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Senataxin homolog Sen1 is required for efficientective termination of RNA polymerase III transcription.

Julieta Rivosecchi, Marc Larochelle, Camille Teste,Frédéric Grenier, Amélie Malapert, Emiliano P. Ricci, Pascal Bernard, François Bachand and Vincent Vanoosthuyse.

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Transaction Report:

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

1st Editorial Decision 5th April 2019

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received three referee reports on your study, which are included below for your information.

As you will see, the reviewers express an overall interest in the study, but also raise several major concerns regarding conceptual, as well as technical issues. In particular, in addition to pointing out several specific issues, referees #1 and #3 both argue that it would be important to compare Sen1 to Dbl8, at least for some central experiments as referee #3 points out. In addition, all referees agree that the issues regarding inconsistent nomenclature and inaccurate citations must be addressed, and that a comprehensive discussion of the work in context with published studies should be included.

Should you be able to adequately address the key concerns, as well as the various more specific points, then we would be happy to consider this study further for publication. I would therefore like to invite you to prepare and submit a revised manuscript.

REFEREE REPORTS.

Referee #1:

The paper by Rivosecchi et al investigates the function of Sen1 protein in fission yeast. They demonstrate that it is required for transcriptional termination of Pol III genes. This function of Sen1 seems to be independent of R-loop resolution. Therefore, the authors propose that previous view of Pol II terminating autonomously is not fully correct.

In my opinion, this paper does provide some initial experimental data into the function of Sen1 in transcription in fission yeast, however the story is not fully developed and requires further experiments to understand the molecular details of this process. Previously the same group has published that Sen1 is antagonising Pol III transcription in fission yeast (Legros et al Plos Genet

2014. RNA processing factors Swd2.2 and Sen1 antagonize RNA Pol III-dependent transcription and the localization of condensin at Pol III genes.). The authors do not refer to this observation and do not put their data in the context of this study, since these are two opposing conclusions. The authors keep referring to some pieces of their data which are not actually shown in the paper (see my comments below). I also do not fully agree with some of interpretations to the data presented (see my comments below). For this reason, I think that in the current format this paper is not suitable for publication in EMBO J.

Major comments:
1. The authors propose that Sen1 is a co-factor of Pol III transcription in S.pombe. They need to present the data to support this (I.e. Sen1 co-IP with different Polymerases)?

2. When looking at the level of transcription in Sen1 depleted cells the authors need to measure the levels of nascent RNA (and not mature RNA). Their Northern blot experiment in Figure 5C shows a strong defect on nascent transcription for one specific gene. For this reason the authors need to present genome-wide nascent RNA seq (or NET-seq) to clearly demonstrate what is happening with the nascent transcription at the gene bodies and termination regions of the genes. This will give a clear view on role of Sen1 in transcription and it should be presented alongside the Pol III chip data (Figure 4 A-C).

3. The authors need to provide a quantification of their R-loop slot blots (Figure 3A). I can see a clear decrease of R-loops in sen depleted strains especially at 1/16 and 1/8 dilutions of genomic DNA. This will be in line with a transcriptional defect at gene bodies. In my opinion, this is also observed to some degree in Figure 3b for high R-loop signals. Alongside, the normalisation ssDNA slot blot should be presented (and quantitated similarly to R-loop slot blot). The authors need to re-consider their conclusions based on the quantifications of these blots. I think the decrease of the R-loop signal possibly reflects decrease in nascent transcription and Pol III accumulation in the gene bodies in Sen 1-depleted strains. If that is this case, how do these conclusions relate to their own data showing that Sen1 antagonises Pol III transcription from PLos Genetcis 2014?

4. In addition to Sen1, there is a second S.Pombe Senataxin homologue called Dbl8. Both of the proteins, Sen1 and Dbl8, are non-essential. In one of the assays the authors suggest that this protein is not involved in transcriptional termination of Pol III genes. It would be nice to see the experiments carried out with this protein alongside with Sen1 for other type of assays in this paper. Does it ChIp to tRNA genes in general or does it bind to Pol II/Pol I genes? I think this is important from the biological point of view, as S.pombe is the organism which has two senataxin homologue proteins. Are their functions redundant? In the discussion they state the Dbl1 interacts with Pol I - they need to show this data.

5. The authors need to elaborate more on their view of how Sen1 may be involved in transcription of Pol III genes in budding yeast (in the gene bodies and termination regions) and refer to their own paper in Plos Genetics 2014.

Minor comments:

1. The abbreviation used throughout the paper is very confusing. 'SenataxinSen1' should be substituted by a conventional names for fission or budding yeast proteins.

2. The authors should pay more attention to the precision of the citations. Page 13 - the paper Yuce and West shows the interaction of Senataxin with three RNA Pols. This was done in budding yeast cells, so the authors should use S.cerevisiae Sen1 name (and not SenataxinSen1 which is extremely confusing). Also the authors should state that in this paper the authors have not found the interaction of mammalian Senataxin with Pol I or Pol III subunits.

3. Page 16 - The authors statement about Senataxin from Cristini et al paper is incorrect. They have confirmed Senataxin as a positive R-loop interacting factor in R-loop IP experiments (Fig.1).

4. WB panels should include additional control panels: Fig 2 A (Sen1 should be included); Figure 3 (Sen1, Rnase A, RNAse H1 and 2 should be included).

5. The presentation of the peak over-laps between Pol III and Sen1 (Figure 1E) should be presented in more detailed way (i.e. type of genes; gene features etc..). I would be great to see Dbl8 on this figure as well.

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The manuscript from Rivosecchi et al. reports the interesting and unexpected finding that S. pombe Sen1 is associated with RNA polymerase III (RNAP3) transcription units and promotes efficient transcription termination on many of these genes. In the absence of Sen1, which is non-essential in S. pombe, RNAP3 accumulates over and downstream of its transcription units, consistent with a defect in termination. Read-through transcripts of several RNAP3 terminators are also detected. The experiments are thorough and the results are convincing. The synthetic lethality of the rpc37-V189D allele and sen1 deletion is a compelling result. However, the authors should be more precise and restrained in their conclusions, as detailed below.

1) I think the novel nomenclature used by the authors, "SenataxinSen1", is cumbersome and misleading. It's use for the S. cerevisiae protein is tautological given that Sen1 is the founder protein and senataxin is short for "Sen1 homolog associated with ataxia". Also, senataxin should not be capitalized. The authors should use the name given to the protein in the organism being discussed. Similarly, the authors state in the introduction that senataxin is implicated in DNA repair, then cite a paper (Li et al., 2016) that studied S. cerevisiae Sen1.

2) The abstract states that their results "challenge the pre-existing view that RNAP3 terminates transcription autonomously". Yet their results show that sen1 deletion has little or no effect on strong RNAP3 terminators, such as the U6 terminator (TTTTTTTTTCT). It would be more accurate to say that sen1 promotes or enhances RNAP3 termination on weaker terminators. The same comment applies to the section heading at the bottom of page 8 and the sentence near the top of page 14 "In contrast to this view...".

3) To my knowledge, in vitro RNAP3 transcription systems do not "often rely on the artificial assembly of an elongating RNAP3 without the need for TFIIIB or TFIIIC" (page 14). The yeast U6 gene is unique in not requiring TFIIIC in a purified system. All other transcription units I'm aware of require TFIIIC for initiation in vitro. In such systems, termination is efficient in the absence of other factors.

4) The cited Wang and Roeder 1998 paper (page 14) does not appear in the reference list.

5) I think the accumulation of RNAP3 over the transcription unit that the authors observe in the sen1 deletion strain could potentially be due to a defect in recycling of RNAP3 rather than a defect in elongation. The authors may wish to consider this alternative.

6) Steinmetz et al. 2006, referenced by the authors, showed that RNAP2 accumulates over tRNA genes in an S. cerevisiae strain with a hypomorphic mutation in Sen1 (Figure S4). Given the results presented here, this finding suggests that in S. cerevisiae Sen1 may remove RNAP2 from RNAP3 transcription units. I think this is worth mentioning, since it supports the authors proposed general role for Sen1 in RNAP removal.

7) The parallels that the authors draw between Sen1 and Mfd1 (also called TRCF) are quite interesting. It certainly seems possible that Sen1, like Mfd, directly interacts with RNAP and pushes on it. However, they ignore the fact that Mfd1 is a double-stranded DNA translocase, not a helicase like Sen1. I am not aware of any evidence that Mfd1 has helicase activity or that Sen1 can act as a dsDNA translocase. The authors should address this disparity.

8) The raw or processed reads from the ChIP-seq experiments should be deposited in an appropriate database and the accession number provided in the published paper.

Referee #3:

This study describes the role of the S. pombe homologue to S. cerevisiae Sen1 in Pol III transcription. In particular it shows that S.pombe Sen1 accumulates over Pol III transcribed genes in a Pol III dependent manner. Sen1 deletion leads to reduced Pol III transcript levels yet increased Pol III occupancy on Pol III transcribed genes. This suggests Sen1 may facilitate Pol III template release. However, Sen1 deletion does not result in higher amounts of R-loops, as might be expected from analogous observations with S. cerevisiae Sen1 or H. sapiens Senataxin, but it does lead to a strong termination and possible elongation defect at tRNAs. Insertion of a strong terminator complements tRNA gene dependency on Sen1, suggesting that Sen1 acts as a Pol III termination auxiliary factor.

Overall, I feel that this study is an interesting addition to the field and one that should be published following some revision suggestions as listed.

1) Introduction: The use of Senataxin/Sen1 is a bit inconsistent. I would recommend not to use a superscript, but to use one name and denote other homologues together with their species. As to the effect of Senataxin mutations, two previous studies unpick the functional consequences of different mutations in Senataxin which should be cited: Chen, Muller, Sundling Brow 2014; Richard, Feng, Manley 2013.

2) Generally, the S cerevisiae literature is not well cited - rather than referring to newer papers that repeat old findings, the authors should cite original publications.

3) Figure 1 is a bit confusing - Figure 1C should precede Figure 1A and B to allow the strong statements made with those figures. This figure would also benefit from comparison with ChIP-seq data from the second Sen1 ortholog Dbl8. Especially given that the authors in later parts of the paper turn to comparing both Sen1 homologs, it would be helpful if both proteins were compared side by side from the beginning. The dissection of Sen1 recruitment through Pol III is nice.

4) Figure 2: the observation in Figure 2B is very interesting but not taken any further - nor much discussed.

5) Figure 3: "Sen1 is believed to antagonize....." which Sen1? S. cerevisiae, pombe or Senataxin? Also, the original publication for this is not cited. The R-ChIP here clearly shows that Sen1 deletion doesn't cause any more R-loops (instead a subtle reduction of R-loops, which may be associated with reduced transcription elongation rates), but the authors omit to discuss why the RNase H double mutant does show reduced R-loop accumulation at these loci?

6) Figure 4: the termination defect at tRNA genes is convincing, but again should be compared to the already published RNA-seq data from the Dlb8Δ strain (Larochelle et al. 2018).

7) Figure 6: the insertion of the T stretch is very neat - but would benefit from some more mechanistic elucidation. Is Sen1 recruitment changed at the T23 in Arg10?

In conclusion the data presented in this study is clearly interesting and new. However, it would be helpful to better integrate these data with previously published and related results detailed in Legros et al. 2014 and Larochelle et al. 2018. This would lead to a fully picture of how S.pombe tRNA gene are transcribed and terminated

1st Revision - authors' response 10th May 2019

Referee #1:

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We thank the referee for pointing out that our work is potentially interesting. We have endeavoured to address the referee's constructive criticisms in our revised manuscript.

Major comments:
1. The authors propose that Sen1 is a co-factor of Pol III transcription in S.pombe. They need to present the data to support this (I.e. Sen1 co-IP with different Polymerases)?
Our conclusion that Sen1 is a cofactor of RNAP3 in fission yeast is based on a number of concordant observations. We had shown previously that Sen1 physically associates with RNAP3 and not with RNAP1 or RNAP2 (Legros et al., 2014) and that lack of Sen1 had no impact on RNAP2 transcription termination (Larochelle et al., 2018). We show here that RNAP3-transcribed genes represent the main binding sites of Sen1 on chromosomes and that the association of Sen1 with RNAP3-transcribed genes requires optimum RNAP3 loading. To strengthen our conclusion further, we now show that the Sen1-RNAP3 association is resistant to a benzonase treatment, demonstrating that it is mediated by direct protein contacts and not by DNA or RNA (Appendix Figure S1). In addition, we have extended our AP-MS studies to compare the proteomes associated with Sen1 and Dbl8 and these new data indicate that Sen1, but not Dbl8, associates with subunits of RNAP3, whereas Dbl8 associates with RNAP1 (Figure EV1 and Appendix Table S1 of the revised manuscript). Taken together, these data strongly support the idea that Sen1 is a cofactor of RNAP3.

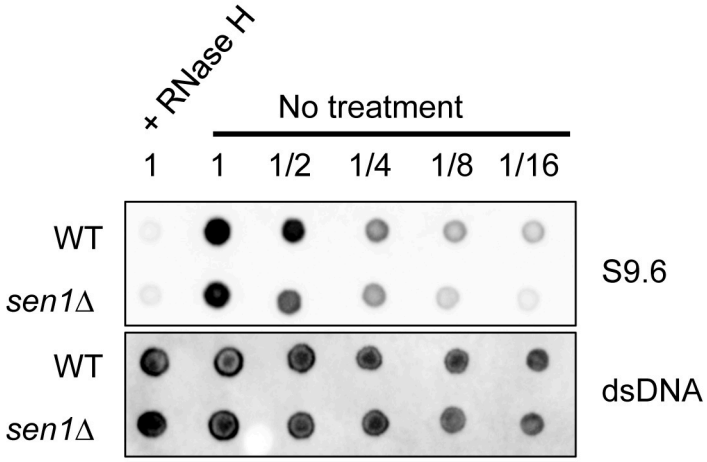
2. When looking at the level of transcription in Sen1 depleted cells the authors need to measure the levels of nascent RNA (and not mature RNA). Their Northern blot experiment in Figure 5C shows a strong defect on nascent transcription for one specific gene. For this reason the authors need to present genome-wide nascent RNA seq (or NET-seq) to clearly demonstrate what is happening with the nascent transcription at the gene bodies and termination regions of the genes. This will give a clear view on role of Sen1 in transcription and it should be presented alongside the Pol III chip data (Figure 4 A-C).
We do not believe that such experiments are necessary to support our conclusion that the greater enrichment of RNAP3 on its target genes does not result in increased RNA production in the absence of Sen1. We feel strongly that the addition of CRAC or NET-seq experiments is unnecessary for the current manuscript. As far as we know, a NET-seq protocol for RNAP3, with the appropriate spike-in controls required to make the experiment both calibrated and quantitative, has not yet established. The fact that RNAP3 transcripts are present in multiple identical copies also renders the subsequent bioinformatics analysis of such experiments challenging. Importantly, we believe that such an experiment would not necessarily give us greater mechanistic insights into the role of Sen1 in transcription termination and would mainly describe with greater resolution what we have already shown in the current study.

3. The authors need to provide a quantification of their R-loop slot blots (Figure 3A). I can see a clear decrease of R-loops in sen depleted strains especially at 1/16 and 1/8 dilutions of genomic DNA. This will be in line with a transcriptional defect at gene bodies. In my opinion, this is also

observed to some degree in Figure 3b for high R-loop signals. Alongside, the normalisation ssDNA slot blot should be presented (and quantitated similarly to R-loop slot blot). The authors need to re-consider their conclusions based on the quantifications of these blots. I think the decrease of the R-loop signal possibly reflects decrease in nascent transcription and Pol III accumulation in the gene bodies in Sen 1-depleted strains. If that is this case, how do these conclusions relate to their own data showing that Sen1 antagonises Pol III transcription from PLos Genetcis 2014?
This is an interesting point. It is important to note however that the focus of the current work is not to establish whether or not Sen1 regulates R-loop formation in fission yeast. This is an entirely independent question that will require more than dot-blots to be properly addressed and that will be the focus of future studies. The focus of the current study is to determine whether or not Sen1 contributes to RNAP3 transcription termination in an R-loop dependent manner, without any prejudices about the putative role of Sen1 in R-loop stabilization. For this, we have destabilized R-loops by expressing *E. coli* RnhA at high levels. The aim of the dot blot was only to confirm that RnhA expression was sufficient to remove R-loops in the absence of Sen1, as we have shown previously in other genotypes (Legros et al., 2014; Hartono et al., 2018). To simplify the manuscript and to avoid confusion, we have now reduced the emphasis on this particular conclusion both in the title and in the manuscript and we have decided to remove the dot blot from the manuscript. However, for the referee's information, we provide below a new dot-blot with the loading control requested, which confirms our former observation.

In our response to point 5 made by the referee, we explain why the conclusion of our current work is different from the conclusion from our 2014 Plos Genetics paper (please see below).

4. In addition to Sen1, there is a second S.Pombe Senataxin homologue called Dbl8. Both of the proteins, Sen1 and Dbl8, are non-essential. In one of the assays the authors suggest that this protein is not involved in transcriptional termination of Pol III genes. It would be nice to see the experiments carried out with this protein alongside with Sen1 for other type of assays in this paper. Does it ChIp to tRNA genes in general or does it bind to Pol II/Pol I genes? I think this is important from the biological point of view, as S.pombe is the organism which has two senataxin homologue proteins. Are their functions redundant? In the discussion they state the Dbl1 interacts with Pol I - they need to show this data.
In the first version of the manuscript, we had already shown using two different assays (the colour assay and the gene-specific RT-qPCR) that Dbl8 does not participate in RNAP3 transcription termination. As mentioned above, and to follow the referee's recommendation, we now provide evidence that Sen1 and Dbl8 associate with a different set of proteins (Figure EV1 and Appendix Table S1) and that Dbl8 associates with RNAP1 but not with RNAP3 (Figure EV1). We also show using ChIP-qPCR that Dbl8 is not recruited to RNAP3-transcribed genes, in contrast to Sen1 (Figure 1F of the revised manuscript). This strongly suggests that Sen1, contrary to its paralog Dbl8, has a specific role at RNAP3-transcribed genes. We believe that these new data strengthen our



conclusions and reveal specificity between Sen1 and Dbl8. For these reasons, we feel that a complete functional characterization of Dbl8 is out of the scope of the current study and should be the focus of a future manuscript.

5. The authors need to elaborate more on their view of how Sen1 may be involved in transcription of Pol III genes in budding yeast (in the gene bodies and termination regions) and refer to their own paper in Plos Genetics 2014.
We agree with the referee that it is indeed an important point to discuss and we apologize for not discussing this question in the original version of our manuscript. We have now extensively corrected this point in the revised version of our manuscript (p16). We demonstrate that we previously reached a different conclusion in our Plos Genetics 2014 paper because we used RT-qPCR primed with random hexamers rather than gene-specific primers to monitor the steady-state levels of tRNAs. We have now added new data (Figure EV5) in which we use our super-terminator mutant to demonstrate that RT-qPCR with random hexamers preferentially reverse transcribed the long read-through transcripts produced in the absence of Sen1, thereby giving the false impression that tRNA were more abundant in *sen1*Δ than in sen1+ cells. We thank the referee for helping us make that point clear.

Minor comments:

1. The abbreviation used throughout the paper is very confusing. 'SenataxinSen1' should be substituted by a conventional names for fission or budding yeast proteins.
We have changed the text accordingly.

2. The authors should pay more attention to the precision of the citations. Page 13 - the paper Yuce and West shows the interaction of Senataxin with three RNA Pols. This was done in budding yeast cells, so the authors should use S.cerevisiae Sen1 name (and not SenataxinSen1 which is extremely confusing). Also the authors should state that in this paper the authors have not found the interaction of mammalian Senataxin with Pol I or Pol III subunits.
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3. Page 16 - The authors statement about Senataxin from Cristini et al paper is incorrect. They have confirmed Senataxin as a positive R-loop interacting factor in R-loop IP experiments (Fig.1).
For simplicity, and in line with our decision to reduce the emphasis on R-loops in the revised manuscript, we have now removed this sentence.

4. WB panels should include additional control panels: Fig 2 A (Sen1 should be included); Figure 3 (Sen1, Rnase A, RNAse H1 and 2 should be included).
Antibodies specific to *S. pombe* Sen1, Rnh1, and Rnh201 have never been generated. We must insist that we worked with deletion strains that are not able to produce the proteins of interest. We have however added to Figure 3C a blot showing the expression of Flag-tagged RnhA in the relevant strains as requested.

5. The presentation of the peak over-laps between Pol III and Sen1 (Figure 1E) should be presented in more detailed way (i.e. type of genes; gene features etc..). I would be great to see Dbl8 on this figure as well.
It should be noted that the correlation matrix shown in Fig. 1E is not the result of a peak calling analysis but from pairwise genome-wide correlations of read coverage at a resolution of 10 bp, showing that the genome-wide binding profiles of Sen1 and RNAP3 are highly similar (high correlation coefficients), whereas the binding profiles of Sen1 and RNAP2 are very different (low correlation coefficient). As requested by the reviewer, we have now assessed the pairwise correlation between Sen1 and Rpc1/Rpc2 (RNAP3) for different types of genes. This data is now presented in Appendix Figure S2 of the revised manuscript and confirms the very strong correlation between Sen1 and RNAP3 at all loci. As mentioned above, the functional characterization of Dbl8 is out of the scope of the current study and will be the focus of a future manuscript.

6. Figure 3B R-Chip should include sen1 delta- double RNAse 1 and H2 mutant, which was used in Figure C.
We must emphasize that the triple mutant *sen1*Δ*rnh1*Δ*rnh201*Δ is dead and that we did not use it in Figure 3C (now Figure 3B). We cannot carry out R-ChIP in the *rnh1*Δ*rnh201*Δ mutant because R-ChIP relies on the presence of Rnh1. We have previously demonstrated using various approaches that R-loops accumulate at tRNA genes in the *rnh1*Δ*rnh201*Δ mutant (Legros et al., 2014; Hartono et al., 2018).

Referee #2:

The manuscript from Rivosecchi et al. reports the interesting and unexpected finding that S. pombe Sen1 is associated with RNA polymerase III (RNAP3) transcription units and promotes efficient transcription termination on many of these genes. In the absence of Sen1, which is non-essential in S. pombe, RNAP3 accumulates over and downstream of its transcription units, consistent with a defect in termination. Read-through transcripts of several RNAP3 terminators are also detected. The experiments are thorough and the results are convincing. The synthetic lethality of the rpc37-V189D allele and sen1 deletion is a compelling result. However, the authors should be more precise and restrained in their conclusions, as detailed below.
We are grateful to the referee for their positive appraisal of our work and for their interesting comments, which we fully addressed below.

1) I think the novel nomenclature used by the authors, "SenataxinSen1", is cumbersome and misleading. It's use for the S. cerevisiae protein is tautological given that Sen1 is the founder protein and senataxin is short for "Sen1 homolog associated with ataxia". Also, senataxin should not be capitalized. The authors should use the name given to the protein in the organism being discussed. Similarly, the authors state in the introduction that senataxin is implicated in DNA repair, then cite a paper (Li et al., 2016) that studied S. cerevisiae Sen1.
We have changed the text according to the referee's recommendations.

2) The abstract states that their results "challenge the pre-existing view that RNAP3 terminates transcription autonomously". Yet their results show that sen1 deletion has little or no effect on strong RNAP3 terminators, such as the U6 terminator (TTTTTTTTTCT). It would be more accurate to say that sen1 promotes or enhances RNAP3 termination on weaker terminators. The same comment applies to the section heading at the bottom of page 8 and the sentence near the top of page 14 "In contrast to this view...".
We have toned down both the title and our conclusions according to the referee's recommendations.

3) To my knowledge, in vitro RNAP3 transcription systems do not "often rely on the artificial assembly of an elongating RNAP3 without the need for TFIIIB or TFIIIC" (page 14). The yeast U6 gene is unique in not requiring TFIIIC in a purified system. All other transcription units I'm aware of require TFIIIC for initiation in vitro. In such systems, termination is efficient in the absence of other factors.
As far as we know, most RNAP3 transcription termination assays rely on the artificial assembly of elongation complexes (ECs) using purified RNAP3, a radiolabeled RNA primer and short oligonucleotides mimicking the template strand and the non-template strand. As far as we understand, no purified TFIIIB or TFIIIC is added to the reaction (see for example Arimbasseri AG, Maraia RJ. Methods Mol Biol. 2015;1276:185-98).

4) The cited Wang and Roeder 1998 paper (page 14) does not appear in the reference list.
The reference was included in the original manuscript. Yet, it was not separated from the previous reference in the list by a free space and it was therefore difficult to see. We have now corrected this mistake.

5) I think the accumulation of RNAP3 over the transcription unit that the authors observe in the sen1 deletion strain could potentially be due to a defect in recycling of RNAP3 rather than a defect in elongation. The authors may wish to consider this alternative.
We have now modified our discussion to include this possibility. This new section can now be found on p.16&17 of the revised manuscript.

6) Steinmetz et al. 2006, referenced by the authors, showed that RNAP2 accumulates over tRNA genes in an S. cerevisiae strain with a hypomorphic mutation in Sen1 (Figure S4). Given the results presented here, this finding suggests that in S. cerevisiae Sen1 may remove RNAP2 from RNAP3 transcription units. I think this is worth mentioning, since it supports the authors proposed general role for Sen1 in RNAP removal.
We have now modified our discussion to include this possibility. This new section can now be found on p. 18 of the revised manuscript.

7) The parallels that the authors draw between Sen1 and Mfd1 (also called TRCF) are quite interesting. It certainly seems possible that Sen1, like Mfd, directly interacts with RNAP and pushes on it. However, they ignore the fact that Mfd1 is a double-stranded DNA translocase, not a helicase like Sen1. I am not aware of any evidence that Mfd1 has helicase activity or that Sen1 can act as a dsDNA translocase. The authors should address this disparity.
We have now made clearer distinctions between Mfd and Sen1 in the Discussion (see p. 18).

8) The raw or processed reads from the ChIP-seq experiments should be deposited in an appropriate database and the accession number provided in the published paper.
The raw and processed reads from the ChIP-seq experiments have now been deposited on the Gene Expression Omnibus (GEO) under accession number GSE130709.

Referee #3:

This study describes the role of the S. pombe homologue to S. cerevisiae Sen1 in Pol III transcription. In particular it shows that S.pombe Sen1 accumulates over Pol III transcribed genes in a Pol III dependent manner. Sen1 deletion leads to reduced Pol III transcript levels yet increased Pol III occupancy on Pol III transcribed genes. This suggests Sen1 may facilitate Pol III template release. However, Sen1 deletion does not result in higher amounts of R-loops, as might be expected from analogous observations with S. cerevisiae Sen1 or H. sapiens Senataxin, but it does lead to a strong termination and possible elongation defect at tRNAs. Insertion of a strong terminator complements tRNA gene dependency on Sen1, suggesting that Sen1 acts as a Pol III termination auxiliary factor.

Overall, I feel that this study is an interesting addition to the field and one that should be published following some revision suggestions as listed.
We are grateful to the referee for their positive appraisal of our work and we have taken into account their comments in the revised version of our manuscript.

1) Introduction: The use of Senataxin/Sen1 is a bit inconsistent. I would recommend not to use a superscript, but to use one name and denote other homologues together with their species. As to the effect of Senataxin mutations, two previous studies unpick the functional consequences of different mutations in Senataxin which should be cited: Chen, Muller, Sundling Brow 2014; Richard, Feng, Manley 2013.
We have corrected the manuscript accordingly and added the requested references. We thank the referee for reminding us that those studies should have been included in the first version of the manuscript.

2) Generally, the S cerevisiae literature is not well cited - rather than referring to newer papers that repeat old findings, the authors should cite original publications.
We have now revisited many of the cited findings to refer to original publications, including the original work from the budding yeast literature.

3) Figure 1 is a bit confusing - Figure 1C should precede Figure 1A and B to allow the strong statements made with those figures.
We always show gene-specific examples of ChIP-seq data before presenting the genome-wide profiles. We believe that this is in fact the most frequent way of presenting genome-wide analyses: gene-specific examples followed by the global view.

This figure would also benefit from comparison with ChIP-seq data from the second Sen1 ortholog Dbl8. Especially given that the authors in later parts of the paper turn to comparing both Sen1 homologs, it would be helpful if both proteins were compared side by side from the beginning. The dissection of Sen1 recruitment through Pol III is nice.
It is important to note that our aim in this manuscript is to focus on the role of Sen1 in RNAP3 transcription termination and not to carry out a functional characterization of Senataxin homologues in fission yeast. However, to address the referee's comment, we have added a substantial amount of new data to further demonstrate that Dbl8, contrary to Sen1, does not function in RNAP3 transcription termination. We now show that Dbl8 interacts with subunits of RNAP1, whereas Sen1 associates with many RNAP3 subunits (Figure EV1 and Appendix Table S1). In addition, we show

that Dbl8 is not recruited to RNAP3-transcribed genes (Fig. 1F) and we confirmed at other genes that lack of Dbl8 does not result in read-through transcription (new Figure EV3). We believe that these new data strengthen our conclusions and reveal specificity between Sen1 and Dbl8. The complete functional characterization of Dbl8 is out of the scope of the current study and should be the focus of a different manuscript.

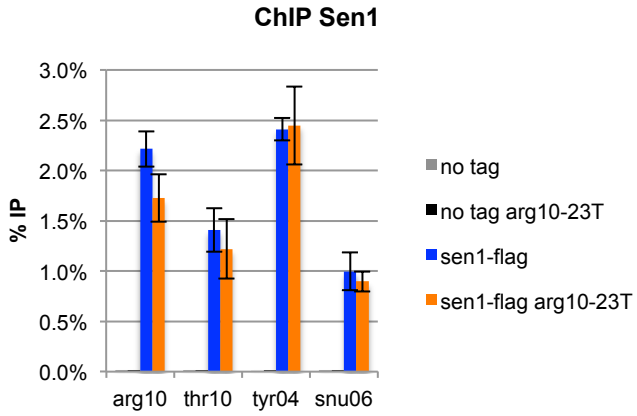
4) Figure 2: the observation in Figure 2B is very interesting but not taken any further - nor much discussed.
We have improved the description and the discussion of Figure 2B in the revised version of our manuscript (see p. 16 of the revised manuscript).

5) Figure 3: "Sen1 is believed to antagonize....." which Sen1? S. cerevisiae, pombe or Senataxin? Also, the original publication for this is not cited.
We have rephrased our manuscript accordingly.

The R-ChIP here clearly shows that Sen1 deletion doesn't cause any more R-loops (instead a subtle reduction of R-loops, which may be associated with reduced transcription elongation rates), but the authors omit to discuss why the RNase H double mutant does show reduced R-loop accumulation at these loci?
We think that there may be a misunderstanding here. We did not show that the RNase H double mutant caused reduced R-loop accumulation. In contrast, we previously reported using both R-ChIP and DRIP as well as both gene-specific (Legros et al., 2014) and genome-wide studies (Hartono et al., 2018) that lack of RNase H stabilizes RNA-DNA hybrids at RNAP3-transcribed genes. What we show here is that lack of RNase H (*rnh1*Δ*rnh201*Δ) reduces the enrichment of RNAP3 (rpc25) over its target genes. Although we do not have a rigorous mechanistic explanation for this result, it clearly rules out the stabilization of R-loops as the cause for the greater enrichment of RNAP3 detected in the absence of Sen1.

6) Figure 4: the termination defect at tRNA genes is convincing, but again should be compared to the already published RNA-seq data from the Dlb8Δ strain (Larochelle et al. 2018).
The study by Larochelle et al. published in Nature Communications did not include RNA-seq data of the *dbl8*Δ mutant, but rather presented ChIP-seq analyses of RNAP2. Importantly, the new data that is included in our revised manuscript clearly rule out a role for Dbl8 in RNAP3 transcription termination (see Fig. 1F, Fig. EV1, Fig. EV3, and Fig. 5A). Notably, we show that Dbl8 does not significantly associate with RNAP3-transcribed genes and that lack of Dbl8 does not result in the production of read-through tRNA transcripts, contrary to what is seen in the sen1 mutant.

7) Figure 6: the insertion of the T stretch is very neat - but would benefit from some more mechanistic elucidation. Is Sen1 recruitment changed at the T23 in Arg10?
As requested, we have examined Sen1 recruitment at the T23 terminator of *SPCTRNAARG.10*. The results show (see below) that the presence of the super-terminator reduces the association of Sen1 by about 20% at *SPCTRNAARG.10*, without affecting its recruitment at other RNAP3-transcribed genes. We are not sure however how to interpret this small change, which is why we prefer not to include these data in the manuscript. It could be that the strength of the terminator influences the turnover of RNAP3 and hence the turnover of Sen1. At this stage, we think that it is more appropriate to simply conclude that Sen1 acts in complement to the intrinsic transcription termination mechanism, which would be somehow boosted by the presence of the super-terminator.



In conclusion the data presented in this study is clearly interesting and new. However, it would be helpful to better integrate these data with previously published and related results detailed in Legros et al. 2014 and Larochelle et al. 2018. This would lead to a fully picture of how S.pombe tRNA gene are transcribed and terminated.
We have endeavoured throughout the manuscript to better integrate our new data with our previously published data.

2nd Editorial Decision 28th May 2019

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below). I am pleased to say that the referees find that their comments have in principle been sufficiently addressed and now support publication. Nonetheless both still raise several points, which will likely require textual changes only, and which should be addressed in the revised final version.

REFEREE REPORTS

Referee #2:

The authors have adequately addressed my concerns in the revised manuscript with the following few exceptions:

Previous point #2: The authors have removed most statements that S. pombe Sen1 is required for RNAP3 termination, but the legend to Figure 4 should read "Sen1 is required for efficient RNAP3 transcription termination" or something similar.

Previous point #3: The use of artificial scaffolds for assembling elongation complexes with purified RNAP3 is a relatively new method. Prior to that, transcription termination in vitro by RNAP3 was typically studied by factor-dependent initiation on plasmid-borne genes, either with purified factors or whole cell extracts. The authors should view studies from the E.P. Geiduschek, S. Hahn, and A. Sentenac labs, for example.

Previous point #7: The authors should not refer to Mfd as a helicase (see page 17). It is not a helicase, and this term is not used in the paper they cite.

In addition, a few other minor errors need to be corrected:

1) Missing "of" in 7th line of the Abstract.

2) In Figure S1B, the antibody used for the Western blot should be stated.

3) In Figures 1 and S4, the snu6 gene has an intron, which should be indicated. Also, it should be explained that this is the gene for U6 snRNA.

4) On page 17, the authors state that the fact that the "super-terminator" in the arg.10 tRNA gene corrects the termination defect but does not return RNAP3 to the normal level indicates that RNAP3 retention is not due to inefficient termination (see Figure 6B). However, the excess RNAP3 they observe may well come from the adjacent ser.09 gene, which continues to experience read-through, and not the arg.10 gene. Thus, their conclusion is not substantiated. In addition, it is highly unlikely that the p values for arg.10 and ser.09 are both 0.028, as shown in Figure 6C. Perhaps the brackets were cut-and-pasted without then changing the values.

5) I believe the Figure 3 legend should read: "Sen1 regulates RNAP3 recruitment in an R-loop independent manner", since transcription is not assayed in the experiments shown. Also, in part B the panels should labeled "left" and "right" rather than "top" and "bottom".

Referee #3:

Overall, this revised manuscript is now written in a way that is more consistent and congruent with previously published data. This allows the reader to fully appreciate the new findings in this paper. It is also significantly enhanced by the new added data. Apart from a few minor concerns, I therefore consider this manuscript ready for publication. It clearly quite nicely enhances our understanding of Sen1 function in various organisms.

Detailed minor remaining points:

Abstract: It has not been shown that Senataxin contributes to genome stability.

Figure 1G: Position of primers and position of deletion needs to be shown in higher resolution.

Figure 2B: Lanes require labelling.

Figure 3B: I apologize for misunderstanding this figure previously - it is now very clearly explained.

It would be useful to include an arrow indicting the direction of transcription for the genome browser shots. Currently termination regions are only indicated by a dashed box, which is not immediately clear.

2nd Revision - authors' response 3rd June 2019

Referee #2:

The authors have adequately addressed my concerns in the revised manuscript with the following few exceptions:

Previous point #2: The authors have removed most statements that S. pombe Sen1 is required for RNAP3 termination, but the legend to Figure 4 should read "Sen1 is required for efficient RNAP3 transcription termination" or something similar.

This has been changed according to the referee's suggestion (page 33).

Previous point #3: The use of artificial scaffolds for assembling elongation complexes with purified RNAP3 is a relatively new method. Prior to that, transcription termination in vitro by RNAP3 was

typically studied by factor-dependent initiation on plasmid-borne genes, either with purified factors or whole cell extracts. The authors should view studies from the E.P. Geiduschek, S. Hahn, and A. Sentenac labs, for example.

We had already said in the Discussion that transcription termination assays often rely on the artificial assembly of elongation complexes. Implicitly, this meant that this was not the only way to assay transcription termination. We have now made that point clearer (see p 15). This does not change the point of Discussion that we want to make.

Previous point #7: The authors should not refer to Mfd as a helicase (see page 17). It is not a helicase, and this term is not used in the paper they cite.

We have replaced the term “helicase” by “translocase” (see p17).

In addition, a few other minor errors need to be corrected:

1) Missing "of" in 7th line of the Abstract.

This has been corrected.

2) In Figure S1B, the antibody used for the Western blot should be stated.

This has been corrected.

3) In Figures 1 and S4, the snu6 gene has an intron, which should be indicated. Also, it should be explained that this is the gene for U6 snRNA.

The position of the intron has been added on the figure (see new Figure 1 and Appendix Figure S4). We have now made it clearer that the *snu6* gene transcribes the U6 snRNA (p 7).

4) On page 17, the authors state that the fact that the "super-terminator" in the arg.10 tRNA gene corrects the termination defect but does not return RNAP3 to the normal level indicates that RNAP3 retention is not due to inefficient termination (see Figure 6B). However, the excess RNAP3 they observe may well come from the adjacent ser.09 gene, which continues to experience read-through, and not the arg.10 gene. Thus, their conclusion is not substantiated.

We have toned down our conclusion to take the referee’s remark into consideration (see page 17).

In addition, it is highly unlikely that the p values for arg.10 and ser.09 are both 0.028, as shown in Figure 6C. Perhaps the brackets were cut-and-pasted without then changing the values.

The p values are correct here (see the raw data in the table below):

genotype	strain	date	Arg.10	Ser.09
WT	112	14/06	0.091%	0.021%
	113	14/06	0.089%	0.020%
	112	06/07	0.072%	0.019%
	113	06/07	0.087%	0.020%
	average		0.085%	0.020%
	st dev		0.008%	0.001%
arg10-23T	5724.1	14/06	0.024%	0.027%
	5724.2	14/06	0.033%	0.026%
	5724.1	06/07	0.022%	0.024%
	5724.2	06/07	0.017%	0.021%
	average		0.024%	0.024%
	st dev		0.006%	0.003%
sen1Δ	4523	14/06	2.571%	0.565%
	4524	14/06	2.361%	0.437%
	4523	06/07	2.391%	0.415%
	4524	06/07	2.250%	0.397%
	average		2.393%	0.454%
	st dev		0.133%	0.076%
sen1Δ arg1023T	5961	14/06	0.033%	0.687%
	5962	14/06	0.087%	0.791%
	5961	06/07	0.016%	0.664%
	5962	06/07	0.014%	0.837%
	average		0.038%	0.745%
	st dev		0.034%	0.083%

5) I believe the Figure 3 legend should read: "Sen1 regulates RNAP3 recruitment in an R-loop independent manner", since transcription is not assayed in the experiments shown. Also, in part B the panels should labeled "left" and "right" rather than "top" and "bottom".

This has been corrected as suggested (see page 33).

Referee #3:

Overall, this revised manuscript is now written in a way that is more consistent and congruent with previously published data. This allows the reader to fully appreciate the new findings in this paper. It is also significantly enhanced by the new added data. Apart from a few minor concerns, I therefore consider this manuscript ready for publication. It clearly quite nicely enhances our understanding of Sen1 function in various organisms.

Detailed minor remaining points:

Abstract: It has not been shown that Senataxin contributes to genome stability.

We are not sure that we understand the referee's comment here. There are a number of studies reporting that Senataxin contributes to genome integrity. For example, the group of Gaëlle Legube showed recently that Senataxin prevents illegitimate translocations (PMID 29416069). We have replaced "stability" by "integrity" in the abstract to be more accurate.

Figure 1G: Position of primers and position of deletion needs to be shown in higher resolution.

Figure 1G has been changed accordingly. We have also added the sequence of the mutations that we introduced to disrupt the TATA box.

Figure 2B: Lanes require labelling.

Figure 2B has been changed accordingly.

Figure 3B: I apologize for misunderstanding this figure previously - it is now very clearly explained.

It would be useful to include an arrow indicting the direction of transcription for the genome browser shots. Currently termination regions are only indicated by a dashed box, which is not immediately clear.

The direction of transcription was already indicated both by a colour code and small arrows in the genome browser snapshots. To follow the referee's recommendations, we have however added new arrows to Figures 1 and 4.

Accepted 11th June 2019

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

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: VANOOSTHUYSE VINCENT
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2019-101955

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
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- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was adapted to the type of experiments and to the field's best practice.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
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5. For every figure, are statistical tests justified as appropriate?	When relevant, a Wilcoxon-Mann Whitney statistical test was applied to determine whether or not the distributions of the quantitative value of interest were similar in two independent groups of measurements (genotypes).
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	In a Wilcoxon-Mann Whitney statistical test, the null hypothesis is that the distributions of a quantitative value are similar in the two populations of interest. The null hypothesis was reached when the p-values were >0.05.

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Is the variance similar between the groups that are being statistically compared?	The Wilcoxon-Mann Whitney statistical test is non parametric and does not assume that the data follow a normal distribution

C- Reagents

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13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	This has been done accordingly.
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