which was significantly improved with addition of new experiments (in particular DRIP-qPCR) and quantification. The work is clearly written, the data is well presented in figures, and the overall model introduced here is sensible and represents a true step forward in the field.

Referee #3:

A very nice manuscript that has been improved even further with the additional experiments. Congratulations on an important piece of work and furthering the understanding of how R-loop processing is important for replication restart.

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4th Oct 2023

Re: EMBOJ-2022-113104R1
RNase H2 degrades toxic RNA:DNA hybrids behind stalled forks to promote replication restart

Dear Philippe,

Thank you for submitting your final revised manuscript and explanations for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

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

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

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

With kind regards,

Hartmut

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

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Table EV2

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	HeLa cells (ATCC, CCL-2) in Materials and Methods
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.	Yes	Cells are regularly tested for mycoplasma by PCR

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.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

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.	Yes	Saccharomyces cerevisiae W303 background (Materials and Methods + Table EV1)

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?	Not Applicable	

Design

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

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

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	Materials and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Samples randomization procedure to count fibers lenght (DNA combing + fiber assay)
Include a statement about **blinding** even if no blinding was done.	Yes	Samples randomization procedure to count fibers lenght (DNA combing + fiber assay)
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	Mann-Whitney rank sum test was used to compare DNA combing measurements.Two-way ANOVA was used for ChIP data. T-test was used for fiber assay and CRAC-seq data. For DNA combing data, we observed a normal distribution of track lenght in every conditions measured. Normal distribution is assessed by plotting the frequency of tracks length grouped in bins. SEM or SD are presented in each group of

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	Figures legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figures legends

Ethics

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

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

Reporting

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

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

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data availability Section (GEO ; GSE215896)
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**.	Yes	Figure EV2B