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Genome-wide function of THO/TREX in active genes prevents R loop-dependent replication obstacles

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1st Editorial Decision 19 January 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below, as you will see the referees express interest in the study especially the role of THO/TREX complex in replication fork progression and require further experimentation and analysis before the manuscript is suitable for publication in The EMBO Journal. These include further controls and analysis of the ChIP experiments, comparison with replication fork data and analysis of replication fork progression via 2-D gels. Referee #3 suggests removing significant amounts of data to the supplement, I am happy to discuss this matter further after you have had time to consider these points. Given the interest in the study should you be able to satisfactorily address these issues, we would be happy to consider a revised version of the manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Gomez-Gonzalez et al. reports a genome-wide characterization of the THO/TREX complex. The authors compare the genome-wide distribution of this complex to RNAPII in ChIP-chip profiling. They also investigate gene expression regulation in THO/TREX complex. They find that the two investigated members of THO/TREX complex, Hpr1 and Sub2, bind RNAPII genes. In contrast to RNAPII binding (Rpb3 subunit) that has a similar binding profile along the total length of ORFs, Hpr1 and Sub2 are accumulated more in the 3' end of ORFs. They conclude that the recruitment of THO/TREX complex is global and not cell cycle dependent because they do not detect any changes in binding profile throughout the cell cycle. To study THO complex in replication fork progression, they investigate if the Rrm3 helicase, that has been shown to be required for replication past stable non-nucleosomal protein-DNA complexes, is recruited to the same sites as hpr1 and if this recruitment is enhanced in hpr1 deleted mutants. By performing ChIP-chip with Rrm3 in WT or hpr1 deleted background, they observe an enhanced recruitment of Rrm3 in hpr1 deleted cells compared to WT cells. In addition they detect that hpr1 recruitment in WT cells overlaps with Rrm3 recruitment in hpr1 deleted cells. Finally, to investigate if Rrm3 accumulation in hpr1 deleted cells is due to formation of transcription dependent R-loop formation, they performed ChIP in RNaseH1 overexpressed cells. They detected lower levels of Rrm3 to the FAS2 ORF when RNaseH1 is overexpressed and conclude that the genomewide binding profile of THO/TREX is to prevent R-loop formation in transcription that may impair replication fork progression.

The manuscript is clearly written and the study is interesting. However, I have several concerns about the analysis of the data and the absence of important control experiments. For acceptance, the authors need to reevaluate their ChIP-chip data and also show data for unspecific binding for their antibodies in No tag strains. They need to perform ChIP in a Sub2 helicase dead mutant and describe how the spatial distribution of the THO/TREX complex along the ORF is determined. They also need to perform more quantitative 2D gels. These points are described in more detail below. Although the ideas in this paper are very interesting for general readers, there are for the moment not sufficient information to establish the interpretation favored by the authors. My specific comments are as follows:

Major comments:

1. One major concern is that the authors do not use very stringent criteria in the Chip-chip analyses. They considered positive binding in ChIP-chip when log2 ratio is 0.75 and above (which is only 1.68 fold higher than background), P values <0.025 and binding to one probe or more within one ORF. Together, these are all quite low cut offs and may therefore give too large false-positive results. In addition, unless I somehow missed it, a critical control is missing: ChIP-chip experiments in a No-tag strain as control, to examine the actual unspecific binding of the antibodies used in the immuno-precipitation experiments. The authors should reevaluate the ChIP-chip data with higher threshold and add the No-tag strain controls to demonstrate the specificity of the THO/TREX complex, Rpb3 and Rrm3 in the ChIP-chip analysis.

2. Important details are missing in both the experimental procedures and results sections. For example details regarding how experiments were done in the sub2 deleted strain (since sub2 deleted cells are described as inviable or very sick) and also about the growth behavior of the tagged strains compared to WT cells. Information on the average shearing size of the ChIP DNA and how this was determined should be provided so readers can judge the resolution of their ChIP-chip microarray data. This information is particularly important since they make conclusions about the spatial distribution of the THO/TREX complex along the ORFs. Given the small size of most yeast genes and the typical resolution of array studies, it's not clear to me that the resolution is high enough to

see regional differences in ChIP within ORFs that they report.

3. To answer the issue that they raise regarding the major abundance of THO complex toward the 3'-end of genes (page 14, last paragraph), the authors should perform ChIP with Sub2 helicase-dead mutant (if viable) which may enhance binding profiles in the genome, since it might reduce its movement along the genome and therefore reveal the immediate binding sites for Sub2.

4. From the appearance of the 2D gel data, there seems to be some slower fork progression in hpr1 deleted cells as the fork reaches the end of the ORF (suppl fig 8c), which the authors do not mention even though it helps their argument. However it is hard to reach a clear conclusion from the data presented both because the 2D gels for the WT and hpr1 deleted cells have very different background intensities and the gels were not quantified. The 2D gels should be repeated to get better quality and quantitated to see if they can document differences in fork progression as forks move through the ORFs. Moreover, they should present 2D gels on at least 3 ORFs for all strains to demonstrate the generality of their findings. Finally they should also do 2D gels with hpr1rrm3 double mutants as these would provide a better test of their conclusion regarding THO/TREX's role in preventing collision between transcription and replication machineries, when both the replication machinery and the transcription machinery "obstacle removers" are absent. These experiments are necessary to make the paper suitable for publication.

Minor comments:
1. The authors should clarify how hits per segment (%) is calculated in fig 3 and fig6.
2. What does the color grey, yellow and orange correspond to in fig 2, 4 and 5?
3. Improve the resolution of suppl. fig 7.
4. The authors should add more detailed information in Material & methods.
5. I could not find detailed information about the references added in suppl. table 2. These references should be added.

Referee #2 (Remarks to the Author):

This is an interesting study on the role of THO/TREX complex in gene transcription and genome replication. It shows that that Hpr1 and Sub2 components of this complex are co-diributed with ORFs genome-wide. Deletion of the corresponding genes down-regulate expression of the long, GC-rich yeast genes. Furthermore, deletion mutants are characterized by over-recruitment of the Rrm3 helicase to the actively transcribed genes, which is reduced upon RNase H overexpression. This leads the authors to propose that the role of THO/TREX complex is to prevent transcription indiscretions, such as R-loop formation, which could serve as obstacles for the replication fork progression.

The data are fairly straightforward and their interpretation is, by-and-large, sound. I have, however, several concerns/questions that need to be addressed in somewhat more depth.

(1) I am aware of two groups of data on the effect of R-loops in DNA replication. One group have suggested that R-loops are responsible for fork stalling during their head-on collisions with transcription, while another group proposed that co-directional collisions are primarily affected by the R-loop formation. Given that the direction of replication is known for every gene in S. cerevisiae, it would be great if the authors explored this matter in some depth in their system.

(2) While the final mechanisms for the R-loop formation remains to be understood, it is quite clear that it depends on the strength of the RNA-DNA hybrid. The latter is not simply a function of a gene's GC content, but results from the sequence bias towards guanines in the sense strand for transcription. Do the authors see such bias within the genes where they expect R-loop formation, such as PM1, ACT1, DPB2, etc.

(3) I didn't get from the paper, what is the authors' idea on how THO/TREX precludes R-loop formation.

Referee #3 (Remarks to the Author):

Summary
Andres Aguilera's lab has made major contributions to understanding mitotic recombination hotspots, the THO/TREX complexes, R-loops, and several important related topics. This manuscript continues work exploring the function of the THO complex, this time focusing on the THO subunit Hpr1 and the RNA-dependent ATPase Sub2, part of the TREX complex. The authors use a high-throughput, genome-wide approach to determine where THO/TREX binds and correlate this to binding of an RNA PolII subunit, Rpb3. Additionally they determine localization of the DNA helicase Rrm3, known to relieve replication fork pauses, in the presence and absence of Hpr1 and find that loss of Hpr1 results in more Rrm3 recruitment, potentially due to more transcription-induced replication fork stalls. Furthermore, their data raise the possibility that R-loop formation due to loss of Hpr1 may be the reason more Rrm3 is recruited to these regions.

General Comments
This paper has several interesting aspects (especially Figure 7, and Suppl. Fig. 8). Other parts of the paper are descriptive, and the paper may be need to be re-organized.
Figure 7 of the paper is quite interesting and would seem to be the basis for a short paper on the point that loss of the THO complex causes accumulation of Rrm3 in the 3' half of the gene. One wonders if a paper that concentrated more on this might have greater focus. However, the authors may need to tighten up their thinking and interpretation of this point. For example, Figure 7A and 7B do not seem to match so well at what one assumes is the middle region probed. That is, the difference between the grey and black lines in Figure 7A is small, but the histogram (which is real-time PCR) is large. Likewise Figure 6C shows that the WT and hpr1 mutant versions are similar in the body of the gene, but are different in Figure 7B. Such differences between the genome-wide approach and the more quantitative real-time data cause one to wonder how general is the genome-wide ORF examination.

Specific Comments

1. While the values in figure 1c show interesting trends, the differences between the two groupings are rather small, and it is unclear if they are statistically significant. Also, the authors never describe how they are measuring expression or define the "AU" units.

2. Section 2 of the results contains the what may be inconsistent statements "THO is mostly recruited to the same genes as Rpb3" and "the THO complex is recruited to all RNAPII transcribed genes". Examining the ChIP on chip it does appear that there are regions with strong recruitment of Rpb3 and no Hrp1 or Sub2.

3. The R2 values for the linear comparisons of Rpb3-Sub2 and Hpr1-Sub2 seem low. For Rpb3-Sub2 there are almost as many Sub2 hits that don't correlate with Rpb3 as there are ones that do. Perhaps a defined P value would bolster the argument that this is a distinct overlap in localization of these components.

4. The setup for the composite ORF analysis is a bit confusing and a better explanation should be given. Is this every single ORF put together and divided into 10 segments? The trend seen from this analysis is interesting and it appears quite clear that enrichment of Hpr1 and Sub2 occurs further away from the promoter. It would be better to see more specific examples of this, such as those provided in Fig 7, and also include some examples of genes with low and medium gene expression.

5. Figure 5 could be moved to supplemental.

6. Figures 1 to 6 are descriptive. Some of this might be moved to a supplement. It is difficult to draw clear conclusions from this part of the paper.

7. Suppl. Figure 8 might be moved to the main text, since it might correspond well with Figure 7.

8. The implications of the data in 6A that THO may have role with stabilizing heterochromatic regions during replication is potentially interesting. One wonders if HML or HMR on ChrIII show a similar enrichment of Rrm3 in the absence of Hpr1. It is unclear from supplemental figure 7 if this is the case. If there is a correlation, perhaps the analysis of ChrIII could be moved from the supplement

to the text.

9. It is unclear to what the 22-fold increase in Rrm3 enrichment described in section 6 of the results refers. The largest change in figure 7B appears to be about 5-fold

10. The data in supplemental figure 4 is compelling as there are more Hpr1 hits in intragenic regions for genes with convergent transcription than for divergent transcription, indicating a possible role for Hpr1 in relieving the torsional strain created when two transcription bubbles converge. Perhaps this could be incorporated into the paper or as made part of another study.

11. The title seems a bit broader than the data.

Minor

1. Although stated in the figure legend, the y-axis in figures 2A, 4, and 5A should indicate that they are in log2 scale.

2. Figure 3B, x-axis "length" is misspelled.

3. In Figure 7B, it should be unambiguously stated that the small black rectangles correspond to the regions examined by RT PCR

4. Also, in supplemental figure 8B you may want to state that GLY1 is transcribed on the Crick strand or else rearrange the order of the RT results so that they mesh with the results in figure 7B, clearly showing enrichment of the Rrm3 at the 3' end of the gene.

1st Revision - authors' response 25 March 2011

Referee #1:

QUERY 1. *One major concern is that the authors do not use very stringent criteria in the Chip-chip analyses. They considered positive binding in ChIP-chip when log2 ratio is 0.75 and above (which is only 1.68 fold higher than background), P values <0.025 and binding to one probe or more within one ORF. Together, these are all quite low cut offs and may therefore give too large false-positive results.*
In addition, unless I somehow missed it, a critical control is missing: ChIP-chip experiments in a No-tag strain as control, to examine the actual unspecific binding of the antibodies used in the immuno-precipitation experiments. The authors should reevaluate the ChIP-chip data with higher threshold and add the No-tag strain controls to demonstrate the specificity of the THO/TREX complex, Rpb3 and Rrm3 in the ChIP-chip analysis.

ANSWER 1: The No-tag control and the re-evaluation of data have been performed as requested. As a result a new Table (Supplementary Table II) and new Supplementary Figures 2 and 14 have been included. The text has been changed accordingly in Materials and Methods (pages 21-25) and Results (pages 7-8). In detail:

The no-tag strain control was performed as requested by the referee. As expected, this control showed no enrichment in hits (**Supplementary Figure 2**), which is consistent with our conclusion that the hits reported for Hpr1, Sub2, Rpb3, and Rrm3 represent specific sites for recruitment of these proteins.

We performed the analysis following more stringent criteria as suggested by the referee and found that the results and conclusions do not change, as it is explained below. However, we kept the criteria previously published for this type of analysis (Bermejo et al, 2009; Bermejo et al, 2007; Katou et al, 2006; Katou et al, 2003) in the manuscript. These criteria are as indicated in Katou et al, 2003: 'For the discrimination of positive and negative signals for the binding, we compared ChIP fraction with supernatant fraction by using three criteria. First, the reliability of strength of signal was judged by detection P-value of each locus ($P \leq 0.025$). Second, reliability of binding ratio was judged by change P-value ($P \leq 0.025$). Third, clusters consisting of at least three contiguous loci that filled the above two criteria were selected, because it was known that a single site of protein–DNA

interaction will result in immunoprecipitation of DNA fragments that hybridized not only to the locus of the actual binding site but also to its neighbours.'

Conclusions cannot be based on the enrichment value. p-values are very important to state the significance of the binding. It is only with the aim of identifying the genes to which Rrm3 preferentially binds that we apply the cut off of 0.75 $\log_2$ ratio as a further stringent criteria in addition to the p values. The genes thus identified are taken as the top Rrm3-bound genes (Figure 6B). The statistical analyses revealed that high Rrm3 accumulation occurs preferentially in genes with high levels of transcription in both WT and *hpr1*Δ strains, but the average length of genes affected is larger in the *hpr1*Δ mutant. These differences are statistically significant as indicated now in the Figure 6B. Similar results are obtained if we compare the total genes showing Rrm3 binding in WT and *hpr1*Δ.

From our new analysis according to the reviewer suggestion, we produced new binding maps for all the ChIP-chip experiments presented in the manuscript using different statistical analysis with more stringent criteria. Briefly, we defined protein-binding clusters as ranges within the chromosome respecting the following previously described parameters (Bermejo et al, 2009): positive signal log ratio in the whole range, change P-value < 0.2 in the whole range (except for segments within the range shorter than 600 bp). As shown in **Figure for referees 1**, the binding profiles obtained with this analysis show similar results to those obtained using the previous parameters. This is: (i) a high correlation between Rpb3, Hpr1 and Sub2 clusters in asynchronous conditions, (ii) a high correlation between the clusters defining binding of Hpr1 in G1, S or G2 phases (α factor, hydroxyurea, or nocodazol arrested conditions) and those defining the binding of Rpb3 under the same conditions, and (iii) a high correlation between the clusters of Rrm3-binding in WT and those of *hpr1* cells. The significance of such protein-cluster distributions was evaluated by confrontation against a null hypothesis model generated with a Montecarlo-like simulation as described in the new Materials and Methods section. Detailed statistical analysis of the correlations are given in the new **Supplementary Table II.**

Second, we repeated our analysis with higher p-value restrictions ($p<0.01$ for both "change" and "detection"). We observed a very slight decrease in the number of hits and genes showing binding, but, importantly, the main conclusions of the manuscript do not change at all. This is, we still observe the correlation between Rpb3 IP, Hpr1 IP and Sub2 IP and for Hpr1 IP and Rpb3 IP in every cell cycle phase as well as in Rrm3 IP binding in WT and *hpr1*Δ. Also, the patterns of length, G+C and expression levels are still observed and the differences still statistically significant. More importantly, the distribution of hits per segment resists even when we add an even more stringent criteria such as a signal log ratio >0.5, >0.75 or even >1 (see new **Supplementary Figure 14**).

Q2. *Important details are missing in both the experimental procedures and results sections. For example details regarding how experiments were done in the sub2 deleted strain (since sub2 deleted cells are described as inviable or very sick) and also about the growth behavior of the tagged strains compared to WT cells...*
Information on the average shearing size of the ChIP DNA and how this was determined should be provided so readers can judge the resolution of their ChIP-chip microarray data.
This information is particularly important since they make conclusions about the spatial distribution of the THO/TREX complex along the ORFs. Given the small size of most yeast genes and the typical resolution of array studies, it's not clear to me that the resolution is high enough to see regional differences in ChIP within ORFs that they report.

A2: More detailed information about how experiment were done has been included as requested. As the referee indicates *sub2*Δ cells are viable in a number of genetic backgrounds. They are viable in the W303 genetic background, in which we have worked in the past (Jimeno et al, 2002). Even though they grow poorly we could get them to grow to a concentration of 1 x 10^7 cells/ml although using longer incubation times. This has been included in Materials and Methods, as requested.
A new **Supplementary Figure 13** showing that growth of the tagged strains is not affected has been incorporated to the manuscript to respond to the reviewer question.
The information regarding the ChIP method used so that resolution of the data can be judged has been included in Materials and Methods as requested.

Q3. *To answer the issue that they raise regarding the major abundance of THO complex toward the 3'-end of genes (page 14, last paragraph), the authors should perform ChIP with Sub2 helicase-dead mutant (if viable) which may enhance binding profiles in the genome, since it might reduce its movement along the genome and therefore reveal the immediate binding sites for Sub2.*

A3: This is an interesting suggestion, but to our knowledge the predicted Sub2 helicase-dead mutants are lethal (Zhang and Green, 2001, *Genes & Dev* **15**:30-35). We are not aware of any allele in which it has been shown experimentally that the sub2 helicase is inactive. In addition, in our hands it is not possible to obtain reliable ChIP-chip data with very poorly growing strains, as the hit signals are very low. Our study shows that the actual data are sufficiently clear and significant to support our conclusions without any need to enhance even further the binding of THO. Having said this, it is worth noting that it has not been shown that *sub2* helicase-dead or null mutants cause a stronger binding of THO to chromatin as suggested. There is evidence suggesting that *sub2* mutations do not affect THO recruitment to chromatin (Zenklusen et al, 2002). Indeed, we would have expected, in the case that the experiment with a *sub2* helicase-dead mutant had been possible, that abortive elongation caused premature termination and, consequently, the disentangling of the whole transcription unit, in which case the *sub2* mutation could have shown the opposite effect making THO binding looser.

In any case, as mentioned for point 1 to confirm the reliability of our composite profiles, we have tested the distribution of hits per segment when considering only hits with a signal log ratio >0.5, >0.75 or even >1 and $p<0.01$ instead of $p<0.025$ for both change and detection with the concomitant decrease in the number of genes considered to have binding and we have observed that it remains the same, thus bolstering our conclusions. We have added these analyses as **Supplementary Figure 14**.

Q4. *From the appearance of the 2D gel data, there seems to be some slower fork progression in hpr1 deleted cells as the fork reaches the end of the ORF (suppl fig 8c), which the authors do not mention even though it helps their argument. However it is hard to reach a clear conclusion from the data presented both because the 2D gels for the WT and hpr1 deleted cells have very different background intensities and the gels were not quantified. The 2D gels should be repeated to get better quality and quantitated to see if they can document differences in fork progression as forks move through the ORFs.*
Moreover, they should present 2D gels on at least 3 ORFs for all strains to demonstrate the generality of their findings.
Finally they should also do 2D gels with hpr1rrm3 double mutants as these would provide a better test of their conclusion regarding THO/TREX's role in preventing collision between transcription and replication machineries, when both the replication machinery and the transcription machinery "obstacle removers" are absent. These experiments are necessary to make the paper suitable for publication.

A4: It is not easy to find out a region where transcription is high and close enough to a relatively early replication origin as to be able to see the type of differences by 2D gels. We performed 2D gel analyses in 3 ORFs as suggested, trying to cover three origins firing at different times. Unfortunately the third origin was too weak and we could not see it sufficiently fired in our conditions as to get a reliable conclusion. The data of the 2 regions analyzed are shown in the new **Figure 7**. As can be seen, there is a higher accumulation of replication forks along the ORFs. This is clear in *hpr1*Δ *versus* wild-type cells. The facts that specific sites for replication stalling was not expected and indeed was not seen as spots in the Y- or bubble-arcs, and that our 2D gels confirm the previously published 2D-gel analysis in a *lacZ* construct (Wellinger et al, 2006), support therefore the conclusions.

Despite our results being consistent with the impact of *hpr1*Δ on fork progression, it is important to remark that while ChIP-chip have shown that Rrm3 as well as Pol2 are accumulated in highly transcribed genes, 2D gels replication patterns were not affected by deleting RRM3 in the wild-type (Azvolinsky et al, 2009). This implies that the regions where replication fork is slowed down (as recorded by Rrm3 accumulation) do not necessarily accumulate replication fork pauses in the absence of Rrm3. In this manuscript, we use Rrm3 as a measurement of fork progression as we could have used Pol2, since Rrm3 travels with the replisome, but, in agreement with Zakian's lab results, we do not claim that highly transcribed genes have Rrm3 sensitive sites. Therefore, we do not really expect a stronger effect as determined by 2D gels. In addition, as we already reported in

the past, the defect in replication fork progression observed in specific constructs is not observed as an accumulation of spots in the 2D-gel reflecting specific pausing sites (Wellinger et al, 2006). As it is discussed in the manuscript, a replication retardation is observed throughout an extended region that would not be detected as an enriched spot. In one case the *hpr1 rrm3* mutants leads to a further detectable retardation, whereas in the other case this is not clear.

Minor comments:

q1. *The authors should clarify how hits per segment (%) is calculated in fig 3 and fig6.*

a1: Clarified as requested.

q2. *What does the color grey, yellow and orange correspond to in fig 2, 4 and 5?*

a2: The meaning of the different colors is now indicated in the Figure Legends, as requested.

q3. *Improve the resolution of suppl. fig 7.*

a3: In order to improve the resolution of Supplementary Figure 7 (now **Suplementary Figure 11**), we have added a high resolution image as a separate pdf file.

q4. *The authors should add more detailed information in Material & methods.*

a4: Performed as requested. Thank you for helping us to improve the quality of our manuscript.

q5. *I could not find detailed information about the references added in suppl. table 2. These references should be added.*

a5: The reference have been added as requested.

Referee #2:

Q1. *I am aware of two groups of data on the effect of R-loops in DNA replication. One group have suggested that R-loops are responsible for fork stalling during their head-on collisions with transcription, while another group proposed that co-directional collisions are primarily affected by the R-loop formation. Given that the direction of replication is known for every gene in S. cerevisiae, it would be great if the authors explored this matter in some depth in their system.*

A1: This is indeed an interesting question. Nevertheless, at present there are no genome-wide direct measures of replication fork direction and most ORFs in the genome can be replicated by both leftward and rightward moving forks (i.e. from different origins in different cells). In order to analyze the proportion of genes in our lists that are placed in a putative co-directional orientation to replication *versus* a head on orientation to replication, we have considered all the genes which are within 10 kb of an early and active ARS as described by Yabuki et al, 2002. Consistent with previous results by Azvolinsky et al, 2009 in a wild type, we have observed similar proportions of genes with co-directional or head-on replication *vs* transcription among the genes that accumulate high levels of Rrm3 in both WT and *hpr1*Δ. Therefore, our results suggest an orientation independent role of R-loops to stall replication fork progression (measured as Rrm3 accumulation in *hpr1*Δ). These data are now included in the Results and discussed in the Discussion sections. Materials and Methods have been modified accordingly.

Q2. *While the final mechanisms for the R-loop formation remains to be understood, it is quite clear that it depends on the strength of the RNA-DNA hybrid. The latter is not simply a function of a gene's GC content, but results from the sequence bias towards guanines in the sense strand for transcription. Do the authors see such bias within the genes where they expect R-loop formation, such as PM1, ACT1, DPB2, etc.*

A2. We agree that this is an interesting question even though we expect R-loop formation in all transcribed genes in the *hpr1Δ* mutant due to the theory that it is a suboptimal mRNP formation what makes RNA more reactive. Consequently, the level of detection of hybrids will be determined by both the ability to be formed plus the strength of the interaction RNA-DNA once formed.

Following the reviewer suggestion, we have tested the GC bias for the whole genome as well as for genes in which a replication fork impediment is presumably stronger (as determined by Rrm3 binding). The data indicates that a higher proportion of G in the non-transcribed strand is not required for R-loop formation, subsequent replication fork stalling, and Rrm3 accumulation in *hpr1*Δ cells. This is consistent with our conclusions that it is the suboptimal mRNP formation what stimulates instability regardless of which strand is the G-rich strand, in contrast to wild-type cells with optimal mRNP packaging (Ruiz et al, 2011). These results have been included and appropriately discussed in the Results section (page 12).

Q3. *I didn't get from the paper, what is the authors' idea on how THO/TREX precludes R-loop formation.*

A3: The text has been modified in the Introduction section (page 4) to make this point clear. Thank you.

Referee #3:

General Comments

This paper has several interesting aspects (especially Figure 7, and Suppl. Fig. 8). Other parts of the paper are descriptive, and the paper may be need to be re-organized.

Figure 7 of the paper is quite interesting and would seem to be the basis for a short paper on the point that loss of the THO complex causes accumulation of Rrm3 in the 3' half of the gene. One wonders if a paper that concentrated more on this might have greater focus. However, the authors may need to tighten up their thinking and interpretation of this point. For example, Figure 7A and 7B do not seem to match so well at what one assumes is the middle region probed. That is, the difference between the grey and black lines in Figure 7A is small, but the histogram (which is real-time PCR) is large.

Likewise Figure 6C shows that the WT and hpr1 mutant versions are similar in the body of the gene, but are different in Figure 7B. Such differences between the genome-wide approach and the more quantitative real-time data cause one to wonder how general is the genome-wide ORF examination.

We agree that ChIP data seem more informative than ChIP-chip data at a specific region, but it gives us no clues on a global scale. In our study, the ChIP studies are meant to indicate that the results obtained in the ChIP-chip are reliable and this is the case as we are even underestimating the real difference existing between Rrm3 accumulation in wild type and *hpr1*Δ. The ChIP-chip data are consistent and they are informative from a general point of view, but a detailed ChIP in some regions can, of course, be more informative and therefore, the analysis by ChIP-chip may lead to loss of information in particular regions. Our analysis likely underestimate the sites of Rrm3 accumulation probably due to the stringency of the criteria used to define a hit and, therefore, to identify sites of high Rrm3 binding.

With respect to the differences in Figures 6C and previous 7B, we probably did not explain it appropriately and apologize for this. We have clarified it by including more details in Materials and Methods. Figure 6C refers to an average distribution of binding. It represents the percentage of hits per segment as compared to the total number of hits for each ORF. From these results we cannot make any conclusion about the binding differences between wild-type and *hpr1*Δ. It shows the slight tendency of the binding to be increasing toward the end of the ORF in average. By contrast, previous Figure 7B (now 8B) shows the actual values of binding in a particular gene (*FAS2*). It allows, therefore, to compare between the binding of Rrm3 in both wild-type and *hpr1*Δ mutant conditions.

We believe that this is now better explained since the way of doing the distribution of binding has been now clarified in the Materials and Methods section (see also point 4 and minor point 1 from referee 1).

Specific Comments

Q1. *While the values in figure 1c show interesting trends, the differences between the two groupings are rather small, and it is unclear if they are statistically significant. Also, the authors never describe how they are measuring expression or define the "AU" units.*

A1: We have performed statistical analyses of the results obtained in Figure 1C and Supplementary Figure 1 and we found that the differences between the down-regulated genes and the genome are statistically significant ($P < 0.0001$, Mann-Whitney U test). This is now accordingly stated in the text and the statistical significance as compared to wild type values is now depicted in all the Figures (asterisks).

With respect to the "AU", we thank very much the referee for the comment. In Figures 1C and Supplementary Figure 1, AU referred to model-based expression units (Li and Wong, 2001) obtained from 8 independent microarray experiments performed with the wild-type W303 strain. Nevertheless, in Figures 6 and Supplementary Figure 3, AU referred to molecules per cell (as taken from Beyer et al, 2004). The referee's comment made us realize that, despite that the same tendencies are observed, it could be easier and more accurate to use our own expression measurements in W303 background in both analyses. Accordingly, we have changed the values in the figures and stated clearly where these values come from in the in the "Statistical Analysis" section of Materials and Methods.

Q2. *Section 2 of the results contains the what may be inconsistent statements "THO is mostly recruited to the same genes as Rpb3" and "the THO complex is recruited to all RNAPII transcribed genes". Examining the ChIP on chip it does appear that there are regions with strong recruitment of Rpb3 and no Hrp1 or Sub2.*

A2: We just didn't want to say adamantly that all genes recruiting Rpb3 are also recruiting THO. Despite we believe that this is the case and in fact, we show now new evidence for the strong correlation existing between each pair of datasets (see the next comment), our maps showed some slight differences in particular regions as pointed out by the referee. We think this might be due to the variability of experiments as well as to the fact the we are using different antibodies for Rpb3 and Hpr1 or Sub2. Nevertheless, we agree that it may sound inconsistent and we have therefore changed the text as follows 'the THO complex is recruited to most RNAPII transcribed genes'.

Q3. *The R2 values for the linear comparisons of Rpb3-Sub2 and Hpr1-Sub2 seem low. For Rpb3-Sub2 there are almost as many Sub2 hits that don't correlate with Rpb3 as there are ones that do. Perhaps a defined P value would bolster the argument that this is a distinct overlap in localization of these components.*

A3: As indicated for referee 1, we have repeated the binding analysis for all the ChIP-chip experiments presented in the manuscript with different statistical parameters, which are based on more stringent criteria and define 'clusters' of binding for each protein. Then, we have evaluated the significance of the protein cluster distribution by confrontation of the actual distribution with respect to randomly simulated positions generated with a Montecarlo-like simulation as has been previously described (Bermejo et al, 2009). The detailed results of the correlations can be found in the new **Supplementary Table II.**

Q4. *The setup for the composite ORF analysis is a bit confusing and a better explanation should be given. Is this every single ORF put together and divided into 10 segments? The trend seen from this analysis is interesting and it appears quite clear that enrichment of Hpr1 and Sub2 occurs further away from the promoter. It would be better to see more specific examples of this, such as those provided in Fig 7, and also include some examples of genes with low and medium gene expression.*

A4: As requested, the way this analysis has been performed is now better explained in the Material and Methods section. We agree that a better definition of the binding profile could help the reader and apologize for our lack of clarity.
Additional examples of low and medium gene expression genes have been included in new **Supplementary Figure 6** as requested.
It is worth noting that in all these types of diagrams the Y axis plot the number of positive hits on each segment calculated over the total number of hits on all genes, regardless of the amount of protein recruited corresponding to each hit. In the data presented in **Supplementary Figure 6**, we represent the average of the $\log_2$-ratio for the positive hits and, interestingly, as expected it gives higher values when expression levels are higher.

Q5. *Figure 5 could be moved to supplemental.*

A5: Following the referee suggestion we have moved Figure 5B to the new **Supplementary Figure 9**. The plot of the recruitment profile of Figure 5A remains there, because we believe that, as in Figure 2, it is important to show a fragment of the original data.

Q6. *Figures 1 to 6 are descriptive. Some of this might be moved to a supplement. It is difficult to draw clear conclusions from this part of the paper.*

A6: We agree that some results may be descriptive, but they are needed to reach the main conclusions of the paper. Following the referee's suggestion we have moved half of the previous Figures 2, 4 and 5 to **Supplementary Figures 4 and 9.**

Q7. *Suppl. Figure 8 might be moved to the main text, since it might correspond well with Figure 7.*

A7: Performed as requested. New Figure 7 now includes the new 2D gel analysis plus data previously shown in Supplementary Figure 8. New examples of genes with low and medium gene expression showing more hits of Rrm3 in *hpr1*Δ than in wild-type cells are shown in new **Supplementary Figure 6**.

Q8. *The implications of the data in 6A that THO may have role with stabilizing heterochromatic regions during replication is potentially interesting. One wonders if HML or HMR on ChrIII show a similar enrichment of Rrm3 in the absence of Hpr1. It is unclear from supplemental figure 7 if this is the case. If there is a correlation, perhaps the analysis of ChrIII could be moved from the supplement to the text.*

A8. We agree that this is an interesting possibility. We have increased the resolution of the Figure, and indeed a correlation can be seen. It is clear for the centromeres and sub-telomeric regions as well as for HMR, but it is not that clear for HML (see **Figure for referees 2**). The possibility is now discussed in the text, remarking the need for further studies as suggested.

Q9. *It is unclear to what the 22-fold increase in Rrm3 enrichment described in section 6 of the results refers. The largest change in figure 7B appears to be about 5-fold*

A9: Thank you for this comment; we made a mistake and it is corrected in our revised manuscript.

Q10: *The data in supplemental figure 4 is compelling as there are more Hpr1 hits in intragenic regions for genes with convergent transcription than for divergent transcription, indicating a possible role for Hpr1 in relieving the torsional strain created when two transcription bubbles converge. Perhaps this could be incorporated into the paper or as made part of another study.*

A10: This is an interesting suggestion (the referee likely means intergenic region). This is now discussed in the Discussion section of the new version of the manuscript. Thanks.

Q11. *The title seems a bit broader than the data*.

A11: We are not sure what the referee is really suggesting. We believe that the title covers the main findings of the manuscript, taking into account the character number limitations of EMBO J. We do not find an easy way to find a better title, but we are open to discuss suggestions.

Minor

q1. *Although stated in the figure legend, the y-axis in figures 2A, 4, and 5A should indicate that they are in log2 scale.*

a1: This has been changed as requested.

q2. *Figure 3B, x-axis "length" is misspelled.*

a2: Corrected. Thank you.

q3. *In Figure 7B, it should be unambiguously stated that the small black rectangles correspond to the regions examined by RT PCR*

a3: Changed as requested.

q4. *Also, in supplemental figure 8B you may want to state that GLY1 is transcribed on the Crick strand or else rearrange the order of the RT results so that they mesh with the results in figure 7B, clearly showing enrichment of the Rrm3 at the 3' end of the gene.*

a4: As suggested, this is now stated in the Figure Legend.

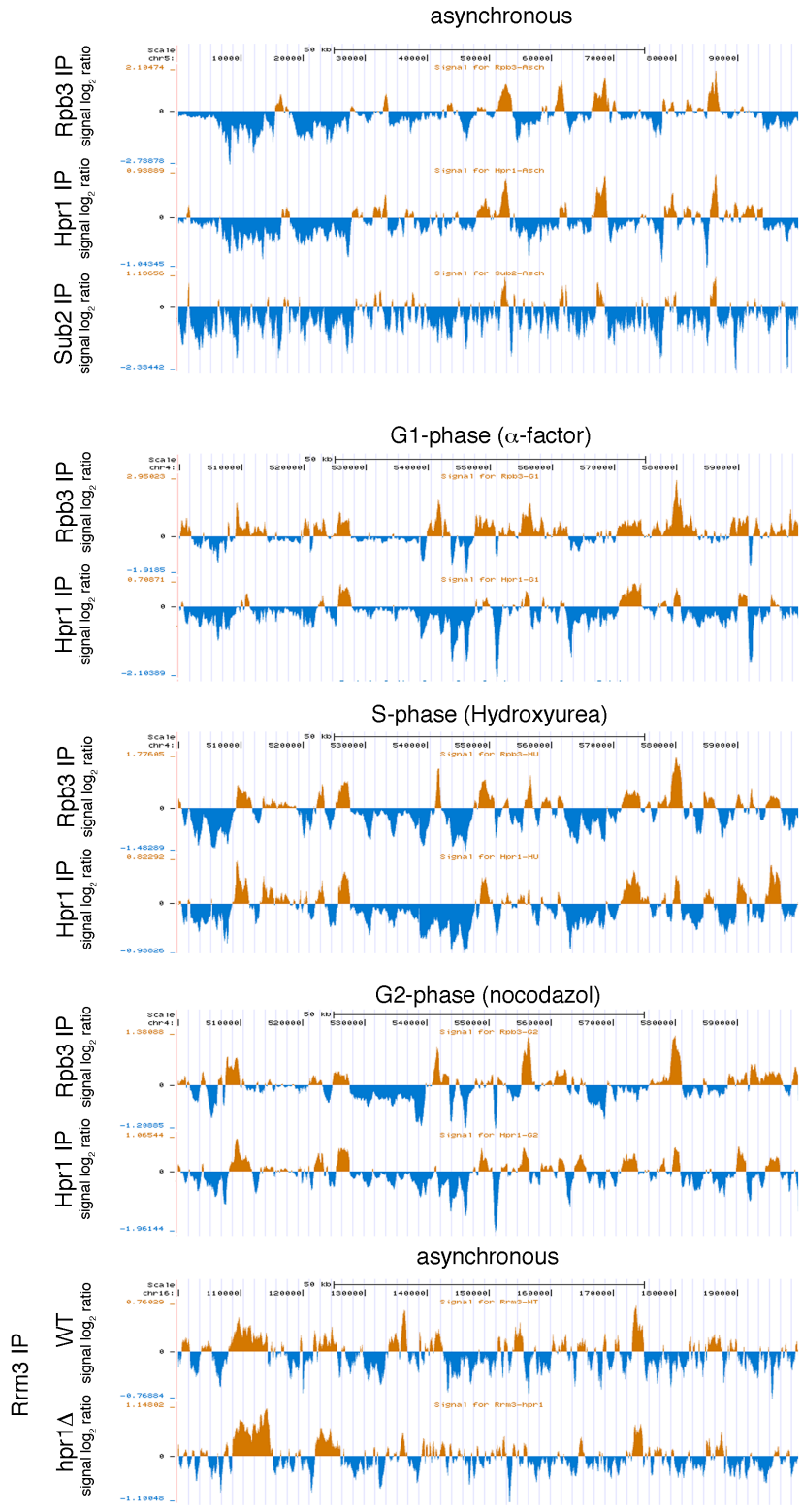


Figure for referees 1. Binding profiles for all ChIP-chips performed in this study using different statistical analysis with more stringent criteria. Protein-binding clusters were defined as ranges within the chromosome respecting the following previously described parameters (Bermejo et al, 2009): positive signal log ratio in the whole range, change P-value < 0.2 in the whole range (except for segments within the range shorter than 600 bp).

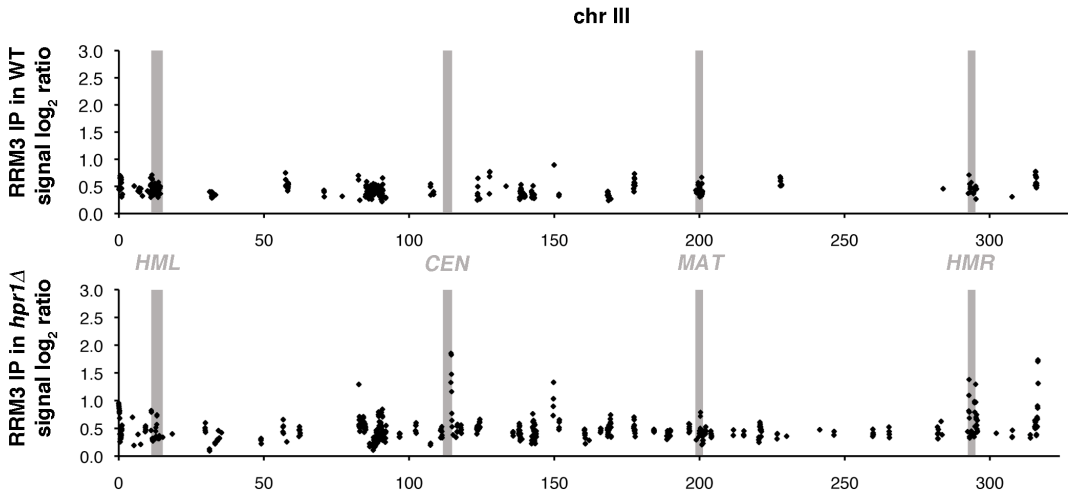


Figure for referees 2. Genomic view of Rrm3 recruitment on chromosome III in wild-type and *hpr1*Δ cells. The positions of the *HML*, *CEN*, *MAT*, and *HMR* loci are indicated.

2nd Editorial Decision 19 April 2011

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Your study has been evaluated by all three original referees and I enclose their reports below. As you will see from their comments they clearly appreciate the work that has gone into the new manuscript. However, there are a number of remaining concerns especially with the 2-D gels and the statistical significance that needs to be addressed as does further discussion of the R-loop concept. At The EMBO Journal we normally only allow a single round of revision, however, referee #1 and #2 feel that these remaining concerns need to be addressed before publication can be recommended Therefore, I am willing to allow a second round of revision to give you an opportunity to address these remaining concerns

Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Because their initial 2D gel analysis looked promising, I asked the authors to improve this part of the paper. The authors have performed 2D gel analysis in one additional site although at least 3 ORFs were suggested, due to this and unclear data interpretation, it is still difficult to establish a significant conclusion from the 2D gel analysis.

The revisions on the rest of the paper correct the problems I noted in the first review.

Below are suggestions and comments regarding Fig 7.

Major comments:
I still have significant problems with the 2D gel analysis. These remaining problems need to be addressed to make the paper suitable for publication.
1. Although the 2D gels are quantified, statistics are missing in the quantification graph to establish if the differences are statistically significant. Without statistics, the 2D gels are not acceptable.
2. The authors claims that there is higher fork accumulation at "R1" in hpr1Δ cells compared to WT cells, however visually it looks like that it is more accumulation at "R1" in WT cells compared to hpr1Δ in SPF1 gene. Therefore, I cannot yet agree with the authors that the accumulation is higher in hpr1Δ cells compared to WT cells and statistics will be important to add to the quantification data.
2. The cells used for the 2D gel analysis were grown in HU, a point I missed on my previous reading of the manuscript. If this was there before, I apologize for missing it. No reason is given for HU use. Moreover, the concentration used is quite high and will probably activate the replication checkpoint. In fact, it has been shown that Rad53 is phosphorylated after 30 min when released in 200mM HU (Puddu et al. Sensing of Replication Stress and Mec1 Activation Act through Two Independent Pathways Involving the 9-1-1 Complex and DNA Polymerase ε). In order for the authors to make the points they wish to make, they must redo these gels using cells that are not HU treated. I also wonder why the 2D gels are performed with DNA from synchronous cells after 30 min release. Has it been shown if GLY1 and SPF1 genes are transcribed and replicated in 200mM HU 30 min after release from alpha factor arrest? These points should be clarified in the text. I suggest that the authors perform the experiments in untreated logarithmically growing asynchronous cells, for capturing all S phase events, similarly to their corresponding ChIP-chip experiments in fig 5.

3. "As it is discussed in the manuscript, a replication retardation is observed throughout an extended region that would not be detected as an enriched spot. In one case the hpr1 rrm3 mutants leads to a further detectable retardation, whereas in the other case this is not clear".) What they write here in their letter, should also be mentioned in the manuscript (in the current manuscript, they refer to only one case. Both cases should be mentioned in the text. The retardation in the double mutant may also be due to only rrm3 single mutation and because this data is not shown, it should at least be mentioned in the manuscript.

4. Fig 7A seems to contain some errors, because PstI site does not exist in the 3' of the GLY1 but rather in the 5' end of the gene. In addition, parts of other ORFs are located in both investigated regions which are not indicated in the figure. These need to be corrected.

5. The authors should clarify both in the figure and in the corresponding text, which region they are analyzing in the 2D gels, because there are other ORFs in the investigated fragments. In the case of SPF1, they cleave a fragment that consists of larger part of SPF1 (3kb fragment) some intergenic region + 5' region of another gene, if my calculations are correct, the fragment they are investigating is about 5.3 kb. The region they mark as R1, might be approx. 2 kb, therefore please clarify in detail which region in the fragment this corresponds to?

6. The same clarification as in point 5 should be performed for GLY1.

7. The region where their probes bind should be indicated, as well as the size of the fragments that

they digest.
The data in this paper do not support the subheading "Impaired replication fork progression and..." at page 13 is for the moment not supported by the data shown and should be removed. The same is true, when discussing the 2D gels in page 18.

Minor comments:
1. Using the word "overaccumulation" in page 13 in the context of Rrm3 arrest should be replaced to a more suitable word, maybe "binding of Rrm3".

2. I could not find in the manuscript how the different cell cycle phases in the ChIP-chip data are performed. For example, concentration of HU or Nocadozole used in the experiments and when the DNA was collected.

Referee #2 (Remarks to the Author):

The revised MS addresses most of the reviewers' comments and is much improved. I still feel, however, that the R-loop concept needs to be discussed in quite more depth.

First, it is surprising that the authors don't see an orientation dependency in the effect of the proposed R-loops on the replication fork stalling, given that head-on collisions with transcription stall fork progression irrespective of R-loops (Takeuchi et al. Genes Dev. 17: 1497-1506, 2003; Mirkin & Mirkin Mol Cell Biol. 25: 888-895, 2005).

Second, as is evident from numerous crystal structures, there is no place for an R-loop within the elongating RNA polymerase. Thus, RNA exiting from the elongation complex must re-invade the upstream DNA duplex to make the R-loop formation feasible. This re-inasion process is clearly energetically unfavorable unless it is payed off by: (i) the unusually high stability of an RNA-DNA hybrid, i.e. local G-richness of the nascent RNA (Belotserkovskii et al. Proc Natl Acad Sci USA 107: 12816-12821, 2010), or (ii) the anchoring of nascent RNA around the upstream DNA duplex generating negative supercoiling sufficient to unwind the duplex (Belotserkovskii & Hanawalt Biophys J. 100:675-684, 2011). Since the authors don't see the bias towards Gs in the transcripts affiliated with the R-loop formation, the anchoring model could be worth discussing. In fact, it is consistent with the observations that the effects of THO-Sub2 deletions are mostly pronounced for long, high GC-content genes.

Referee #3 (Remarks to the Author):

The authors have clearly put forth a lot of effort in producing both the original and this revised manuscript. However, some fundamental issues still remain that were not fully addressed in this revision. One point that the authors themselves raise, but do not fully address, is the stoichiometry problem. It is unclear how there is enough THO to be present at all RNAPII transcribed genes because there are only about 500 molecules/cell of Tho2 and 1000 molecules/cell of Hpr1p while there is about 10,000 molecules/cell of Rpb3p (a key RNA pol II subunit) [Ghaemmaghami S, et al. (2003) Global analysis of protein expression in yeast. Nature 425(6959):737-41], raising an inconsistency with the ChIP-chip data.

While the additional 2D gel experiments undoubtedly took substantial effort, it is not clear that they help to establish that there are more fork pauses in an hpr1-null mutant as the gels themselves look very similar in the wild-type and hpr1-null and the quantitation does not demonstrate a vast change between the two. The argument that the Rrm3-mediated aberrations are distributed throughout the ORF and, therefore, would not give rise to discrete regions of fork pausing would be difficult to prove.

Lastly, the idea of R-loop-dependent Rrm3 recruitment needs to be addressed more thoroughly. It is still not clear from Figure 8 that Rrm3 recruitment is greatly enhanced in the absence of Hpr1. Also, the decrease in Rrm3 enrichment following expression of RNaseH is small and still leaves enrichment 2-5 fold higher than WT, almost suggesting that there is another reason besides R-loop formation for Rrm3 recruitment to these regions. For the argument that loss of THO is leading to R-

loop formation throughout the genome, the authors should at least verify R-loop formation at one of the sites they look at in more depth, such as FAS1, GLY1 or SPF1. This would also more fully address Referee #2's comments about whether specific G-rich regions need to be present on the nontemplate DNA strand to initiate R-loop formation in the absence of THO.

While aspects of this work warrant publication, perhaps a more focused paper targeted to a more specific audience would be appropriate.

2nd Revision - authors' response 10 May 2011

Referee #1:

QUERY 1. *Although the 2D gels are quantified, statistics are missing in the quantification graph to establish if the differences are statistically significant. Without statistics, the 2D gels are not acceptable.*

ANSWER 1: Statistics have been included in Figure 7 as requested.

Q2a. *The authors claims that there is higher fork accumulation at "R1" in hpr1; cells compared to WT cells, however visually it looks like that it is more accumulation at "R1" in WT cells compared to hpr1; in SPF1 gene. Therefore, I cannot yet agree with the authors that the accumulation is higher in hpr1 cells compared to WT cells and statistics will be important to add to the quantification data.*

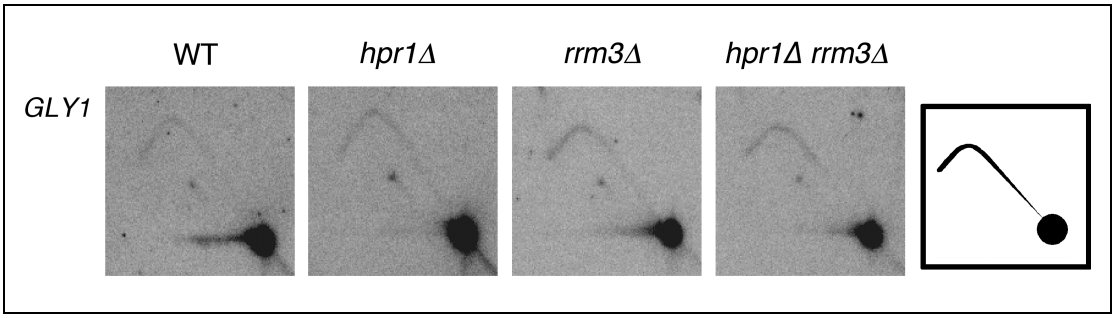
A2a: Quantifications come from three different experiments, but we only showed a representative gel for each genotype. The overall quantification is made considering all signals seen in the gel and shows a statistically significant difference in *hpr1* or in *hpr1 rrm3* depending on the gene analyzed. It is not possible to make direct conclusions by the visualization of specific arcs, without considering all other signals in the gel. In any case we have changed the gel in the figure for another one in which the differences might be clearer.

The result indicates that the sensitivity of 2D gels is limited. As we show and discuss all over the manuscript, the 2D gel data are less sensitive than the Rrm3 binding data. This is a situation that has been already pointed out by others (see Azvolinsly et al 2009: '*Why do 2D gels and ChIP-microarray analyses yield different views of the replication landscape in terms of fork progression? 2D gels are best at detecting pauses that are localized to discrete regions of 100–200 bp, such as the pauses at tRNA genes and centromeres and the arrest at the RFB. In contrast, fork slowing throughout the ORF of a highly transcribed RNA Pol II gene (Figure 3C) is more difficult to detect by 2D gels'*). This is one of the reasons why the present study was performed using Rrm3-FLAG to follow the replication forks. This was previously discussed in the text in pages 13 and 19. To better focus this issue we changed the subheading in Discussion to "*Rrm3-dependent replication obstacles in RNAPII-transcribed genes in THO mutants"* and Discussion has been extended with a new paragraph between pages 18 and 19.

Q2b. *The cells used for the 2D gel analysis were grown in HU, a point I missed on my previous reading of the manuscript. If this was there before, I apologize for missing it. No reason is given for HU use. Moreover, the concentration used is quite high and will probably activate the replication checkpoint. In fact, it has been shown that Rad53 is phosphorylated after 30 min when released in 200mM HU (Puddu et al. Sensing of Replication Stress and Mec1 Activation Act through Two Independent Pathways Involving the 9-1-1 Complex and DNA Polymerase ε). In order for the authors to make the points they wish to make, they must redo these gels using cells that are not HU treated. I also wonder why the 2D gels are performed with DNA from synchronous cells after 30 min release. Has it been shown if GLY1 and SPF1 genes are transcribed and replicated in 200mM HU 30 min after release from alpha factor arrest?*
These points should be clarified in the text. I suggest that the authors perform the experiments in untreated logarithmically growing asynchronous cells, for capturing all S phase events, similarly to their corresponding ChIP-chip experiments in fig 5.

A2b: We used 40mM HU for 2D gels. 200 mM HU were used for ChIP-chips performed in S phase cells. We apologize for this unfortunate "typo". This is now corrected in Materials and Methods.

We indeed had performed the experiments in asynchronous cultures without HU that the referee suggests. The sensitivity of that approach is not sufficient to see anything reliable (a Figure with the result of the *GLY1* gene is included below for the referee, and this result is referred in the text in page 13 as "data not shown"; 2D-gel electrophoreses gave no reliable signals in the *SPF1* gene in two different set of experiments performed; *not shown*). For these reasons 2D gels are performed from synchronous cultures, a much more accurate approach. This guarantees that all cells are replicating at the time of the experiment (the 30 min point after release is taken as a standard in which the adjacent regions are being replicated). Low concentrations of HU (20-40 mM) are used to reduce replication speed in order to visualize better the replication arcs. These conditions are the same as those used in different studies (Moriel-Carretero et al. Mol. Cell. 2011; Gomez-Gonzalez et al. 2009, etc), it is a standard in the field, and does not affect the firing of early replication origins as the ones we study here. Since S phase checkpoint activation does not affect replication progression but late origins firing (Tercero and Diffley, Nature 2001), we do not think that the checkpoint could cause any delay in fork progression in such particular regions.



Finally, we know that both *GLY1* and *SPF1* are transcribed with and without HU treatment as it can be observed in Rpb3 ChIP-chip data and microarray analyses in asynchronous and S-phase conditions respectively (Supplementary Datasets I and II; unpublished results). The fact that these genes are replicated is obvious from the 2D gel results: the Y arcs observed are a demonstration of active replication forks, otherwise no arc would be seen.

Q3. *"As it is discussed in the manuscript, a replication retardation is observed throughout an extended region that would not be detected as an enriched spot. In one case the hpr1 rrm3 mutants leads to a further detectable retardation, whereas in the other case this is not clear".) What they write here in their letter, should also be mentioned in the manuscript (in the current manuscript, they refer to only one case. Both cases should be mentioned in the text. The retardation in the double mutant may also be due to only rrm3 single mutation and because this data is not shown, it should at least be mentioned in the manuscript.*

A3: Text in page 13 has been rephrased to mention all cases, and the experiment made in asynchronous conditions without HU has also been referred.

Q4. *Fig 7A seems to contain some errors, because PstI site does not exist in the 3' of the GLY1 but rather in the 5' end of the gene. In addition, parts of other ORFs are located in both investigated regions which are not indicated in the figure. These need to be corrected.*

A4: Thank you for pointing this to us. It has been corrected.

Q5. *The authors should clarify both in the figure and in the corresponding text, which region they are analyzing in the 2D gels, because there are other ORFs in the investigated fragments. In the case of SPF1, they cleave a fragment that consists of larger part of SPF1 (3kb fragment) some intergenic region + 5' region of another gene, if my calculations are correct, the fragment they are investigating is about 5.3 kb. The region they mark as R1, might be approx. 2 kb, therefore please clarify in detail which region in the fragment this corresponds to?*

A5: Clarified as requested. Thanks.

Q6. *The same clarification as in point 5 should be performed for GLY1.*

A6: Clarified as requested. Thanks.

Q7: *The region where their probes bind should be indicated, as well as the size of the fragments that they digest. The data in this paper do not support the subheading "Impaired replication fork progression and..." at page 13 is for the moment not supported by the data shown and should be removed. The same is true, when discussing the 2D gels in page 18.*

A7: The regions where our probes bind have been included as requested. Subheading in page 13 and text in pages 18 and 19 has been modified as suggested. To be consistent, subheading in page 17 has also been changed accordingly.

Minor comments:
q1. *Using the word "overaccumulation" in page 13 in the context of Rrm3 arrest should be replaced to a more suitable word, maybe "binding of Rrm3".*

a1: Changed as requested. Thanks.

q2. *I could not find in the manuscript how the different cell cycle phases in the ChIP-chip data are performed. For example, concentration of HU or Nocadozole used in the experiments and when the DNA was collected.*

a2: Detailed information was included as requested. Thanks.

Referee #2:

Q1: *First, it is surprising that the authors don't see an orientation dependency in the effect of the proposed R-loops on the replication fork stalling, given that head-on collisions with transcription stall fork progression irrespective of R-loops (Takeuchi et al. Genes Dev. 17: 1497-1506, 2003; Mirkin & Mirkin Mol Cell Biol. 25: 888-895, 2005).*

A1: The results are consistent with our previous work and the work of others - including the ones referred by the reviewer - and in agreement with what we expected. To clarify this better, a new paragraph has been included in pages 18 and 19. For the reasons explained below and in the text we preferred to maintain the discussion on this issue to a minimum:

We have previously shown that whereas convergent transcription-replication collisions strongly compromise instability, the effect is lower in co-directional events in wild-type cells in which there is no evidence for R-loops (Prado and Aguilera EMBO J 2005). This is similar to the results from Mirkin and Mirkin (MCB 2005). However, in *hpr1* cells - in which R-loops are formed - the orientation of replication versus transcription makes no difference. This is expected from the fact that we see a strong overlap between Rrm3 recruitment in *hpr1* and WT cells, in the latter of which transcription-replication impairment has been already reported to be orientation-independent by the Zakian's group (Azvolinsly et al, 2009) and as discussed in the text. Please note that the referred paper of Kobayashi's group (Takeuchi et al. 2003) refers to the rDNA region in which the existence of the FOB sites already announces the relevance of orientation in this region extensively studied by other authors (i.e., El Hage et al, Genes Dev 2010; Kobayashi and Ganley, Science 2005). Instability at the rDNA region, as has also been shown in *E. coli* (Boubakri et al, EMBO J. 2010) show specific requirements not observed for mRNA genes and that needs further work to be understood. In addition, the instability of THO mutants may be related with a suboptimal formation of the mRNP particle, and this does not apply either to the rDNA regions or mRNA genes in wild-type cells.

Finally, it is worth noting that whereas in the examples cited by the referee, and others not cited, the collisions give rise to a clear replication fork pause observable in 2D gels, the collisions in *hpr1* cells do not lead to replication fork pause at specific sites but rather the collisions should be produced all over the gene as observed with the Rrm3 analysis (see pages 18 and 19). Any intent to extrapolate from systems leading to a defined and specific RFP to our study is therefore premature. For these reasons, we would like to maintain the discussion on this issue to a minimum.

Q2. *Second, as is evident from numerous crystal structures, there is no place for an R-loop within the elongating RNA polymerase. Thus, RNA exiting from the elongation complex must re-invade the*

upstream DNA duplex to make the R-loop formation feasible. This re-inasion process is clearly energetically unfavorable unless it is payed off by: (i) the unusually high stability of an RNA-DNA hybrid, i.e. local G-richness of the nascent RNA (Belotserkovskii et al. Proc Natl Acad Sci USA 107: 12816-12821, 2010), or (ii) the anchoring of nascent RNA around the upstream DNA duplex generating negative supercoiling sufficient to unwind the duplex (Belotserkovskii & Hanawalt Biophys J. 100:675-684, 2011). Since the authors don't see the bias towards Gs in the transcripts affiliated with the R-loop formation, the anchoring model could be worth discussing. In fact, it is consistent with the observations that the effects of THO-Sub2 deletions are mostly pronounced for long, high GC-content genes.

A2: We certainly agree that the R-loops must be formed by the re-invasion of the DNA duplex and that re-invasion must be favored by the negative supercoiling created behind RNAPII. We have discussed these and other arguments in previous works (see Aguilera, EMBO 2002; Huertas and Aguilera, Mol Cell 2003; Wellinger et al, MCB 2007; Gomez-Gonzalez and Aguilera, PNAS 2007; Aguilera, Nat Str Mol Biol 2005; Gomez-Gonzalez and Aguilera, 2009). Roy and Lieber (MCB 2009) have nicely shown indeed the relevance of G-density in stabilization of R-loops *in vitro*. In addition, it has been shown that *hpr1* shows synthetic growth defect with *top1* mutants (Aguilera and Klein, 1990).

Following the referee's recommendations we have expanded the discussion on this issue, including the anchoring model, in a new paragraph included between pages 20 and 21. Thank you.

Referee #3

Q1: *One point that the authors themselves raise, but do not fully address, is the stoichiometry problem. It is unclear how there is enough THO to be present at all RNAPII transcribed genes because there are only about 500 molecules/cell of Tho2 and 1000 molecules/cell of Hpr1p while there is about 10,000 molecules/cell of Rpb3p (a key RNA pol II subunit) [Ghaemmaghami S, et al. (2003) Global analysis of protein expression in yeast. Nature 425(6959):737-41], raising an inconsistency with the ChIP-chip data.*

A1: This was discussed in previous page 16, but we have extended and clarified it better in new page 17 following the reviewer suggestion. It is important to note that the data of Ghaemmaghami by themselves raise different questions. Different subunits of the THO complex show abundances from 521 (Tho2) to 5910 (Mft1) molecules/cell. However, as mentioned in Introduction, the biochemistry of the complex indicates that these proteins are in stoichiometric amounts, and the whole THO complex is degraded if one subunit is deleted (Chavez et al. 1997; Huertas et al., MCB 2006). This is also the case for Rpb2 (18000 molecules) or Pfcf11 (2800), which a priori should be also similar to Rpb3 (10000). Having said this, it seems clear that the Hpr1:Rpb3 ratio is different, as indicated by the referee. This difference may be explained by a different stability of the proteins (Ub of Hpr1 seems to play a role in each cycle of transcription), but most likely by a transient and dynamic role of THO/TREX. As has been proposed previously, THO may be required to load Sub2 (Zenklussen et al. 2002), which is highly abundant (~50,000 molecules/cell) and can be stably recruited. The fact that THO is recruited in a gradient manner along the genes and it is not present in promoters or in the early part of the transcript, where Rpb3 is present, may explain part of the difference in abundance between THO and RNAPII. Our data suggests that the loading of THO is transient. THO is likely more stably loaded at the 3'-end, in which it may also be important for polyA+ processing (Rougemaille et al. 2008).

Q2. *While the additional 2D gel experiments undoubtedly took substantial effort, it is not clear that they help to establish that there are more fork pauses in an hpr1-null mutant as the gels themselves look very similar in the wild-type and hpr1-null and the quantitation does not demonstrate a vast change between the two. The argument that the Rrm3-mediated aberrations are distributed throughout the ORF and, therefore, would not give rise to discrete regions of fork pausing would be difficult to prove.*

A2: We certainly agree, and this is what we discussed in the previous revised version of the manuscript. As said before (see A1 of Referee #2), the 2D gels are less sensitive for these types of measurements, and this is why the study of the progression of replication via the Rrm3 gene is much more informative in this case.

Q3. *Lastly, the idea of R-loop-dependent Rrm3 recruitment needs to be addressed more thoroughly. It is still not clear from Figure 8 that Rrm3 recruitment is greatly enhanced in the absence of Hpr1. Also, the decrease in Rrm3 enrichment following expression of RNaseH is small and still leaves enrichment 2-5 fold higher than WT, almost suggesting that there is another reason besides R-loop formation for Rrm3 recruitment to these regions. For the argument that loss of THO is leading to R-loop formation throughout the genome, the authors should at least verify R-loop formation at one of the sites they look at in more depth, such as FAS1, GLY1 or SPF1. This would also more fully address Referee #2's comments about whether specific G-rich regions need to be present on the non-template DNA strand to initiate R-loop formation in the absence of THO.*

A3: The effect of RNH1 overexpression is clear (Fig. 8) despite suppression being partial, as pointed out by the referee. Suppression has not been observed to be complete in any assay, as far as we are concerned (see for examples Huertas and Aguilera 2003, Mischo et al 2011, Li and Manley 2005, Drolet et al, 2005, Gomez-Gonzalez et al, 2009; Paulsen et al, Mol. Cell 2009) even when working in an *rnh1 rnh2* double mutant background in which the effect is stronger (Huertas and Aguilera 2003). It is possible that most of the RNaseH overproduced *in vivo* is not sufficient to remove DNA-DNA hybrids for many reasons: inability to access the site of action, interference with Okazaki fragments, regulation by limiting co-factors, etc. However, in our original report (Huertas and Aguilera 2003) we already proposed that R-loops might not be the only cause of genome instability. Here we show the same, and we think there is no need to reiterate this.

On the other hand, we have shown the same effect of RNAse H on Rrm3 recruitment in three sites at the *FAS2* gene (Fig. 8). So far the only direct way to show the formation of RNA-DNA hybrids at specific sites in vivo would be by ChIP with monoclonal anti-DNA-RNA antibody. Unfortunately the sensitivity of the available antibody is not yet appropriate in our hands nor, to our knowledge, in the hands of others in the field, unless a strong and quick activation of expression is achieved such as for example in the *GAL1p:YRL454w* fusion construct where we are able to see a signal above the wild-type level after transcription activation, but not in steadily transcribing endogenous genes (B. Pardo and A. Aguilera, unpublished; Mischo et al. Mol. Cell 2011). We agree that further work would be required for a better comprehension of this phenomenon.

3rd Editorial Decision 24 May 2011

Your manuscript has been reviewed by one of the original referees, who finds that you have addressed all their remaining concerns. I am happy to accept the manuscript for publication in The EMBO Journal. You will receive the official acceptance letter in the next day or so.

Yours sincerely,

Editor
The EMBO Journal

