THE EMBO JOURNAL

The Torpedo Effect in *Bacillus subtilis*: RNase J1 Resolves Stalled Transcription Complexes

Michaela Šiková, Jana Wiedermannová, Martin Převorovský, Ivan Barvík, Petra Sudzinová, Olga Kofroňová, Oldřich Benada, Hana Šanderová, Ciarán Condon and Libor Krásný.

Review timeline:

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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 15th June 2019

Thank you for submitting your manuscript on a role for RNaseJ1 in processing stalled transcription complexes for consideration by The EMBO Journal. Please apologize the delay in communicating the decision to you, which is also due to a currently high manuscript submission rate to our office. We have received three referee reports on your study, which are included below for your information.

As you will see, the reviewers express interest your study, but also raise several concerns that would need to be addressed in order to further consider publication here. In addition to a number of more specific technical issues, referee #3 in particular is not fully convinced that RNaseJ directly interacts with RNAP and displaces it from DNA. Similar concerns are brought up by referee #2 (points 2-6), and are thus aspects that would need to be addressed and clarified during a revision. In addition, a more detailed description of the methods, as well as computer code, should be provided as suggested by referee #3. Should you be able to adequately address these key concerns, as well as the more specific issues raised by each of the referees, 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. Please note that it is our policy to allow only a single round of major revision and that it is therefore important to clarify all concerns raised at this stage.

REFEREE REPORTS

Referee #1:

Summary

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The work in this manuscript falls into two major parts. In the first part, a combination of RNAseq and ChIPseq are used to analyze the effect of an rnjA deletion on the transcriptome and transcription. The ChIPseq was performed by pulling down RNA polymerase to determine gene occupancy. The combination of these analyses permits distinguishing between direct effects of RNase J on mRNA stability and indirect effects involving transcription. The results in the first part are by themselves interesting and important.
In the course of the transcriptomic analysis, an unexpected class of messages was discovered in which the level of transcription as determined by ChIPseq was uncoupled from the level of transcript as determined by RNAseq. That is, in this class of messages, increased gene occupancy did not correlate with increased transcript level. This result is paradoxical since the direct effect of the rnjA deletion is to stabilize messages that are targeted by RNase J. Together with other results, the authors postulate that RNase J could have a novel role in removing RNA polymerases that are stalled on DNA.
In the second part of the manuscript, in vitro and in vivo evidence is presented showing that there is a physical and functional interaction between RNase J and RNA polymerase. RNase J interacts with RNA polymerase via the nascent transcript. The functional interaction involves the termination of transcription by the 5' to 3' exonuclease activity of RNase J, which 'strips' the nascent transcript from the RNA polymerase.
The experimental work in the manuscript is of high quality. The conclusions are supported by the results.
The presentation of the work is clear and logical. The manuscript is very well written.

Principal significance
This is the first time that a 'torpedo' mechanism of transcription termination involving an exoribonuclease has been shown in Bacteria. Considering previous work with Eukaryotes, the work in this manuscript suggests that 'torpedo' mechanisms of transcription termination are universally conserved although the enzymes involved in this process are different in different organisms.

Major concerns regarding conclusions
I have no major concerns regarding the conclusions.

Minor concerns that should be addressed.
Fig. 2. Instead of the dashed vertical line, it would be better if the graphs contained an internal grid that corresponds to the major divisions on the x- and y- axes.
Top of page 7, Fig SB? I assume the authors means Fig. 5B.
I do not understand what the authors mean when they say that RNAP and rpsB co-localize similarly, nor the reference to the 'well documented' coupling of translation to transcription. The images in Figure 5A and B strongly suggests that the distribution of RNase J differs significantly from rpsB, which is a marker for the 30 subunit of the ribosome. That a relatively small enzyme such as RNase J can enter into the nucleoid is not a surprise. Introducing translational coupling opens a complex set of issues that have nothing to do with the work in the article. Fig. 5B could be deleted. Fig. 5A, C and D make a coherent data set showing that a proportion of RNase J is localized in the nucleoid and that RNAP can be pulled down by RNase J in a complex that can be destroyed by RNase A treatment.
Consistent with the image in Fig. 5B, work over the past decade has shown that only a small proportion of ribosomes are localized to the nucleoid. The Cramer reference (Science 2017) shows coupling of the leading ribosome to RNAP. There is no evidence for polyribosome formation in nucleoid. Since there are 10- to 30-fold more ribosomes than RNAP, the experimental evidence suggests that polyribosomes are formed after release of a nascent transcript from the nucleoid.
Discussion, page 12. Although E. coli and many other Gram negative bacteria do not have 5' to 3' exoribonucleases, it should be mention the Rho performs a similar 'torpedo-like' function in Rho-independent transcription termination in bacteria that do not have RNase J.
The presentation of the work can be improved. Fig. 1 and Fig. 8 are not essential. They should be moved to the supplemental information. Fig. 1A and B are confirmation of previous work. The result in Fig. 1C can be mentioned as a line in the text. For now, the model in Fig. 8 does not add anything. Is an extended interface necessary for the 'torpedo' model? In the absence of a crystal structure showing a tight interaction and conformation changes in the RNA polymerase that promote transcription termination, it is premature to enter into this line of reasoning.
Moving part of the Discussion to the supplementary information in not satisfactory. By dealing with the above mentioned changes, which should liberate space in the main text, it should be possible,

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with some editing, to make a complete Discussion that covers all the work in the manuscript.

Referee #2:

This is a very interesting report, in which the authors propose that Bacillus RNaseJ1 induces displacement of a stalled RNA polymerase (RNAP), a mechanism analogous to the torpedo model of termination in eukaryotes. Given that J1 is a 5' exo RNase, this scenario is logical and is supported by the authors' data, which include cellular co-localization and in vitro experiments. The Chip data are supportive of this conclusion.
Overall, the manuscript is well written and illustrated. I will list only important points that should, in my opinion, be addressed, in the order of their appearance. A few minor flaws that could be easily fixed by copy editors.
1. I found the arguments on the RNAP abundance and the respective Figure 4 distracting, because only a fraction of the presented data could be explained by an overall increase in RNAP levels. I think that this figure and discussion would be better placed in the supplement. It would be interesting to know how many genes belong to each of the 4 classes shown in Fig. 3 (and listed in the supplementary table, but a figure is much nicer), for example from a Venn diagram.
2. I am somewhat confused by the model of RNAP-J1 interactions. On one hand, the data argue that the nascent RNA is required to bridge the two enzymes, which makes a perfect sense. On the other, the authors build a helerologous model of the complex and propose an extensive interface, with the two proteins "fitting together remarkably well". In the absence of strong supporting experimental data, this model is a stretch, but does not do any harm. However, if I visualize the site correctly, in this model NusA should be located close by, if not sterically competing with, J1. The authors should comment on the implications in the discussion while bringing up flap-tip helix.
3. The authors argue that J1 is specific because Xrn1 fails to digest RNA inside the complex. Yet XrnI actually gets closer to the RNA 3' end in Fig. 8; a 16 nt long transcript is barely out of the exit channel in structures. How does this happen without an intimate contact? Please elaborate.
4. In is not clear that a specific contact is required for the transcription complex dissociation, or why it would be necessarily beneficial. For example, Rho can terminate bacterial and eukaryotic enzymes. In any case, to make a specificity arguments, other RNAPs should be tested. I would use both E. coli and T7 RNAPs that are commercially available.
5. As in E. coli, there are undoubtedly partially redundant factors that remove the stalled RNAP in Bacillus. J1 appears to be one of them, Rho is another. Is the double deletion strain viable, and if yes, is it hypersensitive to DNA damage? With regard to the latter, I am slightly confused by the UV resistance assay in the supplement: the legend states that the strains were grown to OD of 0.5, but delta rnjA strain forms 100x fewer colonies. I do not think that an altered cell morphology can lead to such a dramatic difference in scattering. But the difference in sensitivity is not huge: as far as I can tell, wild type strain loses 2 logs, the deletion - 3 logs. I think that these data should be in the main manuscript, as they are way more important than Fig. 4, and quantified by determining CFUs following irradiation. Plates are cute but less informative.
6. The pulling-out mechanism would arguably require a lot of force, a model that makes sense for Rho which can generate 200 pN, but for RNaseJ1? Even with Rho, the torpedo-like model proposed by Nudler is viable. Perhaps drawing parallels to bacterial factors (Mfd, Rho) is justified.

Referee #3:

The manuscript by Šiková et al. presents an intriguing hypothesis positing the involvement of the widely conserved cellular RNase J1 in removal of stalled transcription elongation complexes in B. subtilis. Given the importance of terminating stalled ECs in maintaining genome stability and protein traffic along DNA, this report would be of interest to a wide readership, beyond merely an interest in bacterial transcription. However, major claims require equally impressive evidence. As such, a number of issues should be addressed before this manuscript can be recommended for publication.

Specific criticisms

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1. Introduction: The reference to Mathy et al. (2007) cited in relation to the discovery of RNase J1 should be replaced by that to an earlier and more relevant report by Evan et al, (2005). Furthermore, while introducing enzymatic properties of RNase J1, the authors focused on its function as the 5',3'-exonuclease and completely overlooked its endonuclease activity, which has a direct bearing on the design and interpretation of their in vitro experiments (particularly the comparison to Xrn1 5',3'-exonuclease lacking similar endonucleolytic properties).

2. The phenotypic characterization of the B. subtilis rnjA deletion essentially repeats earlier studies of the same in B. subtilis and other bacteria and is highly redundant to the published reports.

3. The characterization of the effects of ΔrnjA1 on the cellular transcriptome and genome occupancy by RNAP is massive in scope, but largely minute with respect to the magnitude of the up- and down-regulation of transcripts, and not adequately explained by the reported changes in the RNAP distribution along the genome. The proposed connection to the emergence of the "spiral cells" phenotype is highly speculative. The only relevant observation stemming from these results is the emergence of a class of genes, which exhibited increased RNAP occupancy in ΔrnjA1 cells without a concomitant elevation of the transcription output, consistent with an increase in EC stalling. The authors did not provide any explanation as to the basis of this selective increase in stalling, as their discussion of RNase J1 activity lacks any hint regarding its specificity/selectivity.

4. NET-Seq would be a more appropriate method if the authors are talking about pause relief.

5. Fig. 2 is an interesting way of presenting CHIP-Seq data but it is neither very intuitive nor does it effectively reflect the genome-wide redistribution of RNAP. Perhaps a standard heat map of the CHIP-Seq signal would be more illustrative.

6. Fig, 3 does a nice job of illustrating the RNAP redistribution on a handful of genes, but I'd like to see some metagene analysis in which the fraction of the maximum RNAP signal along the gene body is plotted for each class identified.

7. The descriptions of the CHIP-Seq and RNA-Seq methods need more details. How many reads were obtained? How were they filtered? What was used as a control in CHIP-Seq experiments (input? IgG?) How many biological replicates were used (I am assuming 3, as they mention it later when discussing the comparison between the CHIP-Seq and RNA-Seq data, but it needs to be stated clearly for each case). How was the differential gene expression analysis performed?

8. It would be useful (and it would address some of the above issues) if the exact analysis pipeline was shared as code repo (github or similar) in the form of R markdown of jupyter notebook files.

9. The quantification of cellular RNAP in WT and ΔrnjA1 cells makes a relatively minor point and was carried out using only Western blotting. As the authors had focused on the amount of RNAP in the cell, they left the most pertinent question unanswered: if the amount of RNAP in ΔrnjA1 cells is significantly lower than in WT cells (for no apparent reason), what other proteins became more or less abundant, and which of them could have impacted EC stalling (obvious candidates include transcription elongation factors)?

10. In accord with the genome- and transcriptome-wide assays, a similar proteome-wide interrogation of ΔrnjA1 and WT cells must be performed with respect to protein abundance. Considering the proteomics data, the effects of ΔrnjA1 on cell morphology, RNAP distribution, etc. should also be investigated by contrasting double mutants or strains that overexpress relevant genes (such as Rho, GreAB, etc.).

11. Co-localization of a subset of RNAP and RNase J1 in the cell is neither surprising nor relevant, given the fact that the former produces the substrate for the latter, and at the resolution of the assay these data cannot be used to argue for a "physical linkage" between the two.

12. Similarly, the co-purification of RNase J1 and RNAP does not strictly argue for a direct "physical link", i.e., interaction between the two, especially considering the RNA-dependence of this putative interaction. More direct approaches are needed to support this claim.

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13. The structural model of the putative RNase J1-RNAP complex has a little probative value. If carried out in a transparent, score-driven fashion using high-quality experimental starting structures, such a docking model could be used as evidence of the physical plausibility of the proposed complex architecture, and guide subsequent probative experiments (model-specific disruption of the interface, etc.). However, the authors describe an essentially manual model building, guided by no constraints other than the inferred binding of RNase J1 to the nascent RNA extruded from an EC. The starting structure for the B. subtilis enzyme used in this work is a poor-quality homology model, built over a decade ago. The authors claim that "the model reveals an extensive interface between RNAP and RNase J1, the two enzymes fitting together remarkably well", but provide no quality metrics for the model, nor any other relevant metrics on which this optimistic assessment could be based (buried surface, predicted ΔG upon complex formation, etc.). The fact that the homology model used as the starting structure was built before 2005 based on the low-quality experimental structures of Thermus RNAPs means that it missed all the subsequent improvements in model construction and refinement, as well as the majority of more relevant (less evolutionary distant) templates. At this time we have access to the experimental models of bacterial elongation complexes in various states bound by different sets of elongation factors; this makes the rationale of choosing a non-functional minimal structural model in the context of this manuscript even more obscure.

14. The stated claim that RNase J1 can dissociate RNAP from DNA has not been experimentally demonstrated. No assay indicating that RNAP has dissociated from the DNA immobilized on beads have been reported. Instead, the dissociation of RNAP is inferred from the partial dissociation of the RNA from the complexes. Furthermore, the design and the interpretation of the experiments are significantly compromised by the omission of the RNase J1 endonuclease activity from consideration (the reference enzyme, Xrn1, lacks such activity). The description of the experiments fails to explicitly account for RNA molecules of apparently greater length than the 3'-labeled species (Fig 7B). Altogether, even taken at face value, the experiments do not specifically argue for the "collisional" torpedo model; dissociation of the stalled EC could also be explained by the shortening of the nascent RNA to the point that would incur backtracking (often observed in stalled complexes) to destabilize the complex (due to the shortening of the RNA-DNA hybrid).

1st Revision - authors' response 3rd October 2019

Referee #1:

Summary
The work in this manuscript falls into two major parts. In the first part, a combination of RNAseq and ChIPseq are used to analyze the effect of an rnjA deletion on the transcriptome and transcription. The ChIPseq was performed by pulling down RNA polymerase to determine gene occupancy. The combination of these analyses permits distinguishing between direct effects of RNase J on mRNA stability and indirect effects involving transcription. The results in the first part are by themselves interesting and important.
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The experimental work in the manuscript is of high quality. The conclusions are supported by the results.
The presentation of the work is clear and logical. The manuscript is very well written.

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Major concerns regarding conclusions
I have no major concerns regarding the conclusions.

Minor concerns that should be addressed.
Fig. 2. Instead of the dashed vertical line, it would be better if the graphs contained an internal grid that corresponds to the major divisions on the x- and y- axes.
RESPONSE:
We agree.
ACTION TAKEN:
We have changed the Figure (now Figure 1) and included an internal grid as suggested. Moreover, we normalized the data to input (see comment 7 of reviewer #3) and, therefore, the overall distribution of the dots slightly changed – now, the differences between wt and RNase J1-null strain appear even more pronounced.

Top of page 7, Fig SB? I assume the authors means Fig. 5B.
RESPONSE:
Yes, the reviewer is correct.

ACTION TAKEN:
In the revised version, as suggested by this Reviewer, this Figure panel is deleted (see also the next comment and response).

I do not understand what the authors mean when they say that RNAP and rpsB co-localize similarly, nor the reference to the 'well documented' coupling of translation to transcription. The images in Figure 5A and B strongly suggests that the distribution of RNase J differs significantly from rpsB, which is a marker for the 30 subunit of the ribosome. That a relatively small enzyme such as RNase J can enter into the nucleoid is not a surprise. Introducing translational coupling opens a complex set of issues that have nothing to do with the work in the article. Fig. 5B could be deleted. Fig. 5A, C and D make a coherent data set showing that a proportion of RNase J is localized in the nucleoid and that RNAP can be pulled down by RNase J in a complex that can be destroyed by RNase A treatment.
Consistent with the image in Fig. 5B, work over the past decade has shown that only a small proportion of ribosomes are localized to the nucleoid. The Cramer reference (Science 2017) shows coupling of the leading ribosome to RNAP. There is no evidence for polyribosome formation in nucleoid. Since there are 10- to 30-fold more ribosomes than RNAP, the experimental evidence suggests that polyribosomes are formed after release of a nascent transcript from the nucleoid. Discussion, page 12.
RESPONSE:
The idea here was to show that RNase J1 can be found in the vicinity of the nucleoid as a previous study *(Cascante-Estepa, N., Gunka, K., and Stulke, J. (2016). Localization of Components of the RNA-Degrading Machine in Bacillus subtilis. Front Microbiol 7, 1492.)* claimed that RNase J1 was predominantly localized in the cell pole regions. The ribosomal protein (i. e. ribosomes) was selected as a control because of the known transcriptional-translational coupling. We wished to show that RNase J1 can be at least as close to the nucleoid as the ribosomes are. The Figure shows that this is the case, and, moreover, that RNase J1 is possibly even more in contact with the nucleoid than the ribosomes are.
ACTION TAKEN:
To avoid confusion, we deleted Figure 5B as suggested. The Figure (now Figure 3) clearly demonstrates that RNase J1 is localized also in other areas of the cell than the cell poles and it is found also in the vicinity of the nucleoid.
Although E. coli and many other Gram negative bacteria do not have 5' to 3' exoribonucleases, it should be mention the Rho performs a similar 'torpedo-like' function in Rho-independent transcription termination in bacteria that do not have RNase J.
RESPONSE:
We agree.
ACTION TAKEN:

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The 'torpedo-like' function of Rho *(Epshtein, V., Dutta, D., Wade, J., and Nudler, E. (2010). An allosteric mechanism of Rho-dependent transcription termination. Nature 463, 245-249.)* is now mentioned in Discussion (next to the last paragraph on page 11).

The presentation of the work can be improved. Fig. 1 and Fig. 8 are not essential. They should be moved to the supplemental information. Fig. 1A and B are confirmation of previous work. The result in Fig. 1C can be mentioned as a line in the text.
RESPONSE:
For Fig. 1 we agree. This Figure was intended to show consistency with previous work.
For Fig. 8 , we feel that this Figure is an *in vitro* indication of the specificity of the torpedo effect of RNase J1 compared to a yeast 5' to 3' RNase, Xrn1 and is important to show in the main ms. Reviewer 2 also felt the specificity question was important and asked us to develop it further with Ec RNAP.
ACTION TAKEN:
We moved Fig. 1 to Supplementary information (now it is Fig. S1). Figure 8 is now Fig. 5 and it is in the main text. However, we performed similar experiments addressing the specificity of the torpedo effect with respect to the identity of RNAP, using RNAP of *E. coli*. These results (Fig. S11) are in the Appendix. See also Response B to the next comment.
For now, the model in Fig. 8 does not add anything. Is an extended interface necessary for the 'torpedo' model? In the absence of a crystal structure showing a tight interaction and conformation changes in the RNA polymerase that promote transcription termination, it is premature to enter into this line of reasoning.
RESPONSE:
We are not sure whether the reviewer is referring to the *in silico* model in Figure 6 (A) or suggesting that the experiment with Xrn1 (Fig. 8) is redundant (B)?
ACTION TAKEN:
A. As suggested also by reviewers #2 and #3, the *in silico* model was removed from the manuscript. We agree that it was speculative. Experiments are already planned to rigorously probe the interaction between RNase J1 and RNAP in detail. The results will be reported in due course. Instead, we created a new Figure (Appendix, Fig. S10) where we depict the length of RNA from the active site of RNases J1 and Xrn1, and RNAP to their surface – this partly explains the variation in the lengths of the RNA stubs created by the two enzymes.
B. The experiment with Xrn1 addresses the specificity of the torpedo effect (now Fig. 5). We also performed this type of experiment with *E. coli* RNAP (Figure S11 in Appendix). The results show that while the effect is RNase-specific, it is not RNAP-specific. In other words, the features within RNAP that are important for this mechanism are conserved between the two enzymes and it depends on the RNase how efficiently it is able to elicit the effect. This is consistent with the previously reported observation that yeast Rat1/Rai1 does not terminate *Escherichia coli* RNAP *(Park, J., Kang, M., and Kim, M. (2015). Unraveling the mechanistic features of RNA polymerase II termination by the 5'-3' exoribonuclease Rat1. Nucleic Acids Res 43, 2625-2637)*, probably due to the divergent structure of the yeast enzyme. This is now also mentioned in the manuscript (Page 11, the third paragraph from the bottom).
Moving part of the Discussion to the supplementary information in not satisfactory. By dealing with the above mentioned changes, which should liberate space in the main text, it should be possible, with some editing, to make a complete Discussion that covers all the work in the manuscript.
RESPONSE:
We agree. It was done due to space considerations.
ACTION TAKEN:
We revised the supplementary Discussion and moved it now into the main body of the manuscript as the first part of Discussion (this was possible due to the overall reorganization of the text and deletion of several Figures from the main manuscript).

Referee #2:

This is a very interesting report, in which the authors propose that Bacillus RNaseJ1 induces displacement of a stalled RNA polymerase (RNAP), a mechanism analogous to the torpedo model of termination in eukaryotes. Given that J1 is a 5' exo RNase, this scenario is logical and is supported by the authors' data, which include cellular co-localization and in vitro experiments. The Chip data are supportive of this conclusion.

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Overall, the manuscript is well written and illustrated. I will list only important points that should, in my opinion, be addressed, in the order of their appearance. A few minor flaws that could be easily fixed by copy editors.

1. I found the arguments on the RNAP abundance and the respective Figure 4 distracting, because only a fraction of the presented data could be explained by an overall increase in RNAP levels. I think that this figure and discussion would be better placed in the supplement.

RESPONSE:
This Figure illustrates a relatively minor point and, as such, can be moved into Appendix.
ACTION TAKEN:
We moved the Figure into Appendix (now Fig. S8) and the result is now only mentioned in the text.
It would be interesting to know how many genes belong to each of the 4 classes shown in Fig. 3 (and listed in the supplementary table, but a figure is much nicer), for example from a Venn diagram.
RESPONSE:
We agree.
ACTION TAKEN:
The numbers of genes in each class are now shown in the pie-chart in Fig. 2.

2. I am somewhat confused by the model of RNAP-J1 interactions. On one hand, the data argue that the nascent RNA is required to bridge the two enzymes, which makes a perfect sense. On the other, the authors build a helerologous model of the complex and propose an extensive interface, with the two proteins "fitting together remarkably well". In the absence of strong supporting experimental data, this model is a stretch, but does not do any harm. However, if I visualize the site correctly, in this model NusA should be located close by, if not sterically competing with, J1. The authors should comment on the implications in the discussion while bringing up flap-tip helix.
RESPONSE:
The *in silico* model was an attempt to view how the two enzymes (RNAP and RNase J1) fit together. The actual interaction is still unknown. NusA binds in this area and the flap-tip helix plays regulatory roles during transcriptional pausing *[J Mol Biol. 2019 pii: S0022-2836(19)30446-2.]*. We have designed new experiments that will address the details and mechanistic aspects of the RNAP-RNase J1 interaction. However, these experiments will take sizable amounts of time and will represent an independent project. The results will be reported in due time.
ACTION TAKEN:
We deleted the i*n silico* model from the manuscript. Instead, we created a new Figure (S10) where we depict the length of RNA from the active site of RNases J1 and Xrn1, and RNAP to their surface – this partly explains the variation in the lengths of the RNA stubs created by the two enzymes. This Figure does not address the details of the interaction as they are unknown and will be addressed experimentally in the future.
NusA and the flap-tip helix are now mentioned in Discussion (Page 11, the first paragraph).

3. The authors argue that J1 is specific because Xrn1 fails to digest RNA inside the complex. Yet Xrn1 actually gets closer to the RNA 3' end in Fig. 8; a 16 nt long transcript is barely out of the exit channel in structures. How does this happen without an intimate contact? Please elaborate.
RESPONSE:
Xrn1 most likely also reaches the surface of RNAP. However, it does not appear to be able to strip the RNA or provoke its release by RNAP as efficiently as RNase J1. The specificity comes from the ability to provoke dissociation of the RNA, rather than just making contact (see also response to comment 14 of Reviewer #3,).
ACTION TAKEN:
We performed a new experiment, demonstrating that RNase J1 is indeed capable of dislodging RNAP from DNA and that RNase J1 is more efficient in this activity than Xrn1 (Fig. 6.). This result dispels any lingering doubts whether or not the elongation complex is fully dissociated. We have modified the text and described the idea in more detail: Although both Xrn1 may also get close enough to actually contact RNAP judging by the length of the stubs, it appears that the efficiency of the ‘torpedo’ effect is RNase-specific as RNase J1 and Xrn1 acted with different efficiencies to provoke the release of the RNA/dissociation of the EC. (Page 6, last paragraph; Page 11, the second paragraph.

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4. In is not clear that a specific contact is required for the transcription complex dissociation, or why it would be necessarily beneficial. For example, Rho can terminate bacterial and eukaryotic enzymes. In any case, to make a specificity arguments, other RNAPs should be tested. I would use both E. coli and T7 RNAPs that are commercially available.
RESPONSE:
We agree.
ACTION TAKEN:
We performed the requested experiment with *E. coli* RNAP and the results (Fig. S11) show that with respect to the RNAP enzyme, both exonucleases (J1 and Xrn1) act relatively non-specifically ie the two enzymes behave differently from each other, but similarly with respect to RNAP [*B. subtilis* or *E. coli*]). This is consistent with the previously reported observation that yeast Rat1/Rai1 does not terminate *Escherichia coli* RNAP *[Park, J., Kang, M., and Kim, M. (2015). Unraveling the mechanistic features of RNA polymerase II termination by the 5'-3' exoribonuclease Rat1. Nucleic Acids Res 43, 2625-2637]*, probably due to the divergent structure of the yeast enzyme. This is now also mentioned in the manuscript (Page 6, the last paragraph).

5. As in E. coli, there are undoubtedly partially redundant factors that remove the stalled RNAP in Bacillus. J1 appears to be one of them, Rho is another. Is the double deletion strain viable, and if yes, is it hypersensitive to DNA damage?
RESPONSE:
We agree. Moreover, we also focused on HelD. This helicase-like protein associates with the RNAP core [*Wiedermannova, J., Sudzinova, P., Koval, T., Rabatinova, A., Sanderova, H., Ramaniuk, O., Rittich, S., Dohnalek, J., Fu, Z., Halada, P., et al. (2014). Characterization of HelD, an interacting partner of RNA polymerase from Bacillus subtilis. Nucleic Acids Res 42, 5151-5163*] and helps with RNAP recycling, at least *in vitro*. Our RNAseq experiments revealed that the HelD mRNA was ~5x upregulated in the RNase J1 strain and this possibly could have been because the cell was trying to compensate for the absence of RNase J1.
ACTION TAKEN:
We created new strains (□Rho, □□Rho-RNase J1 [the double-deletion strain is viable and grows similarly to the □RNase J1 strain], and □HelD, □□HelD-RNase J1) and performed UV-sensitivity assay experiments. The results of these experiments are now shown in Figure 7, demonstrating that while the absence of HelD does not appear to have a detrimental effect on the cell's survival after UV irradiation, the absence of Rho further negatively impacts the cell's survival in the absence of RNase J1. The absence of Rho alone does not have a negative effect. The experiment is now described in Results (see the last part of Results, "Effect of Rho") and commented on in Discussion. The additive effect of Rho and RNase J1 on UV-sensitivity suggests they act on non-overlapping RNA substrates.
With regard to the latter, I am slightly confused by the UV resistance assay in the supplement: the legend states that the strains were grown to OD of 0.5, but delta rnjA strain forms 100x fewer colonies. I do not think that an altered cell morphology can lead to such a dramatic difference in scattering.
RESPONSE:
This is a highly reproducible phenomenon. In part, it can be ascribed to a difference in scattering, and, in part to the filamentous phenotype of the RNase J1-null strain. The strain has problems to liberate daughter cells after cytokinesis and this may contribute to decreasing the number of colony forming units.
But the difference in sensitivity is not huge: as far as I can tell, wild type strain loses 2 logs, the deletion - 3 logs. I think that these data sho 11uld be in the main manuscript, as they are way more important than Fig. 4, and quantified by determining CFUs following irradiation. Plates are cute but less informative.
RESPONSE:
We agree.
ACTION TAKEN:
We performed new experiments (see ACTION TAKEN to your comment #5) and created a new Figure (Fig. 7). The Figure shows the number of CFUs. For each strain, we provide the ratio between UV-irradiated and UV-non-irradiated cells normalized to wt (the latter set as 1).

6. The pulling-out mechanism would arguably require a lot of force, a model that makes sense for

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Rho which can generate 200 pN, but for RNaseJ1? Even with Rho, the torpedo-like model proposed by Nudler is viable. Perhaps drawing parallels to bacterial factors (Mfd, Rho) is justified.
RESPONSE:
We agree.
ACTION TAKEN:
We modified Discussion, which now mentions Rho (and our new experiments with the Δ*rho* strain) and Mfd (Page 11, next to the last paragraph).
Referee #3:

The manuscript by Šiková et al. presents an intriguing hypothesis positing the involvement of the widely conserved cellular RNase J1 in removal of stalled transcription elongation complexes in B. subtilis. Given the importance of terminating stalled ECs in maintaining genome stability and protein traffic along DNA, this report would be of interest to a wide readership, beyond merely an interest in bacterial transcription. However, major claims require equally impressive evidence. As such, a number of issues should be addressed before this manuscript can be recommended for publication.

Specific criticisms

1. Introduction: The reference to Mathy et al. (2007) cited in relation to the discovery of RNase J1 should be replaced by that to an earlier and more relevant report by Evan et al, (2005).
RESPONSE:
The Even et al. (2005) reference only refers to the endonucleolytic activity of RNase J1. Since this sentence of the introduction is referring to the discovery of the 5'-3 exoribonuclease activity in bacteria, the Mathy et al 2007 reference is more appropriate here. We have however added the Even et al 2005 ref to the next sentence on the enzyme's endonuclease activity.
ACTION TAKEN:
We now cite both references (Page 2, the third paragraph).
Furthermore, while introducing enzymatic properties of RNase J1, the authors focused on its function as the 5',3'-exonuclease and completely overlooked its endonuclease activity, which has a direct bearing on the design and interpretation of their in vitro experiments (particularly the comparison to Xrn1 5',3'-exonuclease lacking similar endonucleolytic properties).
RESPONSE:
We agree that this is possible. However, the endonuclease activity of RNase J1 is significantly less prominent than its exonuclease activity in vitro (*Mathy, Cell 2007*) and thought to be essentially non-existant *in vivo* (*Condon RNA Biol review 2010*). Moreover RNase J1 increases its 5' exoribonuclease processivity with increasing RNA length (*Dorleans, Structure 2011*), Endonucleolytic shortening of the RNA by RNase J1 might therefore actually decrease the efficiency of the torpedo mechanism, leading to an underestimation of the effects we observed.
ACTION TAKEN:
We have added a paragraph to the discussion, mentioning this aspect as a potential caveat, but that would lead to an underestimation of the *in vitro* experiment (Page 10, the second paragraph).

2. The phenotypic characterization of the B. subtilis rnjA deletion essentially repeats earlier studies of the same in B. subtilis and other bacteria and is highly redundant to the published reports.
RESPONSE:
This was pointed out also by reviewer #1 and we agree.
ACTION TAKEN:
The Figure (now Fig. S1) was moved into Appendix.

3. The characterization of the effects of ΔrnjA1 on the cellular transcriptome and genome occupancy by RNAP is massive in scope, but largely minute with respect to the magnitude of the up- and down-regulation of transcripts, and not adequately explained by the reported changes in the RNAP distribution along the genome. The proposed connection to the emergence of the "spiral cells" phenotype is highly speculative.

RESPONSE:
We agree. Our previous studies with the depletion strain showed that most of the up-regulated genes are due to increases in RNA stability, where you do not expect a correlation with RNAP occupancy.

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This would correspond to class III genes, and likely many class I genes (which also show transcriptional up-effects); see discussion (Page 7, the last paragraph).
We agree that the discussion on the spiral phenotype is speculative, but not completely unsubstantiated. In *Kawai et al (2009) Mol Microbiol* Fig. 3N shows spiral-like morphology of cells overexpressing mreBH.
ACTION TAKEN:
We have changed the discussion on Page 7, the last paragraph to point out that many of the RNase J1 effects are known to have (all class III) or expected to have (some class I) a post-transcriptional component.
We changed the phrasing to: "…possibly explains, at least in part, the 'spiral' phenotype…" (Page 3, the end of the second paragraph in Results).
The only relevant observation stemming from these results is the emergence of a class of genes, which exhibited increased RNAP occupancy in ΔrnjA1 cells without a concomitant elevation of the transcription output, consistent with an increase in EC stalling. The authors did not provide any explanation as to the basis of this selective increase in stalling, as their discussion of RNase J1 activity lacks any hint regarding its specificity/selectivity.
RESPONSE:
One possible cause could be the relatively low expression of these genes that does not allow trailing RNAPs to push forward the leading RNAP if it stalls [Epshtein, V., and Nudler, E. (2003). Cooperation between RNA polymerase molecules in transcription elongation. Science 300, 801-805]. This is mentioned in the next to the last paragraph on page 9.

4. NET-Seq would be a more appropriate method if the authors are talking about pause relief.
RESPONSE:
We agree that NET-Seq might have been an interesting approach in hindsight, but feel it is not necessary at this point; we may employ it in follow up studies of the torpedo phenomenon.

5. Fig. 2 is an interesting way of presenting CHIP-Seq data but it is neither very intuitive nor does it effectively reflect the genome-wide redistribution of RNAP. Perhaps a standard heat map of the CHIP-Seq signal would be more illustrative.
RESPONSE:
We tried representing the data as heatmaps, but they did not convey the coverage distribution shift in the ChIPseq dimension clearly enough. Therefore, we decided to keep the scatterplot presentation of coverage data (ChIPseq ~ RNAseq).

ACTION TAKEN:
We changed the presentation of the graphs as suggested by reviewer #1 to make it more reader-friendly. We did make heat-maps, but they did not illustrate the changes as clearly as our original graph. However (see also the next comment and response), we created new summarizing profiles for Class I-IV genes (a different type of visualization) and these profiles are now part of the manuscript (Fig. 2; see also the next comment).

6. Fig, 3 does a nice job of illustrating the RNAP redistribution on a handful of genes, but I'd like to see some metagene analysis in which the fraction of the maximum RNAP signal along the gene body is plotted for each class identified.
RESPONSE:
We agree; this is an excellent suggestion.
ACTION TAKEN:
We created average gene profiles for Class I-IV genes (both for RNAseq and ChIPseq) where the normalized coverage signal is plotted along the gene length (all genes were normalized to be 1 kb long). The plots nicely illustrate the behavior of individual classes. These profiles are now shown in Figure 2, and the original Figure (showing individual genes) was deleted from the main body of the manuscript and moved to Appendix as Fig. S6.

7. The descriptions of the CHIP-Seq and RNA-Seq methods need more details. How many reads were obtained? How were they filtered? What was used as a control in CHIP-Seq experiments (input? IgG?) How many biological replicates were used (I am assuming 3, as they mention it later when discussing the comparison between the CHIP-Seq and RNA-Seq data, but it needs to be stated clearly for each case). How was the differential gene expression analysis performed?

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RESPONSE:
All of these concerns have been addressed.
ACTION TAKEN:
We have extended the description of ChIPseq and RNAseq in the Methods section to include the required details. All experiments were performed as biological triplicates, which we now state in the Methods. We now provide descriptive library statistics for each sequenced sample as a supplementary Table S8. Library size ranged from ~15M to 46M raw reads. We checked reads for adapter presence, mapped them with HISAT2, and only kept reads with MAPQ >= 10 (to also remove non-uniquely mapped reads). The percentage of mapped reads ranged from ~98% (RNAseq, CHIPseq inputs) to 68% (ChIPseq IPs).
As a control for ChIPseq, we originally used ChIP-qPCR validation, comparing occupancy of RNAP on genes vs intergenic regions (both normalized to input). As the results matched the observed ChIPseq patterns, we did not perform further ChIPseq normalization. Nevertheless, prompted by the referee, we now sequenced the corresponding original inputs used for the ChIP seq experiments and used them for IP coverage normalization. As expected, no major changes were observed for most genes. If anything, the discussed effects of *rnjA* deletion now appear even more pronounced than before.
The original assignment of genes to Classes I-IV was based on fixed thresholds (rnjA KO vs WT) for both ChIPseq and RNAseq data. In the revised manuscript we now base the classification on fixed thresholds in the case of input-normalized ChIPseq data, and on statistical analysis of differential expression in the case of RNAseq data (DESeq2, FDR 5%). Gene classification is now described in detail in a separate section of the Methods.
All details of ChIPseq and RNAseq data processing and analysis are now available as Rmarkdown documents from Github, as requested (see our response to your comment 8 below).
All details of ChIPseq and RNAseq data processing and analysis are now available as Rmarkdown documents from Github, as requested (see our response to your comment 8 below).

8. It would be useful (and it would address some of the above issues) if the exact analysis pipeline was shared as code repo (github or similar) in the form of R markdown of jupyter notebook files.

RESPONSE:
We agree.
ACTION TAKEN:
The scripts used for ChIPseq and RNAseq data processing and analysis are now available as Rmarkdown documents from https://github.com/mprevorovsky/krasny-torpedo

9. The quantification of cellular RNAP in WT and ΔrnjA1 cells makes a relatively minor point and was carried out using only Western blotting. As the authors had focused on the amount of RNAP in the cell, they left the most pertinent question unanswered: if the amount of RNAP in ΔrnjA1 cells is significantly lower than in WT cells (for no apparent reason), what other proteins became more or less abundant, and which of them could have impacted EC stalling (obvious candidates include transcription elongation factors)?
RESPONSE:
The lowered amount of RNAP in Δ*rnjA1* cells (and the decreased expression of many of the down-regulated mRNAs) could be an indirect effect of the slower growth rate of the *rnjA* mutant, as many genes related to the central processes of transcription and translation are expressed in proportion to growth rate.
ACTION TAKEN:
As suggested also by Reviewer 2, we removed this Figure from the main text (now Fig S8). Since the effects of the Δ*rnjA* deletion are very pleiotropic, it is difficult at this point to put a finger on any single factor that might be responsible for the drop in RNAP levels or an effect on stalling. A possible contributing factor to the observed stalling could be the decreased level of translation elongation factors; stalling ribosomes increase stalling of RNAPs due to uncoupling of the transcription-translation machineries (*Buskirk and Green, 2017; Nudler, 2012*). We now mention transcription and translation elongation factors in Discussion [Figure S12; page 8, last two paragraphs].

10. In accord with the genome- and transcriptome-wide assays, a similar proteome-wide interrogation of ΔrnjA1 and WT cells must be performed with respect to protein abundance.

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Considering the proteomics data, the effects of ΔrnjA1 on cell morphology, RNAP distribution, etc. should also be investigated by contrasting double mutants or strains that overexpress relevant genes (such as Rho, GreAB, etc.).
RESPONSE:
Identification of proteomic changes would be a new, independent project that we don't feel is warranted for the main point we want to make in this ms, ie that RNase J1 can act as a torpedo-like protein to release stalled RNAP from DNA. We have shown that this is a plausible explanation for the increased occupation of some genes by RNAP. There may be other explanations, but since a third of the transcriptome changes in the *rnjA* mutant, with both up and down effects, even a proteomic analysis may not bring us that much closer to identifying individual factors that could additionally contribute to RNAP pausing.
ACTION TAKEN:
We included a study of Rho and HelD (HelD was previously shown to assist in transcriptional cycling, possibly by helping dissociate RNAP-nucleic acid complexes *(Wiedermannova, J., Sudzinova, P., Koval, T., Rabatinova, A., Sanderova, H., Ramaniuk, O., Rittich, S., Dohnalek, J., Fu, Z., Halada, P., et al. (2014). Characterization of HelD, an interacting partner of RNA polymerase from Bacillus subtilis. Nucleic Acids Res 42, 5151-5163)* on the UV-sensitivity phenotype of the Δ*rnjA* strain. For details see comment 5 of reviewer #2 and our responses.

11. Co-localization of a subset of RNAP and RNase J1 in the cell is neither surprising nor relevant, given the fact that the former produces the substrate for the latter, and at the resolution of the assay these data cannot be used to argue for a "physical linkage" between the two.

RESPONSE:
Previously, RNase J1 was believed to be present mainly at cell poles (*Cascante-Estepa, N., Gunka, K., and Stulke, J. (2016). Localization of Components of the RNA-Degrading Machine in Bacillus subtilis. Front Microbiol 7, 1492.*). We used a more advanced super-resolution fluorescent microscopy to reveal that it is in fact also in other areas. We believe that it is important to show this because of the conflicting evidence in the literature. The question is not as anodyne as one enzyme generating the substrate for the other. The major endoribonucleases responsible for initiating mRNA decay in *B. subtilis* and *E. coli*, for example, are localized in the membrane.
ACTION TAKEN:
In the revised manuscript, we have changed the title of this section to "RNase J1 and RNAP co-localize *in vivo* and are associated through RNA"**.** The motivation behind the microscopy is mentioned on Page 5 (the first paragraph of the section). Moreover, we removed panel B (ribosomal protein-RNAP colocalization) from the Figure as requested by reviewer #1.

12. Similarly, the co-purification of RNase J1 and RNAP does not strictly argue for a direct "physical link", i.e., interaction between the two, especially considering the RNA-dependence of this putative interaction. More direct approaches are needed to support this claim.
RESPONSE:
The interaction between RNase J1 and RNAP is most likely short-lived as the former likely causes the dissociation of the latter from nucleic acids. Shortening of RNA to 16-19 nt then brings the two enzymes to close proximity where they must physically interact. The details of the interaction, however, are currently unknown. Shortening of RNA below 16 nt is not possible (unless the complex dissociates) as this length of RNA is hidden inside the exit channel of RNAP. That said, we agree with the referee that this interaction was not directly shown by the pull-down experiment, but is rather deduced by the torpedo experiment *in vitro*.
ACTION TAKEN:
We have change the word 'interact' for 'associate' throughout the section describing the pull-down experiment.

13. The structural model of the putative RNase J1-RNAP complex has a little probative value. If carried out in a transparent, score-driven fashion using high-quality experimental starting structures, such a docking model could be used as evidence of the physical plausibility of the proposed complex architecture, and guide subsequent probative experiments (model-specific disruption of the interface, etc.). However, the authors describe an essentially manual model building, guided by no constraints other than the inferred binding of RNase J1 to the nascent RNA extruded from an EC. The starting structure for the B. subtilis enzyme used in this work is a poor-quality homology model,

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built over a decade ago. The authors claim that "the model reveals an extensive interface between RNAP and RNase J1, the two enzymes fitting together remarkably well", but provide no quality metrics for the model, nor any other relevant metrics on which this optimistic assessment could be based (buried surface, predicted ΔG upon complex formation, etc.). The fact that the homology model used as the starting structure was built before 2005 based on the low-quality experimental structures of Thermus RNAPs means that it missed all the subsequent improvements in model construction and refinement, as well as the majority of more relevant (less evolutionary distant) templates. At this time we have access to the experimental models of bacterial elongation complexes in various states bound by different sets of elongation factors; this makes the rationale of choosing a non-functional minimal structural model in the context of this manuscript even more obscure.
RESPONSE:
We agree.
ACTION TAKEN:
We deleted the i*n silico* model from the manuscript. Instead, we created a new Figure (S10) where we depict the length of RNA from the active site of RNases J1 and Xrn1, and RNAP to their surface, which partly explains the variation in the lengths of the RNA stubs created by the two enzymes. This Figure does not address the details of the interaction as they are unknown and will be addressed experimentally in the future.

14. The stated claim that RNase J1 can dissociate RNAP from DNA has not been experimentally demonstrated. No assay indicating that RNAP has dissociated from the DNA immobilized on beads have been reported. Instead, the dissociation of RNAP is inferred from the partial dissociation of the RNA from the complexes.
RESPONSE:
Yes, we did not demonstrate dissociation of RNAP from DNA *per se* as we initially focused on RNA degradation, dependent on its release from the elongation complex [EC]. However, the release of RNA from the EC likely causes the EC to collapse *(Park, J.S., and Roberts, J.W. (2006). Role of DNA bubble rewinding in enzymatic transcription termination. Proc Natl Acad Sci U S A 103, 4870-4875).* Moreover, it was previously shown that the eukaryotic torpedo (exonucleolytic Rat1-Rai1 complex) elicits the release of stalled RNA pol II from DNA *(Pearson, E.L., and Moore, C.L. (2013). Dismantling promoter-driven RNA polymerase II transcription complexes in vitro by the termination factor Rat1. J Biol Chem 288, 19750-19759).*
ACTION TAKEN:
To dispel any doubts, we performed a new experiment, challenging elongation complexes with buffer (mock treatment), RNase J1, or Xrn1. The experiment demonstrated that RNase J1 is indeed capable of dislodging RNAP from DNA and that RNase J1 is more efficient in this activity than Xrn1 (Fig. 6.). We modified the text in Results (Page 6, the next to the last paragraph) and in Discussion accordingly (Page 10, the first paragraph).
Furthermore, the design and the interpretation of the experiments are significantly compromised by the omission of the RNase J1 endonuclease activity from consideration (the reference enzyme, Xrn1, lacks such activity).

RESPONSE:
We agree. However, the “endo” activity is relatively minor (see the following text).
ACTION TAKEN:
We modified the text, adding a paragraph discussing this issue (Page 10, the second paragraph): “We also considered the possibility that the endoribonuclease activity of RNase J1 might contribute to the results observed in this study, but a number of arguments favour the idea that the torpedo effect is primarily related to its 5’ -exoribonuclease activity. First, while RNase J1 does have endoribonuclease activity in vitro, it is primarily thought to act an exoribonuclease in vivo. Indeed, most of the endonucleolytic cleavage sites previously ascribed to RNase J1 in vivo are now thought to be performed by RNase Y, which has a similar specificity (Condon, 2010). The enzyme’s preference for exonucleolytic activity has been further confirmed by the crystal structure of RNase J1 bound to RNA (Dorleans et al., 2011). While RNA can easily be threaded through an entry channel to reach the catalytic site in exonucleolytic mode, endonucleolytic cleavage requires dissociation of dimers and then additional separation of the □-CASP from the beta-lactamase domain to allow the RNA to lie across the catalytic site. This likely explains why endonuclease activity is only observed in the presence of a large excess of enzyme over RNA, i.e. by simple probability, only a few isolated RNase J1 molecules are likely to be in a conformation capable of performing endonucleolytic cleavage. Lastly, it has been observed previously that RNase J1 acts

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more processively as an exoribonuclease with increasing length of RNA (Dorleans et al., 2011). Thus, if RNase J1 were to first shorten the RNA endonucleolytically before acting in as an exoribonuclease in our torpedo assay, this would likely result in a decreased efficiency of degradation of the short RNA (<5 nts) buried within RNAP and an underestimation of the torpedo effect."

The description of the experiments fails to explicitly account for RNA molecules of apparently greater length than the 3'-labeled species (Fig 7B).

RESPONSE:

We are not sure what the reviewer means here. There are no RNA fragments of greater length than those in the EC. A possible misunderstanding may stem from the fact that the marker RNA was 30 nt (the length of the longest fragment) and it was 5'-labeled. The RNA fragment in the EC was also initially 30 nt long, but it was 3'-end labeled and extended with 3 consecutive Us, increasing the length of the RNA to 33 nt (some fragments may be 32 nt). This is mentioned in the text and in the Figure legend (now Fig. 5).

Altogether, even taken at face value, the experiments do not specifically argue for the "collisional" torpedo model; dissociation of the stalled EC could also be explained by the shortening of the nascent RNA to the point that would incur backtracking (often observed in stalled complexes) to destabilize the complex (due to the shortening of the RNA-DNA hybrid).

RESPONSE:

We agree. Other scenarios are also possible, but we feel our data supports the torpedo model the best. .

ACTION TAKEN:

We modified the text, adding a paragraph to discuss the possibility of back-tracking in the Discussion (Page 10, the first paragraph, the last two sentences).

2nd Editorial Decision 31st October 2019

Thank you for submitting your revised manuscript for our consideration, it has now been seen once more by the original referees (see comments below). I am pleased to say that the referees overall find that their comments have been satisfactorily addressed. However, referee #1 raises three more minor points that should be addressed in a final revised version.

REFEREE REPORTS

Referee #1:

The authors have revised the manuscript in response to three lengthy and detailed critiques. They have adequately responded to most of the points raised by the referees. In addition to a major revision of the text, the authors have added new data. Altogether, the manuscript has been improved significantly. However, the new work raises three points that the authors should address.

1. Summary. The long sentence dealing with RNase and RNAP specificity is awkward. I suggest either breaking it into two sentences or replacing 'and' with a 'semicolon'.
2. Regarding the new work in Fig. S11 with E. coli RNA polymerase. I am not convinced that this experiment adequately addresses the issue of RNAP specificity since bacterial RNAP sequence and structure is highly conserved. A test with T7 RNAP would have been more meaningful since the structure of the monomeric T7 RNAP is distinct from the structure of the bacterial RNAP. In a comparison of the E. coli and B. subtilis RNAPs, what is the degree of conservation of amino acids in the exit tunnel and the surface surrounding the exit site?
3. Regarding the new work in Fig. 7. The interpretation based on SEMs is not convincing. P-values should be calculated.

Referee #2:

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In the revised manuscript, the authors have adequately addressed all my concerns. The manuscript is easy to follow, the figures have been improved, the conclusions are supported by the data, and discussion is interesting while not overly wild. Clearly, more experiments need to be done to evaluate the model, but this is always the case. In my opinion, the manuscript can be published in its current state because it proposes a very interesting new model and provides sufficient data in support thereof. These findings will be of interest to many researchers working on regulation of gene expression in all life.

Referee #3:

The authors have constructively addressed all my concerns, as well as those of the other two reviewers. I believe the paper is now acceptable.

2nd Revision - authors' response 11th November 2019

Referee #1:

The authors have revised the manuscript in response to three lengthy and detailed critiques. They have adequately responded to most of the points raised by the referees. In addition to a major revision of the text, the authors have added new data. Altogether, the manuscript has been improved significantly. However, the new work raises three points that the authors should address.

1. Summary. The long sentence dealing with RNase and RNAP specificity is awkward. I suggest either breaking it into two sentences or replacing 'and' with a 'semicolon'.
Done. We shortened the sentence. See also response to comment #2.
2. Regarding the new work in Fig. S11 with E. coli RNA polymerase. I am not convinced that this experiment adequately addresses the issue of RNAP specificity since bacterial RNAP sequence and structure is highly conserved. A test with T7 RNAP would have been more meaningful since the structure of the monomeric T7 RNAP is distinct from the structure of the bacterial RNAP. In a comparison of the E. coli and B. subtilis RNAPs, what is the degree of conservation of amino acids in the exit tunnel and the surface surrounding the exit site?

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We agree that RNAPs more divergent from *B. subtilis* RNAP than the *E. coli* enzyme can be found. We have analyzed the relevant exit tunnel region (amino acids) and found that, indeed, these two RNAPs are homologous within this region though not identical (the upper sequence is from *E. coli*, the bottom sequence from *B. subtilis*; alignments of relevant regions in beta [the first alignment] and beta' subunits of RNAP are shown):

Beta

```
4YLN:C|PDBID|CHAIN|SEQUENCE      NFEDSILVSERVVQEDRFTTIHIQELACVSRDTKLGPEEITADIPNVGEAALSKLDESGI
        870
AEW31312.1                       NYEDAIIMSERLVKDDVYTSIHIEEYESEARDTKLGPEEITRDIPNVGEDALRNLDDRGI
        814
                                 *:**:*::***:*::* :*:***:*  . :*********** ******* ** :**: **

4YLN:C|PDBID|CHAIN|SEQUENCE      VYIGAEVTGGDILVGKVTPKGETQLTPEEKLLRAIFGEKASDVKDSSLRVPNGVSGTVID
        930
AEW31312.1                       IRIGAEVKDGDLLVGKVTPKGVTELTAEERLLHAIFGEKAREVRDTSLRVPHGGGGIIHD
        874
                                 : *****..**:********* *:** **:**:******* :*:*:*****:* .* : *
```

Beta'

```
4YLN:D|PDBID|CHAIN|SEQUENCE      IFGPVKDYECLCGKYKRLKHRGVICEKCGVEVTQTKVRRERMGHIELASPTAHIWFLKSL
        120
AAB59112.1                       IFGPTKDWECHCGKYKRVRYKGVVCDRCGVEVTRAKVRRERMGHIELAAPVSHIWYFKGI
        110
                                 ****.**:** ******:::*:**:*::******::**************:*.:***::*.:
```

It is possible that with a more divergent RNAP, such as the single-peptide T7 phage RNAP, the result would be different. Therefore, we modified the claim (both in Abstract [claim deleted] and in Discussion [page 11, third paragraph]), narrowing it down to *B. subtilis* and *E. coli* RNAPs, and added a sentence discussing more divergent RNAPs (page 11, third paragraph).

3. Regarding the new work in Fig. 7. The interpretation based on SEMs is not convincing. P-values should be calculated.

Prompted by this comment, we consulted a statistician. We realized that in the previous version of Fig. 7 we were averaging ratios, which might be misleading [Communications of the ACM - The MIT Press scientific computation series; (1986) 29(3): 218-221, ACM New York, NY, USA]. Therefore, we performed the analysis using the logarithms of ratios instead. The p values for relevant combinations are shown above the bars. We note that for two combinations (wt *vs* Δ*rnjA* and Δ*rnjA vs* Δ*rnjA*Δ*rho*) the p values fall outside the statistically significant interval and this is now mentioned in the text (page 7; "Effect of Rho" paragraph).

Referee #2:

In the revised manuscript, the authors have adequately addressed all my concerns. The manuscript is easy to follow, the figures have been improved, the conclusions are supported by the data, and discussion is interesting while not overly wild. Clearly, more experiments need to be done to evaluate the model, but this is always the case. In my opinion, the manuscript can be published in its current state because it proposes a very interesting new model and provides sufficient data in support thereof. These findings will be of interest to many researchers working on regulation of gene expression in all life.

Referee #3:

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The authors have constructively addressed all my concerns, as well as those of the other two reviewers. I believe the paper is now acceptable.

3rd Editorial Decision 26th November 2019

Thank you for submitting your revised manuscript for our consideration. I am pleased to say we will be happy to formally accept the study for publication after the two final editorial issues are addressed.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Libor Krásný
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2019-102500

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ 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?	Not applicable.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not applicable.
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.	Not applicable.
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Not applicable.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable.
5. For every figure, are statistical tests justified as appropriate?	Error bars s.d. and s.e.m.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	DESeq2 assumes that raw read counts are used as input for identification of differentially expressed genes. This assumption was met.
Is there an estimate of variation within each group of data?	All treatment of RNAseq data during the identification of differentially expressed genes was performed by the DESeq2 package according to its internal statistical model.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-report

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	All treatment of RNAseq data during the identification of differentially expressed genes was performed by the DESeq2 package according to its internal statistical model.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	anti Beta RNAP, clone 8RB13, Santa Cruz; anti RNase J1, gift from Ciaran Condon, Institut de Biologie Physico-Chimique, Paris, France; secondary antibodies conjugated with a fluorophore dye (WesternBrightTM MCF-IR, Advansta, 700nm anti-rabbit (RNase J1) or 800nm anti-mouse (RNAP) antibody)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

D- Animal Models

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

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not applicable.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	ChIP-seq data: ArrayExpress E-MTAB-5659; RNA-Seq data: ArrayExpress E-MTAB-5660
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Not applicable.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable.

G- Dual use research of concern

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