The EMBO Journal  Peer Review Process File - EMBO-2014-88544

Manuscript EMBO-2014-88544

RNA polymerase II contributes to preventing transcription-mediated replication fork stalls

Irene Felipe-Abrio, Juan Lafuente-Barquero, María García-Rubio and André Aguilera

Corresponding author: André Aguilera, Centro Andaluz de Biología Molecular y Medicina Regenerativa CABIMER, Universidad de Sevilla

Review timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>20 March 2014</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>02 May 2014</td>
</tr>
<tr>
<td>Revision received</td>
<td>20 July 2014</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>02 September 2014</td>
</tr>
<tr>
<td>Revision received</td>
<td>29 September 2014</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>08 October 2014</td>
</tr>
<tr>
<td>Accepted</td>
<td>08 October 2014</td>
</tr>
</tbody>
</table>

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.)

Editor: Alexander Kohlmaier

1st Editorial Decision 02 May 2014

Thank you for submitting your manuscript "RNA polymerase II has a function counteracting transcription-mediated replication fork stalling" for consideration to the EMBO Journal. We have now received the comments of two expert referees that you will find copied below.

As you will see, both referees' comments are well-considered and constructive, yet voice a number of substantial concerns. Referee #1 lists a number of considerations that question whether a conclusive model for mechanism can be derived from the presented experiments. In particular, referee #1 (points d and f) worries that given the lack of molecular understanding of the DNA damage sensitivity in Pol II mutations and the absence of strict correlation of these defects with genome instability, the presented evidence does not offer definitive conclusions that were fully satisfying for an EMBO Journal paper. As such, referee #1 as well as #2 (point 2) also concur that the presented work reports more on the pathophysiology of RNAP II mutants, rather than revealing a dedicated endogenous role of the wildtype RNAP II in counteracting stalled replication forks.

On the other hand, several previous studies on TAR were essentially done on episomes or focused on R-loops, eukaryotic mechanisms restricting replication/transcription interference might be very different from prokaryotes, and the fact that specific RNAP II subunits might be involved in this process is interesting in principle. Together, we came to the conclusion that we may be able to consider a revised version of your manuscript further, in case you should be willing to extend the
molecular and mechanistic analysis as suggested by both referees. A precondition to further consideration at the EMBO Journal would be the successful experimental revision of all key experimental concerns, in particular also to support the claims made in the title and abstract.

The points raised by the two referees are well considered and clearly expressed, and I will not go through them in detail here. I just outline a few key inconsistencies in the data presented that would likely involve further-reaching more extensive molecular investigation: 1. rbp2-10 shows genetic interactions with repair factors but no hyper-recombination or replication defects, which does not seem to support the core model (referee #1, specific point d). 2. there is controversy regarding increased genome-wide Pol II stalling in rpb1-1 cells - it would be essential for the proposed model to decisively corroborate genome-wide increased Pol II stalling, while current interpretations only base on analysis of a limited number of loci (referee #1, specific point f). 3. Too few factors were functionally tested in figure 6/E7A-C, and the criteria of their selection from the Chips were not fully conclusive given point 1 above (referee #2, specific point 11).

We understand that the experimental revision is both substantive and the outcome uncertain, and we would therefore understand if you regard this as beyond the scope of the current project and therefore decide to submit your manuscript elsewhere.

Thank you for the opportunity to consider this work, I am looking forward to your revision.

REFEREE COMMENTS

Referee #1:

This manuscript analyzes RNA Polymerase II mutations, specifically in the Rpb1 subunit and the Rpb9 subunits, for their effects on DNA damage repair and replication in yeast. The authors tested how mutations in RNA Polymerase II genetically interact with DNA damage repair factors and how they increase the propensity of DNA replication fork stalling. They propose a model where these mutations increase RNA Polymerase II stalling during transcription, which then leads to increased collisions with DNA replication machinery. Although this is a long-standing model in yeast and prokaryotes, this paper supplies some informative experiments that support the idea. The shortcomings of the paper are that it fails to directly connect the repair defects of the Pol II mutations with stalling as proposed in Fig 7D. In addition, the title is slightly misleading in suggesting a "function", suggesting there is some activity of wild-type Pol II to counteract stalled replication forks, when it is in fact a loss of some Pol II function (i.e., loss of processivity, etc.) that contributes to enhanced replication fork stalling. This would be more simply stated as "mutations in RNA polymerase II that increase genomic instability" rather than the implication that wild-type RNA Polymerase has some direct function in counteracting stalled replication forks. Ultimately, the conclusions aren't that satisfying given that the mechanistic basis for the connection between Pol II mutation and damage sensitivity remains completely unknown. The authors clearly acknowledge this fuzziness when they write in the discussion: "The differential DDR phenotypes of the mutants suggest that, although transcription elongation impairment can be associated with genome instability there is no strict correlation with the DDR defect."

a) For the yeast spot and growth assays it is important to state in the figure legends the temperature and time of growth. Indeed, in many other experiments in the paper (e.g., ChIP experiments) the temperature at which cells were grown is omitted. This is particularly important for rpb1-1 strains, which are temperature sensitive and likely show reduced rpb1 expression as the temperature increases. Also, by cutting out little strips to show the spotting, there's no way to know if all these strains were grown in exactly in parallel for the same amount of time and temperature. It's like running every sample on a different gel. Figure E1B is missing controls for WT, and for mre11 or rad52 alone.

b) Fig 2A - really should have a total H2A control to make conclusions about phosphorylation

c) Fig 3Ba - It would be very helpful to have diagrams to show how the gels species correspond to replication intermediates.
d) rpb2-10 shows the genetic interactions with repair factors, but not hyper-recombination or the replication defect in 2D gels. Is there an obvious explanation for this? Otherwise, this would seem to invalidate the connection the authors try to make between these phenotypes.

e) p9 E4A. I don't understand the argument about epistasis, since the single mutants looks similar to each other. On what basis do you say rpb1 mutant is epistatic?

f) In figure 5A it is impossible to distinguish between the phosphorylated and un-phosphorylated forms of Rad53 (i.e. there is no obvious doublet) making interpretation difficult. The authors should create another blot that shows full separation between phosphorylated and un-phosphorylated forms so they can perform quantification.

g) In figure 5B there seems to be incongruity between "BC" and "CF" in the figure and figure legend.

h) In figure 6C, the authors show increased RNAPII on the PMA1 gene in the rpb1-1 background and use this to claim increased Pol II stalling throughout the genome in rpb1-1 cells. However, a previous whole genome Pol II ChIP in rpb1-1 cells did not report a global increase in Pol II signals (Kim et al., RNA polymerase mapping during stress responses reveals widespread nonproductive transcription in yeast, Genome Biology 2010, 11:R75), so to be convincing the authors would need to assay more genes and address the discrepancy. This point is particularly important for the author's proposed model (Figure 7D, noted in the abstract as well) where increased Pol II stalling in the rpb1-1 mutation is the reason for the DNA damage sensitive phenotype, even though the other Pol II mutants tested, rpb1-S751F and rpb9Δ share similar DNA damage phenotypes but no apparent evidence of increased Pol II stalling (fig. 6C).

i) A bit more information of the rpb1-1 mutation in terms of its location in the Pol II structure and why the authors believe that this mutation may lead to increased stalling and collisions with the replication machinery would be informative. Rpb1-1 is presumably unstable/mis-folded at higher temperatures. Perhaps it forms unstable/partial pre-initiation complexes that actually never start transcription but remain at the promoter? (Zanton SJ, Pugh BF: Full and partial genome-wide assembly and disassembly of the yeast transcription machinery in response to heat shock. Genes Dev 2006, 20:2250-2265.) The appropriate background citations and greater description of the properties of these mutants would be helpful to the reader.

Referee #2:

The starting premise was that mutations in RNAPII itself, and not just RNA processing defects, might cause replication-transcription conflicts. The authors thus queried a panel of RNAPII mutants for synthetic growth defects (alone or with MMR or HU) with HR functions, with the logic being that such conflicts would result in broken RFs that require HR for re-start. This eventually led to a focus on 3 specific mutants: rpb1-1, rpb1-S751F and rpb9Δ. That DSBs occur in these mutants was inferred by accumulation of H2A-P and Rad52 foci, and by enhanced HR and plasmid loss (Fig. 2). This was followed by demonstration that S phase entry (FACS) and RF initiation/progression (2D gels) were delayed (Fig. 3), through an analysis of BrdU incorporation at specific loci and by DNA combing (Fig. 4). Analyses of the rpb1 alleles in rad53 backgrounds suggested a problem with replication re-start, which was interpreted as a more persistent RNAPII obstacle (Fig. 5). A trivial explanation of the effects could be that the RNAPII defects were secondary and reflected a specific change in gene expression. The presentation of the microarray data related to this is problematic (see specific comment below) and should be clarified or eliminated. It is clear, however, that expression patterns of the 3 mutants analyzed is very different - the expectation was that the rpb1 mutants would look similar (and they don’t), and different from the rpb2 mutant (and they do). An examination of RNAPII at the PMA1 locus revealed that it is enriched only in the rpb1-1 mutant, even though transcription (by northerns) was reduced in all mutants analyzed. Finally, the distribution of Rrm3, which is used to removed protein obstacles during replication, was examined genome-wide in the rpb1-1 background. This revealed an accumulation at ORFs and at ARS segments. A huge amount of data is presented, and overall, the data are convincing (but see specific comments below). The conclusion that an elongation effect per se, which all of the mutants have, is not sufficient to trigger genome instability is supported. Based on the rpb1-1 data, it is suggested that the persistence of RNAPII on DNA causes replication problems, and a model is presented in
Fig. 7D. Altogether the data make a nice story. Below are comments that need to be addressed when revising the manuscript.

1. The R-loop data are critical to the interpretation that these mutants cause a very different problem. These data should be moved to the main text.

2. The authors need to refrain from over-attributing the fork progression role directly to RNAPII itself. With regard to this, the title would benefit from a change.

3. p. 6 - would not characterize the growth defect of rpb9 with rad52/mer11 as "strong" (Fig. 1A)

4. Fig. 2B - ts effect only convincing in the rpb1-l mutant

5. Fig. 2E - hyper-rec effect not evident in rpb1-l mutant

6. p. 7, line 23 - "consistent with the idea" infers that this has been previously suggested. If so then please provide a reference. If not, then change to "suggests that."

7. It needs to be clearer in the text (p. 8) that Fig. 4A queries only specified genes; Fig. 4B is the much more global analysis.

8. Representative combing data should be shown in the supplement.

9. p. 9, line 6 - "consistent with the idea" infers that this has been previously suggested. If so then please provide a reference. If not, then change to "suggests that."

10. p. 9 - paragraph describing with the X-molecules on 2D gels is out of place and should be moved to previous page where the other 2D gel data are described.

11. The analysis in Fig. 6A is unclear. It is stated that rpb2-10 is a "control", but then the authors focus on genes that are coordinately up- or down-regulated in all 3 mutant backgrounds. Shouldn't the focus be on the intersection of rpb1-l and rpb1-S751F that does NOT also intersect with rpb2-10? In the text, it is stated that 63 genes were co-regulated in all 3 mutants, but there are only 34 in Fig. 1A (18 up +16 down = 34 total). The textual description on p. 11 (end of 1st paragraph) also does not fit with the analysis in Fig. E6A. Finally, the examination of the 3 "selected" genes from among those whose expression was altered is less than satisfying. Maybe the authors simply did not look the "right" genes. The sweeping conclusions that are drawn for this analysis need to be toned down.

12. The decrease of Rrm3 (in ChIP-chip analyses) at centromeres is not evident because the positions of the centromeres are not indicated in Fig. 7A. In Table E4, the difference is NOT significant.

13. Fig. 7B is not referenced in the text, and is described after Fig. 7C (which is mistakenly referred to as Fig. 7D in the text on p. 13, line 7). Please clarify the definition of "ARS segments" in Fig. 7B, and the orientation of segments (1-12 and 1-11) with respect to transcription.

14. Please explain to the reader what "transcription slippage" is (p. 14).

English/grammar issues
Abstract, line 9: compensated for
p. 3, line 6: to coordinate these processes and guarantee
line 26: sentence that begins here makes no sense as written
p. 4, line 1: indicating that
line 6: been implicated in
line 16: helicases are involved
line 26: but their accumulation is greatly enhanced
Need to define HU, MMS, BrdU, 6-AU and MPA in text
p. 5 sentence beginning on line 4 of Results - correct tenses; line 11 change to we did not find viable double mutants; next sentence chang to rpb1-S751F was tolerated in both...
p. 6, next to last line: delete pretty
It is not clear which promoter the L-lacZ and GL-lacZ constructs have. Please clarify in text or Fig.
p. 7, last sentence - correct tense
p. 8, sentence beginning on line 9 is too long
line 21: other other
line 23: replace provided that they with which
line 31: located near a late...
line 34: replace accused with severe
p. 9, line 19: epistatic to
p. 19, line 21: demand is less
p. 11, line 17: suggesting that a secondary...does not explain
line 31: We found that htt1 prevents...levels were lower...
p. 12 First sentence on p. 12 worded too strongly
6th sentence in middle section is redundant with the following sentence
p. 14, line 26, and does not have a transcription...
p. 15, line 26, clarify what is meant by "movement of the fork away from the fragment..."
line 28, three mutants
line 31, consistently
p. 16, line 31, mutated RNAPII is an inability to resolve RF stalls
lines 31 and 33 - change that to why
p. 17, sentence beginning on line 15 is awkward
line 20, as occurs in
line 28, sentence is too long. Also unclear what is meant by "trapped in the collision"
line 36, constraints serve as a signal to prevent the replisome from entering ...and colliding...or that defective...and become DSBs (refs). Deregulation of...

Figure 1: sideways triangles look like something is being pointed to.
No error bars in part of Fig. 2E
Fig 3A - label Y-axis time after G1 arrest
Fig 3B - provide a schematic for 2D gel

In the references, make sure multiple references in the same year have the designation a,b,etc, as they do in the in-text citations.

1st Revision - authors' response 20 July 2014

Editor

The points raised by the two referees are well considered and clearly expressed, and I will not go through them in detail here. I just outline a few key inconsistencies in the data presented that would likely involve further-reaching more extensive molecular investigation: 1. rpb2-10 shows genetic interactions with repair factors but no hyper-recombination or replication defects, which does not seem to support the core model (referee#1, specific point d). 2. there is controversy regarding increased genome-wide Pol II stalling in rpb1-1 cells - it would be essential for the proposed model to decisively corroborate genome-wide increased Pol II stalling, while current interpretations only base on analysis of a limited number of loci (referee#1, specific point f). 3. Too few factors were functionally tested in figure 6/E7A-C, and the criteria of their selection from the Chips were not fully conclusive given point 1 above (referee #2, specific point II).

These 3 points remarked by the Editor have been addressed with particular care and the answers are indicated below.

Referee #1:

This manuscript analyzes RNA Polymerase II mutations, specifically in the Rpb1 subunit and the
Rpb9 subunits, for their effects on DNA damage repair and replication in yeast. The authors tested how mutations in RNA Polymerase II genetically interact with DNA damage repair factors and how they increase the propensity of DNA replication fork stalling. They propose a model where these mutations increase RNA Polymerase II stalling during transcription, which then leads to increased collisions with DNA replication machinery. Although this is a long-standing model in yeast and prokaryotes, this paper supplies some informative experiments that support the idea. The shortcomings of the paper are that it fails to directly connect the repair defects of the Pol II mutations with stalling as proposed in Fig 7D. In addition, the title is slightly misleading in suggesting a "function", suggesting there is some activity of wild-type Pol II to counteract stalled replication forks, when it is in fact a loss of some Pol II function (i.e., loss of processivity, etc.) that contributes to enhanced replication fork stalling. This would be more simply stated as "mutations in RNA Polymerase II that increase genomic instability" rather than the implication that wild-type RNA Polymerase has some direct function in counteracting stalled replication forks. Ultimately, the conclusions aren't that satisfying given that the mechanistic basis for the connection between Pol II mutation and damage sensitivity remains completely unknown. The authors clearly acknowledge this fuzziness when they write in the discussion: "The differential DDR phenotypes of the mutants suggest that, although transcription elongation impairment can be associated with genome instability there is no strict correlation with the DDR defect."

Thank you for the helpful, constructive and thoroughly revision. We believe that the new data added provides more convincing arguments for the connection of the PolIII mutations with replication stalling, as we eliminate any option that an indirect effect is the main cause of it, adding more specific analysis on the regions analysed by ChIP-chip and new experiments showing that genes whose lower expression, as determined from microarray analyses, cannot be responsible for the phenotypes detected. The title has been changed accordingly.

a) For the yeast spot and growth assays it is important to state in the figure legends the temperature and time of growth. Indeed, in many other experiments in the paper (e.g., ChIP experiments) the temperature at which cells were grown is omitted. This is particularly important for rpb1-1 strains, which are temperature sensitive and likely show reduced rpb1 expression as the temperature increases. Also, by cutting out little strips to show the spotting, there's no way to know if all these strains were grown in exactly in parallel for the same amount of time and temperature. It's like running every sample on a different gel. Figure E1B is missing controls for WT, and for mre11 or rad52 alone.

The information requested has been included. All experiments were performed at semi-permissive temperature for rpb1-1 mutant, 30°C. This is now indicated in Material and Methods. Exceptions are indicated in figure legends (Fig. 2B Rad52 foci 37°C). The "missing controls" of Fig E1B were in Fig 1A, but we agree with the referee and now they are also included in Fig E1B. Thanks.

b) Fig 2A - really should have a total H2A control to make conclusions about phosphorylation

H2A phosphorylation is not a question of levels; either H2A is not phosphorylated at all or a phosphorylation signal is seen at the sites of DNA breaks. In the gel, the undamaged WT control is non-phosphorylated, as it should be; this is why an anti-H2A western is unnecessary and nobody provides it. It is important to keep in mind, in case the referee is thinking on the possibility that the mutants could have extremely high levels of H2A, that changes in histone levels are not tolerated by yeast cells, as the stoichiometry of these proteins are very exquisite and any change would kill cells, and that our microarray analysis confirms this fact. On the other hand, we use the standard loading control used by many labs, β-actin, whose stoichiometry levels in cells is critical for life also. It can be seen in the gel that even being the loading of proteins (as deduced from β-actin) a bit lower in rpb1-1 respect to WT, in the absence of DNA damage H2AP is only visualized in the mutant. Therefore the only interpretation possible for this result is that even if the loaded protein and H2A were a bit lower, we are able to detect phosphorylation. These results are repetitive and certainly consistent with the Rad52-P foci and hyper-recombination.

c) Fig 3Ba - It would be very helpful to have diagrams to show how the gels species correspond to replication intermediates.
Diagrams are included in Fig. 4B (previous Fig. 3B) as requested.

d) *rpb2-10* shows the genetic interactions with repair factors, but not hyper-recombination or the replication defect in 2D gels. Is there an obvious explanation for this? Otherwise, this would seem to invalidate the connection the authors try to make between these phenotypes.

This point is based on an over-interpretation of our conclusions that we do not agree with. We are not proposing a connection between transcription elongation defects and DDR defects in the way the referee is presenting. Indeed as this referee remarks in his first and general paragraph we say: “... although transcription elongation impairment can be associated with genome instability there is no strict correlation with the DDR defect.” Therefore the *rpb2-10* phenotype does not invalidate any of our conclusions. The fact that mutations in RNAPII that show a DDR defect are affected in transcription elongation, does not mean at all, nor we conclude, that whatever defect in transcription elongation has to be accompanied by a DDR phenotype. If that were the case just adding 6-AU to cells should produce a DDR defect and this is certainly not the case. The nature of the transcription defect of *rpb2-10* is different from the other mutations and we do not imply to establish a rule for this association between transcription elongation defects and DDR defects. Specific mutations affecting elongation generates a DDR defect, but this is different in each mutant analysed. We need to understand first the nature of the transcription defect to have a proper explanation on how a specific mutation that affects transcription elongation impacts the DDR response or other phenotypes. Indeed there are many different options from the transcription angle that the transcription community does not understand yet about the effect on elongation of different RPB mutants and mutants of transcription elongation factors. For example, Mason and Struhl (Mol Cell 2005) show different in vivo transcription elongation behaviours of different mutants, but also similar transcription elongation behaviour of mutants that have a completely different effect on RNA processing an export. It would not make sense trying to establish a strict correlation between elongation defects and other phenotypes as a rule, as we do not understand yet the structural and functional nature of elongation defects. Our previous works with some of the mutants analysed by Mason and Struhl indicate indeed that it cannot be established a simple rule connecting transcription elongation defects with DDR defects.

In summary, a transcription defect may affect the rate of elongation, affinity to chromatin, degradation of RNAPII after stalling, response to Ubiquitination, coupling with RNA processing, transcription-coupled repair efficiency, etc.; there are many features that may be specific for different transcription elongation mutants. We have been careful enough in this paper not to establish a rule or strict association between elongation defects and DDR. We identified a specific subset of transcription-defective RNAPIIs that allows us to define a positive role of RNAPII in avoiding the conflicts with replications. It turns out that this subset of mutations is affected in elongation, but this does not mean that all elongation-defective mutants have to have a DDR phenotype.

Attending the question of this referee, we have made sure in any case that the message in the text is the one explained here. Thanks.

e) p9 E4A. I don’t understand the argument about epistasis, since the single mutants looks similar to each other. On what basis do you say *rpb1* mutant is epistatic?

*rpb1-1* and *rad51Δ* mutants show a different cell cycle pattern in the presence of HU. *rpb1-1* cells take longer to enter into S-phase than *rad51Δ* ones (vertical lines are incorporated in the figure to improve comparisons). This indicates that replication in *rpb1-1* is slower than in *rad51Δ*. This result is corroborated by 2D gel electrophoresis, 100 minutes after release replication has passed through the analyzed region in *rad51Δ* but not in *rpb1-1*. *rpb1-1 rad51Δ* double mutant has a profile similar to that of *rpb1-1*. This has been briefly reasoned in the manuscript. Thanks for the clarification.

f) In figure 5A it is impossible to distinguish between the phosphorylated and un-phosphorylated forms of Rad53 (i.e. there is no obvious doublet) making interpretation difficult. The authors should create another blot that shows full separation between phosphorylated and un-phosphorylated forms so they can perform quantification.

As requested, experiments have been repeated, and a new gel clearly showing the Rad53-P band separated from the non-phosphorylated Rad53 has been included in Fig. 6A (previous Fig. 5A). The result is validated by quantification. Thanks for making us improve the manuscript.
The EMBO Journal Peer Review Process File - EMBO-2014-88544

The EMBO Journal

Peers Review Process File

EMBO 2014 88544

© European Molecular Biology Organization

8

8

© European Molecular Biology Organization

8

g) In figure 5B there seems to be incongruity between "BC" and "CF" in the figure and figure legend.

Error corrected. Thanks

h) In figure 6C, the authors show increased RNAPII on the PMA1 gene in the rpb1-1 background and use this to claim increased Pol II stalling throughout the genome in rpb1-1 cells. However, a previous whole genome Pol II ChIP in rpb1-1 cells did not report a global increase in Pol II signals (Kim et al., RNA polymerase mapping during stress responses reveals widespread nonproductive transcription in yeast, Genome Biology 2010, 11:R75), so to be convincing the authors would need to assay more genes and address the discrepancy. This point is particularly important for the author's proposed model (Figure 7D, noted in the abstract as well) where increased Pol II stalling in the rpb1-1 mutation is the reason for the DNA damage sensitive phenotype, even though the other Pol II mutants tested, rpb1-S751F and rpb9∆ share similar DNA damage phenotypes but no apparent evidence of increased Pol II stalling (fig. 6C).

As requested, two other genes have been tested, SPF1 and PDC1 (Fig. E8). In both cases an increase of RNAPII retention is obtained in rpb1-1, as expected. Differences in recruitment values between the three genes analyzed in rpb1-1 mutant are due to differences in the specific transcription level of each gene. Results are repetitive in all cases tested.

We do not find any discrepancy with the results published previously by Kim et al. 2010. They addressed the relationship between RNAPII levels and mRNA levels in response to environmental stimuli. In that paper, rpb1-1 cells were shifted from 24°C to 37°C and did not report a global increase in RNAPII signals. Our ChIP experiments were performed at the semi-permissive temperature of 30°C for rpb1-1, a situation very different and in which RNAPII is affected but it is still functional.

Thank you for making the manuscript more convincing.

i) A bit more information of the rpb1-1 mutation in terms of its location in the Pol II structure and why the authors believe that this mutation may lead to increased stalling and collisions with the replication machinery would be informative. Rpb1-1 is presumably unstable/mis-folded at higher temperatures. Perhaps it forms unstable/partial pre-initiation complexes that actually never start transcription but remain at the promoter? (Zanton SJ, Pugh BF: Full and partial genome-wide assembly and disassembly of the yeast transcription machinery in response to heat shock. Genes Dev 2006, 20:2250-2265.) The appropriate background citations and greater description of the properties of these mutants would be helpful to the reader.

Additional information on rpb1-1 mutant has been added in Discussion (page 16). In addition, we have explained better at the end of Discussion why we consider, on the base of our results, that this mutation may increase collisions.

We do not believe that the formation of unstable pre-initiation complexes may be the explanation for our phenotypes due to the fact that at semi-permissive temperature for rpb1-1 transcription is reduced but not stopped. As we have explained in the previous point, our experiments are performed in a different context, not in temperature-shift conditions. Therefore, we prefer not to speculate in this direction.

Referee #2:

The starting premise was that mutations in RNAPII itself, and not just RNA processing defects, might cause replication-transcription conflicts. ... Altogether the data make a nice story. Below are comments that need to be addressed when revising the manuscript.

Thank you very much for the positive reception of the manuscript and constructive suggestions.

1. The R-loop data are critical to the interpretation that these mutants cause a very different problem. These data should be moved to the main text.

Done as requested.
2. The authors need to refrain from over-attributing the fork progression role directly to RNAPII itself. With regard to this, the title would benefit from a change.

Title changed to “RNA polymerase II contributes to avoid transcription-mediated replication fork stalling”. We believe it is more appropriate now. Thanks.

3. p. 6 - would not characterize the growth defect of rpb9 with rad52/mer11 as "strong" (Fig. 1A)

The growth defect of rpb9 with mre11 strains is only strong in the presence of MMS. We have changed the text accordingly to avoid confusion:

“Also, rpb1-1 and rpb2-10 strains showed strong growth defects in the absence of HR functions. rpb9Δ mutant itself is very sensitive to HU and in the absence of Mre11 presents severe growth defect in MMS.” (Page 6)

4. Fig. 2B - ts effect only convincing in the rpb1-1 mutant

Although rpb1-1 is the most temperature-sensitive mutant tested, in both rpb1-S751F and rpb9Δ strains an increase in Rad52 foci is observed at high temperature. The increments are significant in the three mutants respect to WT strain at 30 and 37ºC and between the two conditions assayed in these three mutants.

5. Fig. 2E - hyper-rec effect not evident in rpb1-1 mutant

The increment in recombination in rpb1-1 between inverted repeats in the pTINV system and between direct repeats in the chromosomal one is statistically significant respect to WT. No difference was observed with the L/GL-lacZ systems, and this could be due to a lower efficiency of HR leading to detectable recombination products, which are different for each assay. This is indicated in page 7.

6. p. 7, line 23 - "consistent with the idea" infers that this has been previously suggested. If so then please provide a reference. If not, then change to "suggests that.”

Changed as requested (now in page 8).

7. It needs to be clearer in the text (p. 8) that Fig. 4A queries only specified genes; Fig. 4B is the much more global analysis.

The text has been clarified accordingly in pages 9-10, as requested.

8. Representative combing data should be shown in the supplement.

As requested, representative DNA fibres of WT and rpb1 mutants are shown in Fig. E4.

9. p. 9, line 6 - "consistent with the idea" infers that this has been previously suggested. If so then please provide a reference. If not, then change to "suggests that.”

Changed as requested (page 10).

10. p. 9 - paragraph describing with the X-molecules on 2D gels is out of place and should be moved to previous page where the other 2D gel data are described.

Moved to page 9 as requested.

11. The analysis in Fig. 6A is unclear. It is stated that rpb2-10 is a "control", but then the authors focus on genes that are coordinately up- or down-regulated in all 3 mutant backgrounds. Shouldn't the focus be on the intersection of rpb1-1 and rpb1-S751F that does NOT also intersect with rpb2-10? In the text, it is stated that 63 genes were co-regulated in all 3 mutants, but there are only 34 in Fig. 1A (18 up +16 down = 34 total). The textual description on p. 11 (end of 1st paragraph) also does not fit with the analysis in Fig. E6A. Finally, the examination of the 3 "selected" genes from among those whose expression was altered is less than satisfying. Maybe the authors simply did not
look the "right" genes. The sweeping conclusions that are drawn for this analysis need to be toned down.

As requested we now focused the analysis on the intersection of rpb1 mutants and text and Fig. 7 (previous Fig. 6) have been changed accordingly and conclusions have been toned down in the text. With the new list of coincident genes down-regulated in rpb1 mutants we have analysed Rad52 foci, recombination and growth in the presence of DNA damaging drugs in mutants of the most relevant genes according to function (Fig. E7).

12. The decrease of Rrm3 (in ChIP-chip analyses) at centromeres is not evident because the positions of the centromeres are not indicated in Fig. 7A. In Table E4, the difference is NOT significant.

The referee is right. We have removed this. Thank you for making us aware of the lack of significance.

13. Fig. 7B is not referenced in the text, and is described after Fig. 7C (which is mistakenly referred to as Fig. 7D in the text on p. 13, line 7). Please clarify the definition of "ARS segments" in Fig. 7B, and the orientation of segments (1-12 and 1-11) with respect to transcription.

Each section of Figure 8 (previous figure 7) is now properly referenced in the text.

Requested clarifications are included in the text (pages 14-15). We would like to emphasize that with the used tool we cannot take into account the orientation respect to transcription of genes and ARSs.

14. Please explain to the reader what "transcription slippage" is (p. 14).

Clarified in page 16 as requested. Thanks

English/grammar issues

All English and grammar issues and text clarification have been performed as requested

Figure 1: sideways triangles look like something is being pointed to. Triangle orientation has been changed for clarification. Thanks

No error bars in part of Fig. 2E

They are present, but they are too small to be seen.

Fig 3A - label Y-axis time after G1 arrest

Performed as requested (arrow included).

Fig 3B - provide a schematic for 2D gel

Included as requested (now Fig. 4B).

In the references, make sure multiple references in the same year have the designation a,b,etc, as they do in the in-text citations.

Updated as requested.

2nd Editorial Decision 02 September 2014

Thank you for submitting the revised version of your manuscript "RNA polymerase II contributes to prevent transcription-mediated replication fork stalls" for consideration to the EMBO Journal. We have now received the assessment of the two referees that you will find copied below.

We are pleased to inform you that both referees concur that you have successfully addressed most of the concerns that had been raised during revision. While referee #2 now thinks that the manuscript is suitable for publication pending some residual changes, corrections and explanations, referee #1 is still concerned that the findings do not yet immediately offer a deeper understanding why only selective RNAP II mutants impede DNA replication fork progression. Similarly, also referee #2
criticizes the very specific claim in the abstract that the data "suggest that RNAPII itself participates in facilitating fork progression" (i.e. in a direct/active role of RNAP II in counteracting stalled forks). Additionally, some clarifications were requested also by referee #2 regarding the occupancy of Rrm3 towards 3' regions in rpb1-1, which was used as additional argument for the more intimate replication-transcription relationship.

At the same time, as expressed by the referees and in-line with the in-depth assessment of an external editorial board adviser, whom we have additionally consulted in this case, we are confident that the presented work is an important contribution to the field. In the adviser's and in our opinion, addressing and understanding the molecular mechanism how RNA and DNA polymerase complexes-dependent processes avoid each other or cross-regulate in the face of transcription-associated recombination due to transcription in S-phase is of high significance, but beyond the scope of the current study.

Together, the editorial team thinks that only a small touch-up in the form of a minor final revision step will be needed at this point before we will proceed to formally accept your manuscript: we invite you to attend to ref#2's points, in particular to a slight textual modification (toning down the conclusion on an active role of RNAPII in counteracting fork progression) as well as to a clarification of Rrm3 association in the ChIP-chip experiment (ref #2). Regarding the latter point the external editorial board adviser also constructively suggested to think about presenting the correlation of Rrm3 enrichment at different classes of genes showing increasing transcriptional activity (in order to show that the frequency of replication fork arrest correlates with gene expression). This re-analysis of the existing might be potentially more informative than showing average profiles for all genes in the genome.

To us, addressing these two points is sufficient for the formal acceptance of your manuscript for publication in the EMBO Journal, that is without the need for generating additional new experimental data.

I congratulate you on your work and am looking forward to receiving the final manuscript after the small modifications requested. Please do contact me regarding any questions related to this decision.

------------------------------------------------

REFEREE COMMENTS

Referee #1:

The revised paper addresses many of the technical comments raised in the first review. However, the paper still presents lots of observations without any clear explanation for why only certain mutations that inhibit transcription elongation lead to increased replication fork collisions. An added paragraph in the discussion provides some ideas about how the affected polymerase mutants might be different, and still suggests some kind of vague active role for polymerase in preventing collisions. But no supporting data are provided, so to me the conclusions of the paper still feel too preliminary and speculative.

Referee #2:

The authors did a nice job revising the manuscript and addressing the concerns of the previous reviews. Below are a few remaining issues that should be considered when making final revisions.

The title is awkward. Change to "contributes to preventing" or "contributes to the prevention of"

In the Abstract, the authors still imply a direct/active function of RNAPII in counteracting stalled forks ("RNAPII itself participates in facilitating fork progression").

p. 2, line 9 compensated for by additional
p. 7, line 27 may be more correct to say that mre11 abrogates rather than abolishes early steps of DSB repair.

Bottom of p. 7 - logic concerning increased SSA and reduced Rad52 foci is not clear.

Figure 3D - might be worth mentioning that hpr1 is a positive control for R-loop formation and reduction by RNH1 overexpression.

p. 8, line 17 schematic of replication intermediates is 4B, not 4C

Fig. E3A - double mutant looks more rad51-like to me.

Fig. 6A - to the naked eye, the persistence of Rad53 phosphorylation in the mutants is clear, but at later times, these look the same as WT

The description of Fig. E6A in the text is confusing. Is the horizontal line the genome average in WT? If so, up- as well as down- regulated genes are shorter.

Table E4 needs an explanatory legend.

p. 14, line 8 - there is no evident increase in Rrm3 occupancy near the 3’ end in rpb1-1

Editorial comment:

Together, the editorial team thinks that only a small touch-up in the form of a minor final revision step will be needed at this point before we will proceed to formally accept your manuscript: we invite you to attend to ref#2’s points, in particular to a slight textual modification (toning down the conclusion on an active role of RNAPII in counteracting fork progression) as well as to a clarification of Rrm3 association in the ChIP-chip experiment (ref #2): Regarding the latter point the external editorial board adviser also constructively suggested to think about presenting the correlation of Rrm3 enrichment at different classes of genes showing increasing transcriptional activity (in order to show that the frequency of replication fork arrest correlates with gene expression). This re-analysis of the existing might be potentially more informative than showing average profiles for all genes in the genome. To us, addressing these two points is sufficient for the formal acceptance of your manuscript for publication in the EMBO Journal, that is without the need for generating additional new experimental data.

Thanks for the positive reception of the manuscript and the positive comments. We believe to have toned down the message and with respect to the second point we present a new Extended Figure E11 where the correlations requested are shown. However, it is important to note that this type of comparison do not take into the account the fact that the expression level values used correspond to mRNA accumulation levels in an asynchronous culture; therefore we do not have the information of the transcriptional activity at the genes during the S phase, which is when Rrm3 is supposed to be present together with the replication fork. With this limitation, however, the results are consistent with our expectations.

Referee #1:

The revised paper addresses many of the technical comments raised in the first review. However, the paper still presents lots of observations without any clear explanation for why only certain mutations that inhibit transcription elongation lead to increased replication fork collisions. An added paragraph in the discussion provides some ideas about how the affected polymerase mutants might be different, and still suggests some kind of vague active role for polymerase in preventing collisions. But no supporting data are provided, so to me the conclusions of the paper still feel too preliminary and speculative.
We certainly disagree with this comment. The paper provides new results that help understand the role of RNA polymerase in the maintenance of genome integrity and new models are discussed up to the level that are well supported. We agree that we need to get deeper in the future, as with any other problem addressed in Molecular Biology.

Referee #2:

"The authors did a nice job revising the manuscript and addressing the concerns of the previous reviews. Below are a few remaining issues that should be considered when making final revisions."

Thank you very much for the positive reception of the manuscript and for the suggestions.

The title is awkward. Change to "contributes to preventing" or "contributes to the prevention of"

Title changed as requested to "contributes to preventing".

In the Abstract, the authors still imply a direct/active function of RNAPII in counteracting stalled forks ("RNAPII itself participates in facilitating fork progression").

Changed.

p. 2, line 9 compensated for by additional

Changed.

p. 7, line 27 may be more correct to say that mre11 abrogates rather than abolishes early steps of DSB repair.

The referee is right. We have changed it accordingly. Thank you.

Bottom of p. 7 - logic concerning increased SSA and reduced Rad52 foci is not clear.

We have changed the sentence and we expect it is clearer now. Thanks.

Figure 3D - might be worth mentioning that hpr1 is a positive control for R-loop formation and reduction by RNH1 overexpression.

Done as requested.

p. 8, line 17 schematic of replication intermediates is 4B, not 4C

Error corrected. Thanks.

Fig. E3A - double mutant looks more rad51-like to me.

Considering both cell cycle pattern and 2D-gel electrophoresis we believe that the double mutant is similar to the rpb1-1 single mutant.

Fig. 6A - to the naked eye, the persistence of Rad53 phosphorylation in the mutants is clear, but at later times, these look the same as WT

For later times we took advantage of Odyssey IR scanner and the Image Studio 2.0 software (LI-COR) and we were able to appreciate the differences between WT and rpb1 mutants.

The description of Fig. E6A in the text is confusing. Is the horizontal line the genome average in WT? If so, up- as well as down- regulated genes are shorter.

The horizontal line is the genome average based on large-scale data for the yeast Saccharomyces cerevisiae (Marin 2003, Beyer 2004). The referee is right, up and down-regulated genes are shorter. We have modified this accordingly.

Table E4 needs an explanatory legend.

Included as requested.

p. 14, line 8 - there is no evident increase in Rrm3 occupancy near the 3' end in rpb1-1.

The mistake has been corrected. Indeed, there is a slight tendency to increase towards the 5'-end in rpb1-1.
Thank you for submitting your revised manuscript ("RNA polymerase II contributes to preventing transcription-mediated replication fork stalls" to The EMBO Journal. I appreciate the introduced changes and I am pleased to inform you that we will accept your manuscript for publication. Your article will be processed for publication in The EMBO Journal by EMBO Press and Wiley.