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Organismal Benefits of Transcription Speed Control at Gene Boundaries

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2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

Fast and slow mutants in RNA polymerase II have been useful tools for analyzing how elongation rates affect RNA processing, histone modifications, and termination. This work has been primarily done in yeast (by replacing the WT copy) or in mammalian cell culture (by expressing the mutant as an alpha-amanitin resistant allele and poisoning the WT polymerase). Although there have been some studies with TFIIS mutants, to my knowledge, these types of polymerase mutants have not been previously studied in plants, or in the context of any full multicellular organism. So I think there is value in these studies. A slow mutant does not produce viable offspring, so not much can be said. The paper makes a good case that the Rpb2 Y732F mutant is faster than normal, although that conclusion rests in part on the corresponding mutant in yeast. Given the very high level of conservation in the Pol II active site, I think it's a pretty safe bet. Genomics experiments show the fast mutant causes clear effects on pausing, splicing, and termination. Similar conclusions have been reached in other organisms, but it's good to document.

I have some comments about statements I found confusing or felt were over-stating what the data shows.

1. Line 117: "plants homozygous for NRPB2-Y732F nrpb2-2." Isn't this a heterozygote by definition?

2. Line 124: These mutants presumably change the elongation rate everywhere, so I don't see how this leads to any conclusions about local or transient elongation regulation.

3. Please better explain the yeast phenotypes:
Line 135: What are the expected MPA and Mn++ phenotypes for fast and slow polymerases? Isn't MPA sensitivity usually indicative of slowed transcription (for example, TFIIS deletion) rather than fast mutants?
137/138: if there's no MPA or Mn phenotype in the Y769A mutant, how does that suggest reduced elongation? Isn't that the same phenotype you see in WT cells?

4. The Fig 2D presentation is confusing. It's unexpected that the more 3' positions appear to come up faster than the 5' probe. How are things normalized here?

5. It seems contradictory to see an increase in 5' cleaved splice sites by NET-seq, but less overall intron by RNA-seq. Please explain how these results are consistent.

6. Line 319: Probably worth repeating here that plants don't have NELF.

7. Lines 337-341: The fast mutant phenotypes may show some correlation with gene expression patterns during defense signaling, but contrary to what is written, this paper doesn't really establish "tight connections". This is just speculation at this point.

Referee #2:

The paper entitled "Organismal Benefits of Transcription Speed Control at Gene Boundaries" by Leng and collaborators is based on an interesting idea. The authors wanted to explore if RNA polymerase II elongation rate ("speed") is a relevant parameter that could determine gene expression regulation and, moreover, phenotype changes in plants. The authors planned to generate transgenic lines with "slow" and "fast" RNA polymerases. They report that a mutation decelerating RNA pol II transcription is not viable; however, due to this phenotype they were not able to assess transcriptional elongation in this line so they rely on observations in yeast to call it "slow". On the other hand, a mutation accelerating transcription is viable, and was further used in the manuscript to assess its effects on gene expression and for "speed" measurements. It is important to note that the phenotype of the mutant line is really strong. These plants are severely affected by the expression of the mutated polymerase. To further analyze the effects of the point mutation in the second largest subunit of the RNA pol II (NRPB2) in overall transcription rates the authors used a cutting-edge methodology, NET-seq, that was set up for plants by the same group in another paper (co-submitted). Though the idea is indeed intriguing, and the methods are likely appropriate, the conclusions lack novelty and need further work and validation. Moreover, since there are many other papers with similar questions in different organisms (Genes Dev. 2014 Dec 1;28(23):2663-76; Nat Commun. 2018 Feb 7;9(1):543. doi: 10.1038/s41467-018-03006-4; Mol Cell. 2019 Mar 7;73(5):1066-1074.e3. doi: 10.1016/j.molcel.2018.12.005; EMBO J. 2015 Feb 12;34(4):544-58. doi: 10.15252/embj.201489478), the originality of the results is mostly based on the novel methods here applied. Since the authors co-submitted a manuscript describing the method and showing changes in elongation caused by cold, this present manuscript loses-at least in the present state-its main strength.
My general vision is that these results could be part of the co-submitted manuscript as they are an example of the use of the plaNET-seq method. My main concern is related to the lack of novelty of the paper since the issue of transcription "speed" and processing/splicing is deeply explored in many organisms and previous papers. Moreover, while the results related to stalling at TSS and PAS regions of the genes are convincing the rest of the conclusions would need further validation.
Mainly, I don't think calling the Y732F mutant "fast" is appropriate from the observations in the manuscript. It is clear this mutant shows differences in initiation (and also in termination) but the changes in gene bodies could be explained by this and not related to changes in elongation.
Since I am aware of the amount of work required for a manuscript like this to be put together and that as a (human) reviewer I could be missing or neglecting the relevance of some results, below I provide a detailed list of concerns and comments that may help improving this manuscript and strengthening its conclusions.
-Figure 1. C. The western blot image and the Stain-Free Blot (loading control) images are quite poor. To my eyes it is not easy to claim that there are no differences in expression (or to claim the opposite). Western blots are not precisely known as a good tool to quantify protein levels. In fact, if the authors analyze the publicity of their "loading control" they will see that, while this stain-free method is quite linear with respect to protein amounts, the house keeping blots are not linear at all. In order to have a better idea of amounts the authors should use fluorescent secondary antibodies and something as Odyssey® Imaging Systems. From these images I would not say these lines have "comparable protein level".
-Figure 1. D. It would be interesting to know if there are significant differences between NRPB2Y732F and NRPB2WT. Related to this, when the authors claim that "'slow' transcription in NRPB2P979S fails to provide the RNAPII activity..." they are assuming that the mutation they

believe to be homologous to P1018S in yeast, provokes the same effect in plants. However, since the authors were not able to obtain these transgenic lines (in the null mutant background) they did not measure any parameter to know if RNAPII elongation is actually affected. Hence, this conclusion is wrong. The authors can just comment that the NRPB2P979S nrpb2-2 genotype cannot be recovered.
-Figure 1. E. The phenotype of the NRPB2Y732F nrpb2-2 is astounding. I don't see it simple to use this mutant line to compare anything with the "wild type". A plant looking like this mutant may have tons of transcriptional differences that might not be direct but a consequence of the extreme phenotype or even stress related.
-Figure S2. B. The double mutant looks the same as the single Y769F mutant, just that all the bands are less intense. If there is an effect of P1018S on Y769F is minor in terms of TSS shifts, then I do not think that concluding the double mutant is neutralizing TSS effects is right.
-Figure 2. D & E. Probe 3 of each locus is at a place where alternative splicing was reported for these genes, hence, these "probes" might be giving a wrong impression (if splicing is affected). Besides this, the candidate genes are too close to one another. The authors should give examples in different locations of the genome, different chromosomes would fit better. Moreover, by looking at figures S2 E-J it seems to me that the way the normalization was done for figs. D & E gives a wrong impression about the results. Faster transcription across the gene body means a higher capacity of reaching the 3' end of a gene in a shorter time. However, the beginning and the middle part of the gene (probes 1 & 2) must be reached by RNA pol II faster than the end since the polymerase has no other way to reach the end than going through the whole gene. In both examples of fig 2 D-E seems that probe 3 is peaking much earlier than probes 1 and 2. In any case, I think the authors should change the way they analyze RNA pol II speed. First, they should choose a final time point in which all the evaluated regions are at the highest possible level. This means that after this time point, the relative expression level in that particular probe is no longer rising, hence, the plateau was reached. By analyzing the supplementary figures, I would say 10 minutes is not looking like that time (plateau time) and the authors should explore longer times. After this, the authors should test how long it takes for the wild type and mutant polymerase to reach that level at each position (region/probe). To be called "fast", a polymerase should be able to reach that level (the plateau level) faster than the WT at each probe, not only at the 3' end of the gene. Besides this, it would be nice to see another way to measure pol II elongation in parallel, a pol II ChIP for example, to be able to compare the results and figure out if this new method is giving any advantage.
-Though it is true that by looking at figs. 3A and S3D it seems that RNA pol II accumulation close to the TSS is reduced in the Y732F mutant, what is also clear is that termination is compromised. Figs. 3A, 4A and S3D are all pointing towards a strong phenotype in termination. Figure 3D should be done considering this so to include the region downstream the PAS. From the general picture I would say that the mutant pol II shows differences in initiation (close to TSS) and termination (after PAS). However, I do not see big differences in gene body distribution. I mean, it is clear that the mutant is giving higher signal in exons and introns but with similar pattern than the WT. The authors conclude that all these changes are related to a higher "speed" of the mutant pol II. However, the differences could be attributed to a higher rate of general transcription. Since initiation seems to be affected, the mutant would have general higher amounts of transcribing polymerases throughout whole gene body while the elongation could remain the same.
-Figure 3. E. In the scheme below the figure it seems the authors are only considering internal exons; however, this is not clear from the text. Is that the case?
-Though it is hard to know this a priori, a faster transcriptional elongation would be expected to result in higher intron retention (less recognition of the 5' and 3' ss). Though, this is not that linear in animal cells, as shown by Fong and collaborators (Genes Dev. 2014 Dec 1;28(23):2663-76. doi: 10.1101/gad.252106.114); in plants, when using the At-ntr1 and TFIISmut, "fast" and "slow" mutant lines respectively, it is clear that fast elongation leads to higher intron retention while slow elongation leads to higher levels of intron splicing (Dolata et al., Genes Dev. 2014 Dec 1;28(23):2663-76. doi: 10.1101/gad.252106.114). Would be easier and more straightforward for the authors to analyze other types of alternative splicing. Exon skipping is probably the easier to correlate as fast elongation results (in general terms) in lower levels of exon inclusion. Since this is not so common in plants, the authors could use 5' and 3'ss in competition as in Dolata et al., 2014.
-For all the analyses, having constitutive and alternative exons and introns, introns with different lengths, exitrons (see Marquez et al., Genome Res. 2015 Jul;25(7):995-1007. doi: 10.1101/gr.186585.114) with canonical introns, as part of the same group is not recommended. The authors should analyze each event as a different category.
-Why to call the time points t0, t1... if these are just minutes... the authors could directly use 0, 1... and use min as unit. It makes it easier for the reader.

Referee #3:

RNA polymerase II (RNAPII) transcription is a highly dynamic and regulated process. While the regulation and importance of appropriate RNAPII speed have been intensively studied in yeast and animals, the situation in plants remains poorly understood. In this manuscript, Leng et. al. modulated RNAPII transcription speed by introducing point mutations in Arabidopsis thaliana. While the putative "slow" RNAPII is lethal to Arabidopsis, plants with fast RNAPII displays several interesting features. These include abolished 5' and 3' stalling, enhanced nascent RNAPII signals over gene bodies, reduced intron retention and defects in transcription termination. This study highlights the importance of RNAPII transcription speed in the regulation of gene expression and plant development.
While the data are overall solid and very well presented, the following comments should to be addressed.
Major concerns:
1. In Fig 2, the authors assayed the transcription speed of two FLG22 responsive genes in NRPBY732F. Validation of NRPBY732F as a fast mutant allele sets the foundation of all the following studies. It is therefore necessary to assay more FLG22 responsive genes and show that they are consistently transcribed at faster speed in the NRPBY732F mutant.
2. In Fig 4E, F, the authors need to clarify if the analysis is based on one or two biological repeats. If it is based on one experiment, a biological repeat and statistical analysis is necessary to validate that the difference observed is consistent and statistically significant.
Minor concerns:
3. The authors claim that "the classic 'slow' point mutation NRPBP979S failed to provide viable RNAPII activity during gametogenesis". However, there is no direct experimental evidence proving that Arabidopsis NRPBP979S is a slow mutant allele. The authors need to clarify that this is a "potential" slow mutation.
4. It would be ideal if the authors can show that the expression of NRPB2WT-FLAG transgene is comparable to the endogenous NRPB2 with anti-NRPB western blot. This will validate that the dynamics observed with NRPB2WT-FLAG reflects the endogenous NRPB2.
5. In Fig S2 E, F and H, the transcription rate in NRPBY732F mutant drops at t10. It would be interesting to discussion why this happens and if normalization of the previous time point to t10 is appropriate.
Additional suggestions:
6. In Fig 3C, the authors calculated the RNAPII stalling index. It would be interesting to know if the stalling index has any correlation with the expression level. i.e. If a higher stalling index correlates with larger FPKM.
7. In Fig 3G-I, the authors analyzed splicing efficiency and intron retention in NRPBY732F mutant. It would be interesting to see if there is any exon skipping from their RNAseq data, because fast transcription usually associates with exon skipping.
8. It would be interesting to look into the RNA seq data and examine what effects NRPBY732F has on global transcription and RNA stability.

1st Revision - authors' response 28 December 2019

Referee #1:

Fast and slow mutants in RNA polymerase II have been useful tools for analyzing how elongation rates affect RNA processing, histone modifications, and termination. This work has been primarily done in yeast (by replacing the WT copy) or in mammalian cell culture (by expressing the mutant as an alpha-amanitin resistant allele and poisoning the WT polymerase). Although there have been some studies with TFIIS mutants, to my knowledge, these types of polymerase mutants have not been previously studied in plants, or in the context of any full multicellular organism. So I think there is value in these studies. A slow mutant does not produce viable offspring, so not much can be said. The paper makes a good case that the Rpb2 Y732F mutant is faster than normal, although that conclusion rests in part on the corresponding mutant in yeast. Given the very high level of

conservation in the Pol II active site, I think it's a pretty safe bet. Genomics experiments show the fast mutant causes clear effects on pausing, splicing, and termination. Similar conclusions have been reached in other organisms, but it's good to document.

Response: We thank referee #1 for appreciating the novelty of our study. We have improved our assay to determine the *in vivo* transcription speed of RNAPII in *Arabidopsis* to strengthen the conclusions in our revised manuscript.

I have some comments about statements I found confusing or felt were over-stating what the data shows.

1. Line 117: "plants homozygous for NRPB2-Y732F nrpb2-2." Isn't this a heterozygote by definition?

Response: We thank referee #1 for pointing out the confusing abbreviated nomenclature. In this case, *NRPB2*$_{Y732F}$ *nrpb2-2* refers to plants homozygous for both, the transgene *NRPB2*$_{Y732F}$ and the *nrpb2-2* mutant allele. We have improved the nomenclature using *NRPB2*$_{Y732F}$ +/+, *nrpb2-2* -/- in the revised text (lines 112-113) to avoid misunderstanding.

2. Line 124: These mutants presumably change the elongation rate everywhere, so I don't see how this leads to any conclusions about local or transient elongation regulation.

Response: We agree with referee #1's point and we have removed the term "locally or transiently" in the revised text.

3. Please better explain the yeast phenotypes: Line 135: What are the expected MPA and Mn++ phenotypes for fast and slow polymerases?

Response: We apologize for the unclear explanation of MPA and Mn^{2+} related yeast phenotype. RNAPII fast mutants are characterized by MPA and Mn^{2+} sensitivity as demonstrated previously (https://doi.org/10.1016/j.molcel.2008.04.023, https://doi.org/10.1371/journal.pgen.1002627, https://doi.org/10.4161/trns.28869 and https://doi.org/10.1371/journal.pgen.1006321). RNAPII slow mutants tend to be MPA and Mn^{2+}-resistant (https://doi.org/10.1371/journal.pgen.1006321). We have improved our manuscript by clearly stating this point in lines 127-134.

Isn't MPA sensitivity usually indicative of slowed transcription (for example, TFIIS deletion) rather than fast mutants?

Response: We take the opportunity to better explain the mechanisms for MPA sensitivity.
In fact, we know now that there are likely multiple mechanisms rendering MPA sensitivity, however the primary mechanism arises from an initiation defect at the *IMD2* gene. This complex transcription unit is sensitive to defects in a large number of factors, and inability to express this gene appropriately results in GTP starvation induced by MPA treatment, thus showing growth defects towards MPA. The commonly assumed mechanism that MPA sensitivity derives from exacerbation of general transcription elongation defects through GTP limitation is erroneous. Most MPA sensitive mutants in fact are differentially GTP-limited from WT in response to the MPA treatment (https://doi.org/10.1002/yea.1300). The major determinant of this difference is due to differential *IMD2* expression, not necessarily RNAPII elongation defects (e.g. in Δdst1) (https://doi.org/10.1093/nar/gkx037). We have improved our manuscript by providing a better explanation to avoid misunderstanding in lines 131-134.

137/138: if there's no MPA or Mn phenotype in the Y769A mutant, how does that suggest reduced elongation? Isn't that the same phenotype you see in WT cells?

Response: We agree that the data on MPA and Mn^{2+} sensitivity by themselves are insufficient to conclude that Y769A is as slow mutant. Other yeast growth and reporter assay support this idea but we have excluded these data from the manuscript for the sake of simplicity. We address this comment by removing the related text from manuscript since the focus of this manuscript is on Y769F.

4. The Fig 2D presentation is confusing. It's unexpected that the more 3' positions appear to come up faster than the 5' probe. How are things normalized here?

Response: We thank referee #1 for raising concerns about the normalization, which leads to misunderstanding. This is the first time an assay for measuring the *in vivo* speed of RNAPII was developed in plants. We have based our analyses on an assay established in yeast, where

normalization to the maximal transcription time point is commonly used (DOI 10.1016/j.molcel.2005.02.017 ; doi: 10.1016/j.molcel.2012.10.014).
In the original Figure 2D and 2E, for each probe, nascent RNA level was first normalized to reference gene *ACT2* and then normalized to the relative nascent RNA level after 10 min flg22 treatment to monitor the relative gain of RNAPII on the candidate genes during treatment. Thus, the "relative gain" of RNAPII is sensitive to the nascent RNAPII level at 10 min, which might be misleading. In the original manuscript, the data for different probes were illustrated together in the same plots, which stimulated invalid comparisons among probes. The key comparison for this assay is to compare the nascent RNA level between mutant and wild type for the same probe during gene induction by flg22 treatment.
Since the normalization we previously used might have led to misinterpretation of the data, we have analyzed the data without normalization to any timepoint. In the revised Figure 2B and 2C, we directly show the nascent RNA level only normalized to reference gene *ACT2* separately. The new illustration clarifies that we observe similar induction level of nascent RNA by wild type and mutant RNAPII. Meanwhile, we also detected higher nascent RNA level in mutant RNAPII compared to wild type in the gene body and towards the end of candidate genes, starting at three minutes of treatment. These data suggest that although the WT and mutant RNAPII were roughly equally induced by flg22, the mutant RNAPII elongates faster toward the 3' end of candidate genes (revised Figure 2B, 2C and EV2E).
As suggested by other referees, we were able to find an additional candidate gene that is relatively long and quickly responsive to flg22 treatment. It is located on a different chromosome (revised Figure 2C). We also revised the position of our probe 3 to avoid any effects from potential alternative splicing events that happen near 3' gene end (revised Figure 2B and Figure EV2E). So, we replaced the previous data for probe 3 with data for new probe 3. We did not find that this changed the conclusion, which confirms the validity of our assay. The additional analyses clearly strengthen our conclusion that NRPB2-Y732F mutant RNAPII elongates faster than WT RNAPII on candidate genes.

5. It seems contradictory to see an increase in 5' cleaved splice sites by NET-seq, but less overall intron by RNA-seq. Please explain how these results are consistent.
Response: We apologize for the confusing text. We detected increased 5' cleaved splicing sites by NET-seq in Y732F mutant (revised Figure 4H), which is consistent with the idea of increased splicing efficiency in the mutant. More efficient splicing correlates with less intronic reads by RNA-seq (Figure 4I). We have better explained this point with revised text 231-234.
6. Line 319: Probably worth repeating here that plants don't have NELF.
Response: We have revised the text accordingly (line 328).

7. Lines 337-341: The fast mutant phenotypes may show some correlation with gene expression patterns during defense signaling, but contrary to what is written, this paper doesn't really establish "tight connections". This is just speculation at this point.
Response: We agree with the referee #1's point and revised our manuscript to conclude more modestly: "Our data proposed a potential connection between plant defense signaling, promoter-proximal RNAPII stalling and the speed of RNAPII transcription." (line: 342-343).

Referee #2:

The paper entitled "Organismal Benefits of Transcription Speed Control at Gene Boundaries" by Leng and collaborators is based on an interesting idea. The authors wanted to explore if RNA polymerase II elongation rate ("speed") is a relevant parameter that could determine gene expression regulation and, moreover, phenotype changes in plants. The authors planned to generate transgenic lines with "slow" and "fast" RNA polymerases. They report that a mutation decelerating RNA pol II transcription is not viable; however, due to this phenotype they were not able to assess transcriptional elongation in this line so they rely on observations in yeast to call it "slow".
Response: We concede that we have perhaps jumped to conclusions prematurely here and revised the text to reflect on what this mutant offers more appropriately.
On the other hand, a mutation accelerating transcription is viable, and was further used in the manuscript to assess its effects on gene expression and for "speed" measurements. It is important to note that the phenotype of the mutant line is really strong. These plants are severely affected by the expression of the mutated polymerase.

We agree that we were fortunate to present a novel mutation in RNAPII that accelerates transcription that confers striking images of growth defects. These images will make it very accessible to a broad scientific audience that the RNAPII transcript speed control is highly biologically significant for the growth and development of multi-cellular organisms. As the reviewer may know, corresponding yeast and mammalian cell cultures with mutant RNAPII share equivalent growth defects. While unfortunately not perfect, our analyses thus follow precedents in the literature where yeast and mammalian RNAPII mutants have been compared to a healthier isogenic "wild type" control.
In addition, we have revised the description of the transcription speed assay to avoid confusion. This assay has not been generated with the material shown in Fig.1. The material used for the transcription speed assay is more clearly pointed out in the revised Figure 2 and Methods (line 695), we could not detect noticeable growth differences in the material we used for this important assay. Our description of this assay has now been improved in the revised manuscript.
We believe that RNAPII point mutants that changed the speed of transcription in yeast and mammalian cell culture revealed important molecular insights even though they had severe growth defects. Our study presents the first mutant of that kind in plants, opening a new field of study.

To further analyze the effects of the point mutation in the second largest subunit of the RNA pol II (NRPB2) in overall transcription rates the authors used a cutting-edge methodology, NET-seq, that was set up for plants by the same group in another paper (co-submitted). Though the idea is indeed intriguing, and the methods are likely appropriate, the conclusions lack novelty and need further work and validation.
Response: We take the opportunity to list novelties in our manuscript that may have been missed.

- **Our manuscript presents the research material to study the effects of accelerated RNAPII transcription in the context of a multi-cellular organism for the first time. The multi-cellular dimension is inaccessible in yeast and human cell culture.**
- **Our data show that the process of organogenesis from stem cell niches can take place repeatedly in the presence of RNAPII with accelerated transcription speed. We consider it a very remarkable result that the gene expression programs dictating cell division, growth and morphogenesis can be executed at all.**
- **Our experimental data address the important question how much of the knowledge and models gained from cellular systems actually scales to the multicellular contexts.**
- **The effect on speed is caused by a novel point mutation in RNAPII second largest subunit directly.**
- **In plants, direct mutations in RNAPII that affects transcription speed have not been reported thus far.**
- **We also present an assay to investigate the *in vivo* transcription speed in *Arabidopsis* seedlings. This is an important achievement in and of itself, and it took us several years to develop this assay. At the same time, we are grateful for the suggestions of the reviewers to improve this assay in our revised manuscript.**

Moreover, since there are many other papers with similar questions in different organisms (Genes Dev. 2014 Dec 1;28(23):2663-76; Nat Commun. 2018 Feb 7;9(1):543. doi: 10.1038/s41467-018-03006-4; Mol Cell. 2019 Mar 7;73(5):1066-1074.e3. doi: 10.1016/j.molcel.2018.12.005; EMBO J. 2015 Feb 12;34(4):544-58. doi: 10.15252/embj.201489478), the originality of the results is mostly based on the novel methods here applied. Since the authors co-submitted a manuscript describing the method and showing changes in elongation caused by cold, this present manuscript loses-at least in the present state-its main strength. My general vision is that these results could be part of the co-submitted manuscript as they are an example of the use of the plaNET-seq method.
My main concern is related to the lack of novelty of the paper since the issue of transcription "speed" and processing/splicing is deeply explored in many organisms and previous papers.

- **The effect of accelerated RNAPII transcription speed and RNAPII pausing was not characterized at the molecular level in multicellular organisms, partly because the**

materials and methods needed to be developed. We have developed the missing materials and methods and combined them in this manuscript.

- **The biological insight that RNAPII transcription speed has such profound effects on the growth and development of a multi-cellular organism is also novel.**
- **The main results of the paper under review (Leng et al) is that the Y732F mutant reduces all major RNAPII stalling sites in plants. We believe this to be a substantial and important finding for the molecular biology of plants and beyond. We note that none of the papers above show mutants in RNAPII that have similar effects, in plants or other species.**
- **Moreover, the conclusions of this paper (Leng et al) are completely distinct from the conclusions in our manuscript that describes the development of the plaNET-seq method (https://doi.org/10.1093/nar/gkz1189). Therefore, we do not think it justified to merge these manuscripts, since the manuscripts are only connected by a common method and would read unfocused.**

Moreover, while the results related to stalling at TSS and PAS regions of the genes are convincing the rest of the conclusions would need further validation. Mainly, I don't think calling the Y732F mutant "fast" is appropriate from the observations in the manuscript. It is clear this mutant shows differences in initiation (and also in termination) but the changes in gene bodies could be explained by this and not related to changes in elongation.

Response: We believe this comment is due to a misunderstanding. In *Arabidopsis*, RNAPII 5'-end stalling is downstream of TSS and centered at the first nucleosome. The reduction of promoter-proximal stalling in *Arabidopsis* Y732F thus should not be confused with changes in transcription initiation. Therefore, we do not observe differences in initiation between *Arabidopsis* Y732F and wild type NRPB2 in our data. What we do observe is that the Y732F mutant reduces RNAPII stalling downstream of TSS and PAS, and that it results in increased RNAPII activity at gene bodies and transcriptional read-through (revised Figure 3, 4 and 5).
If Y732F RNAPII were to increase initiation to give higher RNAPII level in gene bodies without altering the stalling, then the plaNET-seq profile of mutant and WT RNAPII would be indistinguishable due to the FPKM normalization. The increased RNAPII level in gene bodies is the consequence of reduced stalling by Y732F rather than differences in initiation.
We found that Y732F is a fast mutant by showing that it elongates faster than wild type RNAPII on candidate genes. We have now revised Figure 2 to make this point and showed the difference in transcription elongation in the "fast" mutant compared to wild type.

Since I am aware of the amount of work required for a manuscript like this to be put together and that as a (human) reviewer I could be missing or neglecting the relevance of some results, below I provide a detailed list of concerns and comments that may help improving this manuscript and strengthening its conclusions.

-Figure 1. C. The western blot image and the Stain-Free Blot (loading control) images are quite poor. To my eyes it is not easy to claim that there are no differences in expression (or to claim the opposite). Western blots are not precisely known as a good tool to quantify protein levels. In fact, if the authors analyze the publicity of their "loading control" they will see that, while this stain-free method is quite linear with respect to protein amounts, the house keeping blots are not linear at all. In order to have a better idea of amounts the authors should use fluorescent secondary antibodies and something as Odyssey® Imaging Systems. From these images I would not say these lines have "comparable protein level".

Response: We have repeated the western blot experiment. We provide additional loading controls that help to appreciate that we fail to detect large differences in protein levels. These results are similar to experiments in yeast where single substitutions within the RNAPII active site do not derange protein levels on average. LiCor Odyssey is indeed a likely improvement from many other techniques. Unfortunately, we don't have access to one. We hope the reviewer can appreciate the new data and understands that it is beyond the scope of a revision to purchase a new piece of equipment.

-Figure 1. D. It would be interesting to know if there are significant differences between

NRPB2Y732F and NRPB2WT. Related to this, when the authors claim that "'slow' transcription in NRPB2P979S fails to provide the RNAPII activity..." they are assuming that the mutation they believe to be homologous to P1018S in yeast, provokes the same effect in plants. However, since the authors were not able to obtain these transgenic lines (in the null mutant background) they did not measure any parameter to know if RNAPII elongation is actually affected. Hence, this conclusion is wrong. The authors can just comment that the NRPB2P979S nrpb2-2 genotype cannot be recovered.

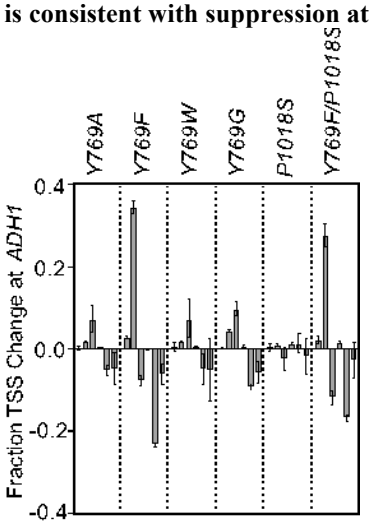
Response: We have included the missing statistical tests. The differences between $NRPB2_{Y732F}$ and $NRPB2_{WT}$ were not statistically significant. We have revised the text related to $NRPB2_{P979S}$ according to referee's suggestion. The text now reads "These data suggest that *$NRPB2_{P979S}$* fails to provide the RNAPII activity necessary for germline development" (line 109).

-Figure 1. E. The phenotype of the NRPB2Y732F nrpb2-2 is astounding. I don't see it simple to use this mutant line to compare anything with the "wild type". A plant looking like this mutant may have tons of transcriptional differences that might not be direct but a consequence of the extreme phenotype or even stress related.

Response: It is important to note that our comparisons are not using "wild type" as a reference, they use a line indistinguishable in appearance from wild type that carries the *NRPB-FLAG* transgene and complements the *nrpb2-2* mutant. So we use an isogenic control, the only difference of this control line to *$NRPB2_{Y732F}$* is one single amino acid substitution that we later show accelerates transcription. Therefore, our data demonstrate carefully the effect of a point mutation in RNAPII that accelerates transcription. RNAPII mutants altering transcription speed in yeast and human also exhibit growth defects, and when their effects on gene expression are analyzed equivalent controls to those in our manuscript are used. The most likely interpretation is that defects in transcription speed leads to the phenotype since this is consistent with how equivalent data in yeast and human are interpreted. In our revision, we have provided a clearer description of the control we used in every experiment.

-Figure S2. B. The double mutant looks the same as the single Y769F mutant, just that all the bands are less intense. If there is an effect of P1018S on Y769F is minor in terms of TSS shifts, then I do not think that concluding the double mutant is neutralizing TSS effects is right.

Response: Thanks for this point. We have now provided a quantitation of our primer extension data (Figure below) as described in https://doi.org/10.1534/g3.114.015180. We agree with the referee that the neutralizing effect of Y769F/P1018S on TSS shift is minor and have now removed the conclusion derived from this assay in the revised text. However, we stress that in Y769F/P1018S double mutant, the suppression of Y769F phenotype on MPA sensitivity is consistent with suppression at the level of initiation of *ADH1* seen by primer extension.



-Figure 2. D & E. Probe 3 of each locus is at a place where alternative splicing was reported for these genes, hence, these "probes" might be giving a wrong impression (if splicing is affected).

Response: We understand the referee's concern about the probe 3 used in the previous manuscript. To avoid potential effects from alternative splicing, we have made new experiments in which the position of probe 3 is changed. The new probe 3 is targeting a

genomic region without alternative splicing potentials near 3'-end of candidate genes (revised Figure 2B and Figure EV2E). The new data using the new probe 3 confirmed the previous conclusions.

-Besides this, the candidate genes are too close to one another. The authors should give examples in different locations of the genome, different chromosomes would fit better.
Response: We successfully identified an additional candidate gene that is relatively long and quickly responsive to flg22 treatment at a different chromosome (revised Figure 2C). The new analysis of this gene in this assay is shown in revised Figure 2. Given the restrictions that limit genes suitable for this assay identifying additional genes is far from a simple task. The equivalent assay in yeast measures repression of only a single transgene (GAL1-YLR454, DOI 10.1016/j.molcel.2005.02.017 ; doi: 10.1016/j.molcel.2012.10.014). The additional analyses strengthened our conclusion that mutant RNAPII elongates faster than wild type RNAPII.

-Moreover, by looking at figures S2 E-J it seems to me that the way the normalization was done for figs. D & E gives a wrong impression about the results. Faster transcription across the gene body means a higher capacity of reaching the 3' end of a gene in a shorter time. However, the beginning and the middle part of the gene (probes 1 & 2) must be reached by RNA pol II faster than the end since the polymerase has no other way to reach the end than going through the whole gene. In both examples of fig 2 D-E seems that probe 3 is peaking much earlier than probes 1 and 2.
Response: Many thanks for raising this important point. The previous analysis was based on the similar assay in yeast, where normalization to the reference time point was used to calculate the relative changes of RNAPII on template (DOI 10.1016/j.molcel.2005.02.017 ; doi: 10.1016/j.molcel.2012.10.014). We were under the impression that this was the most common approach, but the comments by the reviewers suggested otherwise. In the original figure 2D and 2E, normalization was based on the nascent RNA level at 10 minutes. This might have caused confusion as it may have given the impression that probe 3 is peaking faster than probe 1 and 2, possibly due to that in the original manuscript, the data for different probes were shown together in the same plots, which invited readers to invalid comparisons among probes. We apologize for this confusion. The key comparison for this assay is to compare the nascent RNA level between mutant and wild type for the same probe during gene induction by flg22 treatment.
In the revised Figure 2B, 2C and EV2E, we have analyzed the data without normalization to any specific time point. We now directly show the nascent RNA level, only normalized to reference gene ACT2. The new illustration now makes it clearer that we observe similar induction level of nascent RNA by wild type and mutant RNAPII. Meanwhile, we also detected higher nascent RNA level in mutant RNAPII compared to wild type in the gene body and towards the end of candidate genes, starting at three minutes of treatment. These data suggest that although the WT and mutant RNAPII were roughly equally induced by flg22, the mutant RNAPII elongates faster toward the 3' end of candidate genes (revised Figure 2B, 2C and EV2E).

-In any case, I think the authors should change the way they analyze RNA pol II speed. First, they should choose a final time point in which all the evaluated regions are at the highest possible level. This means that after this time point, the relative expression level in that particular probe is no longer rising, hence, the plateau was reached. By analyzing the supplementary figures, I would say 10 minutes is not looking like that time (plateau time) and the authors should explore longer times.

-After this, the authors should test how long it takes for the wild type and mutant polymerase to reach that level at each position (region/probe). To be called "fast", a polymerase should be able to reach that level (the plateau level) faster than the WT at each probe, not only at the 3' end of the gene.
-Besides this, it would be nice to see another way to measure pol II elongation in parallel, a pol II ChIP for example, to be able to compare the results and figure out if this new method is giving any advantage.
Response: We address the three related points above together. We have considered many alternative strategies including the one suggested by the referee and derived in the one used in the manuscript for the following reasons:

- **There appears to be a misunderstanding. Since the induction is transient, it should not be assumed that flg22 induction results in a plateau.**
- **The revised manuscript shows that the induction of candidate genes is synchronized from 0 to 4 minutes after flg22 treatment and mutant RNAPII elongates faster than WT RNAPII toward the 3' end of candidate genes (revised Figure 2B, 2C and EV2E).**
- **Thus, the WT and mutant RNAPII were about equally induced by flg22 at probe 1, the mutant RNAPII showed higher nascent RNA level at probe 2 and probe 3, suggesting faster elongation. We believe that our original data presentation may have been confusing, since we agree with the spirit of the comment of the reviewer in what we should see in this assay to conclude that transcription is accelerated in the mutant.**
- **We started our project several years ago with the idea that ChIP could give us the answer, but technical considerations associated with this assay made it clear that we had to develop this new and improved approach. It is clear to us that using ChIP to investigate native Pol II elongation in plant tissue will not give meaningful results because:**

    1. **ChIP requires tissue fixing in formaldehyde solution for at least 15 minutes. For plant tissues the formaldehyde tissue penetration usually requires vacuum infiltration. The associated treatment and sample handling times exceed the time where this assay can be conducted in a meaningful way. This is key since we are interested in the first few minutes. Therefore, changes of transcription may occur during cross-linking and sample handling that make ChIP not suitable here.**
    2. **ChIP also offers inferior resolution compared to our assay. We chose relatively long genes that may work also for ChIP, but nascent RNA offers higher resolution.**
    3. **Strand-specificity is lost in ChIP. To be sure that our method is not confounded by (frequent) antisense transcription, we rely on strand-specific measurements of nascent RNA.**
    4. **For these reasons, we moved away from ChIP to address this question. In our method, the tissue is snap frozen immediately after flg22 treatment, which preserves nascent fraction of Pol II without any time-lag or cross-linking effects. A similar approach helped us previously to detect nascent RNAPII elongation in Arabidopsis under cold treatment https://doi.org/10.1038/s41467-018-07010-6.**

-Though it is true that by looking at figs. 3A and S3D it seems that RNA pol II accumulation close to the TSS is reduced in the Y732F mutant, what is also clear is that termination is compromised. Figs. 3A, 4A and S3D are all pointing towards a strong phenotype in termination. Figure 3D should be done considering this so to include the region downstream the PAS. From the general picture I would say that the mutant pol II shows differences in initiation (close to TSS) and termination (after PAS). However, I do not see big differences in gene body distribution. I mean, it is clear that the mutant is giving higher signal in exons and introns but with similar pattern than the WT. The authors conclude that all these changes are related to a higher "speed" of the mutant pol II. However, the differences could be attributed to a higher rate of general transcription. Since initiation seems to be affected, the mutant would have general higher amounts of transcribing polymerases throughout whole gene body while the elongation could remain the same.

Response: We agree with this observation. However, we structured the manuscript to focus the results in Figure 3 on RNAPII stalling near 5'-end of genes. We focus on the 3'-end RNAPII stalling in revised Figure 5. We have revised the screenshot in Figure 3A and revised Figure EV3D by only showing the relevant regions to avoid readers focusing on aspects that are presented in the following figure.

plaNET-seq peaks downstream from TSS near 5'-ends of genes are considered as RNAPII stalling sites. These sites do not capture differences in initiation because the stalled RNAPII complex may either continue to productive elongation or terminate prematurely.

Instead, our data show that fast transcription speed results in relocation of RNAPII from stalling sites at 5'- and 3'-ends into gene bodies. The plaNET-seq signal was normalized to 1 million reads in each sample, therefore the metagene plots show only relative changes of the RNAPII profiles. If the Y732F mutation would result in a "general higher amount of

transcribing polymerases" without changing RNAPII stalling preferences, then the metagene profile in the mutant were indistinguishable from the wild type.

-Figure 3. E. In the scheme below the figure it seems the authors are only considering internal exons; however, this is not clear from the text. Is that the case?
Response: Yes, to avoid interference from RNAPII 5' stalling events, these analyses focused on internal exons. We have clarified this in the methods section and the revised figure legend (line 439).

-Though it is hard to know this a priori, a faster transcriptional elongation would be expected to result in higher intron retention (less recognition of the 5' and 3' ss). Though, this is not that linear in animal cells, as shown by Fong and collaborators (Genes Dev. 2014 Dec 1;28(23):2663-76. doi: 10.1101/gad.252106.114); in plants, when using the At-ntr1 and TFIISmut, "fast" and "slow" mutant lines respectively, it is clear that fast elongation leads to higher intron retention while slow elongation leads to higher levels of intron splicing (Dolata et al., Genes Dev. 2014 Dec 1;28(23):2663-76. doi: 10.1101/gad.252106.114). Would be easier and more straightforward for the authors to analyze other types of alternative splicing. Exon skipping is probably the easier to correlate as fast elongation results (in general terms) in lower levels of exon inclusion. Since this is not so common in plants, the authors could use 5' and 3'ss in competition as in Dolata et al., 2014.
Response: We thank referee for the suggestion to analyze more alternative splicing events. In the revised manuscript, we have improved the data analysis accordingly and provide two new figures for alternative splicing analysis (revised Figure 4 and EV5). Deeper analysis on RNA-seq in NRPB2-Y732F nrpb2-2 and NRPB2-WT nrpb2-2 identified differentially expressed (DE) exons and introns (revised Figure 4A). Quantification of DE effects suggest fast RNAPII is strongly correlated with reduced intron retention and slightly correlated with enhanced exon skipping (revised Figure 4B to 4E) in plants. We further investigated the effect of fast transcription on the shift of 5'SS and 3'SS. The results suggest that fast transcription tends to shift 5'SS upstream and 3'SS downstream in plants (Figure EV5).
Reviewer#2 points out a reasonable starting hypothesis that we shared when we started the project: the expectation that intron retention increases when transcription is accelerated. We also point out that this simple model for effects of elongation on co-transcriptional splicing emerged from yeast data, but given differences in splicing from yeast to multicellular organisms it might not be sound to assume this hypothesis to be generally true. Therefore, when examining *Arabidopsis*, we found this hypothesis not to be supported by the data: in our system, intron retention decreases in Y732F plants, concomitant with apparent acceleration of transcription. Our manuscript provides so far the only point mutation in RNAPII that affects intrinsic transcription speed in plants. Arguably, this approach changes RNAPII transcription more directly than using mutations in splicing factors or transcription factors as in the listed publications. Our manuscript also represents the first manuscript that provides a genome-wide comparison of splicing (i.e. RNA-seq) and nascent transcription (i.e. plaNET-seq) through a direct mutation in RNAPII. The effects of transcription speed on splicing are an active area of research and seem far from resolved, particularly when looking at this question in the context of multi-cellularity.

-For all the analyses, having constitutive and alternative exons and introns, introns with different lengths, exitrons (see Marquez et al., Genome Res. 2015 Jul;25(7):995-1007. doi: 10.1101/gr.186585.114) with canonical introns, as part of the same group is not recommended. The authors should analyze each event as a different category.
Response: We followed the referee's suggestion by providing the requested analyses.
We stratified exons and introns by their lengths and investigated the RNAPII activity (plaNET-seq). We found that the overall higher RNAPII level at exons and introns in fast mutant was not affected by their size (revised Figure EV3A to 3F). Likewise, mutant RNAPII showed higher level in both constitutive and alternative exons and introns (revised Figure EV3G to 3J).
In addition, we failed to observe clear effect of fast transcription on expression (RNA-seq) of constitutive and alternative exons and introns

-Why to call the time points t0, t1... if these are just minutes... the authors could directly use 0, 1... and use min as unit. It makes it easier for the reader.

Response: We have improved our nomenclature of the X-axes in revised Figure 2B-C thanks to this suggestion.

Referee #3:

RNA polymerase II (RNAPII) transcription is a highly dynamic and regulated process. While the regulation and importance of appropriate RNAPII speed have been intensively studied in yeast and animals, the situation in plants remains poorly understood. In this manuscript, Leng et. al. modulated RNAPII transcription speed by introducing point mutations in Arabidopsis thaliana. While the putative "slow" RNAPII is lethal to Arabidopsis, plants with fast RNAPII displays several interesting features. These include abolished 5' and 3' stalling, enhanced nascent RNAPII signals over gene bodies, reduced intron retention and defects in transcription termination. This study highlights the importance of RNAPII transcription speed in the regulation of gene expression and plant development.
While the data are overall solid and very well presented, the following comments should to be addressed.
Response: We thank Referee #3 for appreciating the advance provided by our study. We have improved our manuscript by addressing the concerns and additional suggestions as detailed below.

Major concerns:

1. In Fig 2, the authors assayed the transcription speed of two FLG22 responsive genes in NRPBY732F. Validation of NRPBY732F as a fast mutant allele sets the foundation of all the following studies. It is therefore necessary to assay more FLG22 responsive genes and show that they are consistently transcribed at faster speed in the NRPBY732F mutant.
Response: We acknowledge that additional flg22 responsive genes are beneficial. However, we would like to point out that it is far from trivial to select candidate genes suitable for this assay. Target genes need to be quickly responsive to flg22, induced at sufficiently high levels for detection and relatively long to resolve RNAPII transcribing this locus.
We successfully identified an additional locus. AT4G19520 is located on a different chromosome to rule out concerns of chromosome-specific dynamics suggested by reviewer #2. The assay for this gene is presented as revised Figure 2C. The new experiment strengthened our conclusion that NRPB2$_{Y732F}$ mutant RNAPII elongates faster than WT RNAPII.
We have incorporated the data in the revised Figure 2 and Figure EV2D and provided better explanation of this assay.
2. In Fig 4E, F, the authors need to clarify if the analysis is based on one or two biological repeats. If it is based on one experiment, a biological repeat and statistical analysis is necessary to validate that the difference observed is consistent and statistically significant.
Response: The 2 replicates of plaNET-seq showed strong correlation (revised Figure EV3A and B). All the metagene profiles were generated based on 2 biological replicates of plaNET-seq in both mutant and WT. We have revised the text to make this more accessible in the Methods (line 726).

Minor concerns:
3. The authors claim that "the classic 'slow' point mutation NRPBP979S failed to provide viable RNAPII activity during gametogenesis". However, there is no direct experimental evidence proving that Arabidopsis NRPBP979S is a slow mutant allele. The authors need to clarify that this is a "potential" slow mutation.
Response: We have revised the text to avoid overstatement. This mutation is "slow" in yeast (line 63-64). Instead of calling this mutant "slow" in Arabidopsis, we used the mutant name NRPB2$_{Y979S}$ in the revised manuscript and clarified that it is a potential slow RNAPII mutation in *Arabidopsis* due to its conservation to yeast RNAPII slow mutant *rpb2-P1018S* (*rpb2-10*).

4. It would be ideal if the authors can show that the expression of NRPB2WT-FLAG transgene is comparable to the endogenous NRPB2 with anti-NRPB western blot. This will validate that the dynamics observed with NRPB2WT-FLAG reflects the endogenous NRPB2.

Response: We tried hard to follow this suggestion. Unfortunately, antibodies for Arabidopsis proteins are not always available. Most of the total RNAPII antibodies for Arabidopsis are targeting subunit NRPB1 (AS11 1804 Agrisera). There used to be the antibody anti-RPB2 (ab10338, Abcam) that has been used to detect NRPB2 in Arabidopsis (https://doi.org/10.1105/tpc.112.098277; https://doi.org/10.1101/gad.1868009; https://doi.org/10.1104/pp.15.00351). However, this antibody was discontinued by Abcam (https://www.abcam.com/rpb2-antibody-chip-grade-ab10338.html). The replacement ab228933 antibody has never been reported to work for Arabidopsis NRPB2.
Besides, NRPB2WT-FLAG covering nrpb2-2 null allele has been reported previously to rescue mutant allele (https://doi.org/10.1534/genetics.108.090621). This line has previously been used to purify the RNAPII complex from Arabidopsis (https://doi.org/10.1016/j.molcel.2008.12.015), so we feel it is reasonable to consider this line to accurately reflect endogenous RNAPII complexes and serve as a reliable control in our assay.

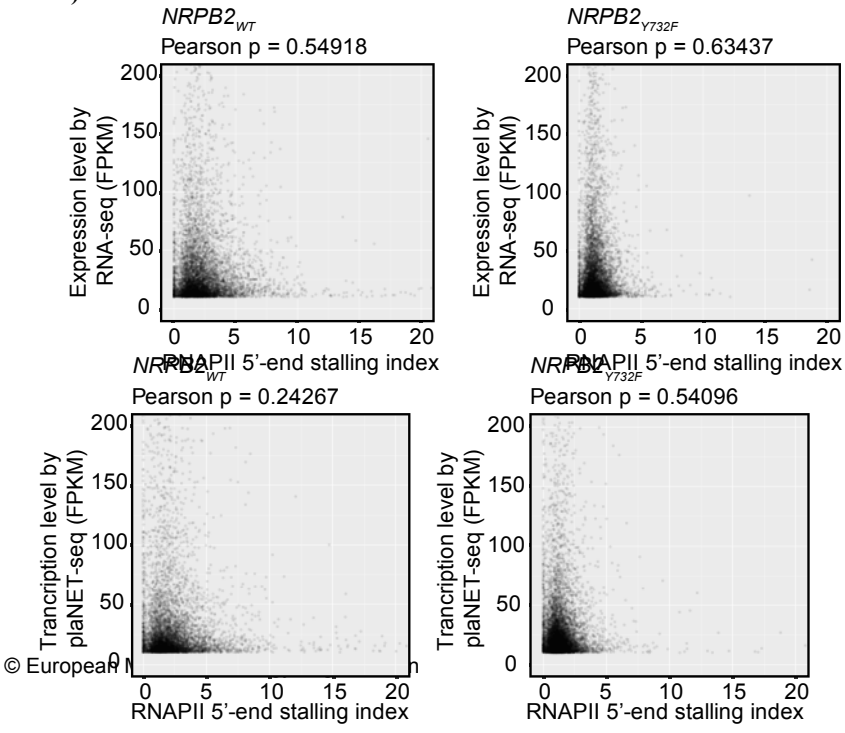
5. In Fig S2 E, F and H, the transcription rate in NRPBY732F mutant drops at t10. It would be interesting to discussion why this happens and if normalization of the previous time point to t10 is appropriate.

Response: We thank Referee #3 for pointing this out. In the original Fig S2 E, F and H, the nascent transcript level dropped at 10 min at the 5' end of genes in mutant compared to WT. This phenomenon might be due to the drop of gene induction at 10 minutes in the mutant compared to WT. This also suggests that the previous way to illustrate data normalized to 10 minutes may have not been ideal and potentially misleading.
We improved the illustration of this data in the revised manuscript by removing normalization to a time point. Instead, we chose to directly show the nascent RNA level (relative to reference gene *ACT2*) at each probe from 0 to 4 minutes after flg22 treatment. We observed similar induction level of nascent RNA by WT and mutant RNAPII at the region near 5'-end of candidate genes.
We also detected higher nascent RNA level in mutant RNAPII compared to WT in gene bodies and towards the 3´-end of candidate genes from three minutes of treatment onwards. These data suggest that the mutant RNAPII elongates faster toward the 3' end of candidate genes. This conclusion can be derived from comparing increased nascent RNA level at probe 2 and 3 compared to WT (revised Figure 2B, 2C and EV2E). We have also revised the manuscript with a better explanation of this assay.

Additional suggestions:

6. In Fig 3C, the authors calculated the RNAPII stalling index. It would be interesting to know if the stalling index has any correlation with the expression level. i.e. If a higher stalling index correlates with larger FPKM.

Response: We thank Referee #3 for the good suggestion to analyze if RNAPII 5'-end stalling is correlated with gene expression level. We now have provided these new analyses (Figure below).



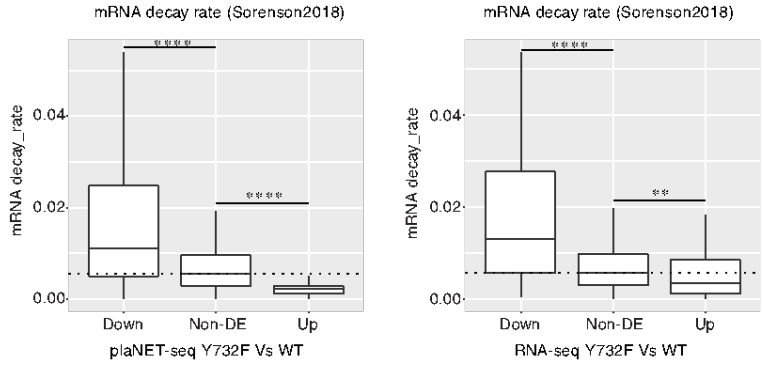
To address this question, we investigated the correlation between RNAPII 5'-end stalling index with transcription activity (by plaNET-seq) and gene expression level (by RNA-seq) in WT and mutants. However, we didn't observe a good correlation between RNAPII stalling index with either the transcription activity or the gene expression level. As we have shown in revised Figure 3D, reduction of RNAPII 5'-end stalling indeed leads to higher RNAPII activity in gene body in the fast mutant. However, RNAPII 5'-stalling may not represent a major determinant of regulating genome-wide mRNA expression in Arabidopsis.

7. In Fig 3G-I, the authors analyzed splicing efficiency and intron retention in NRPBY732F mutant. It would be interesting to see if there is any exon skipping from their RNAseq data, because fast transcription usually associates with exon skipping.

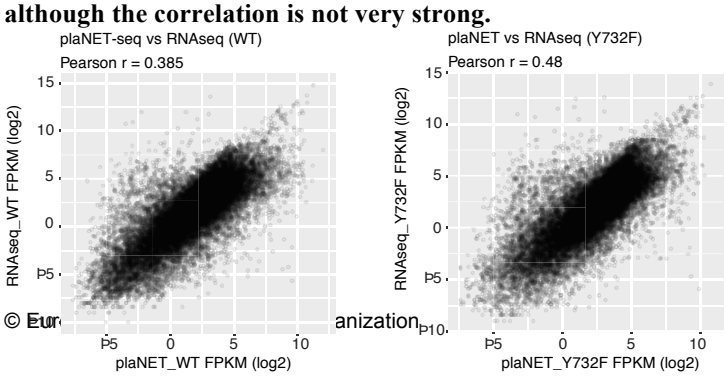
Response: We thank Referee #3 for this excellent suggestion. We have included these new analyses as part of a new Figure, revised Figure 4. Fast transcription is associated with exon skipping as previously shown (https://doi.org/10.15252/embj.201489478; https://doi.org/10.1016/j.molcel.2018.12.005.; https://doi.org/10.1101/gad.252106.114). To address the question, we reanalyzed our RNA-seq data in WT and fast mutant. We identified differentially expressed (DE) exons and introns in fast mutant by DEXSeq (https://doi.org/10.1101/gr.133744.111) (Revised Figure 4A). Our analysis suggests that there are more exon skipping and less intron retention in fast mutant compared to WT (Revised Figure 4B-E). We also found that the decreased intron retention is a more widespread phenomenon than the increased exon skipping.

8. It would be interesting to look into the RNA seq data and examine what effects NRPBY732F has on global transcription and RNA stability.

Response: To address this question, we investigated if differentially expressed genes in fast mutant have different RNA stability by using the published RNA decay rate dataset in Arabidopsis (https://doi.org/10.1073/pnas.1712312115) (Figures below). We found that accelerated RNAPII transcription appears to downregulate genes with less mRNA stability and upregulate genes with higher RNA stability. While potentially interesting, we provide these analyses to satisfy the curiosity of the reviewer as we found it challenging to incorporate these data into the manuscript.



We further investigated the correlation between nascent transcription activity (plaNET-seq) and mature mRNA level (RNA-seq). The results showed that overall nascent transcription activity is positively correlated with mature mRNA level in both WT and fast mutant, although the correlation is not very strong.



We appreciate the referee's interests in the effect of the fast mutant. We have extended our analysis in different alternative splicing events (intron retention, exon skipping, alternative 5'SS and 3'SS) in genome-wide. We found that fast mutant shows less intron retention, increased exon skipping, upstream-shifted 5'SS and downstream-shifted 3'SS. These new data are included as new Figure 4.

2nd Editorial Decision 23 January 2020

Thank you for the submission of your revised manuscript. We have now received the comments from all referees, as well as cross-comments, that are pasted below.

As you will see, while referee 1 is more critical, both referees 2 and 3 support the publication of your revised study. Referee 3, in the cross-comments, suggests another experiment, and you are very welcome to add this to your work, but it is not strictly required, also given that both referees 2 and 3 are satisfied with the current set of data. The novelty of the findings should be toned down though, or better explained.

Before we can proceed with the official acceptance of your manuscript, a few other issues need to be addressed:

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript file using the track changes option.

- Fig 4F+G are called out after 4H, please correct
- There is a callout for Fig 4I, but there is no such panel.
- Fig 6 panels are not called out.

- The DATASET EV titles are missing. Please add a title to the first tab in the excel sheet.

- The resource table can be part of the methods section. In this case the talbe does not need to be called out in the text.

- please label the numerical source data (add a title) and upload a single source data file per figure

- In the figure legends Fig EV4 panels D and E seem to be swapped

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

I deeply acknowledge and appreciate the efforts of the authors to answer all the concerns raised by the 3 different reviewers. I think the manuscript, in this new version, is easier to follow and the results are clearer, however, some of my main concerns are still present. Firstly, though the authors claim that they provide material to study the effects of accelerated RNAPII transcription in a multi-cellular context for the first time, this is not the case. It is true that the mutation they use, affects one of the main subunits of the RNAPII, NRPB2, other authors addressed this issue previously by using pharmacological inhibitors, chromatin modifiers and mutations ("indirect") affecting polymerase

elongation. Hence, the main point of the authors is that they use a "direct mutation". I do not understand why this way of addressing this issue would be better (or "more valid") than those used previously. Secondly, and connected to this, the phenotype of the mutant (NRPB2Y732F on the nrpb2-2 background) is astonishing. I was expressing this before and I still do. I see this as a negative thing for establishing comparisons to whatever that looks like the wild type. I think there was a misinterpretation of my previous comment on this, when I said: "I don't see it simple to use this mutant line to compare anything with the "wild type". A plant looking like this mutant may have tons of transcriptional differences that might not be direct but a consequence of the extreme phenotype or even stress related." I meant that using such a strong phenotype to compare effects of polymerase speed is, from my point of view, wrong. The expression of NRPB2Y732F on the nrpb2-2 background shows an extremely dwarf and affected phenotype that would-most likely-provoke tons of differences, at any parameter tested, with a "wild type" plant (in this case the NRPB-FLAG on the nrpb2-2 background).
I am sorry and apologize to the authors if I'm not getting their point from the manuscript or they response to the reviewers, but these main concerns are substantial from my perspective.

Referee #2:

I am satisfied with the authors' responses to my review.

Referee #3:

The authors addressed my concerns and the paper appears ready to publish.

Cross-comments by referee 3:

I disagree with the referees first point. Perhaps the referee has a point that the paper overstresses the novelty, but I think the authors should just tone down this in the text. It is still the first time any such study has been done in plants, and to be this seems novel enough.

On the second point, I would think this issue is addressable. The plants are dwarfed, etc etc, and so it is hard to compare with wild type which has a normal morphology. So yes, some of the effects will be indirect. So what if the authors parse out different gene groups and see if they can reproduce the difference, for instance only at housekeeping genes that should be the same in tall vs dwarfed plants.

2nd Revision - authors' response 24 January 2020

Referee #1:

I deeply acknowledge and appreciate the efforts of the authors to answer all the concerns raised by the 3 different reviewers. I think the manuscript, in this new version, is easier to follow and the results are clearer, however, some of my main concerns are still present.

We thank reviewer 1 for appreciating the improvements we have made in the revised manuscript thanks to the contributions of all reviewers.

Firstly, though the authors claim that they provide material to study the effects of accelerated RNAPII transcription in a multi-cellular context for the first time, this is not the case. It is true that the mutation they use, affects one of the main subunits of the RNAPII, NRPB2, other authors addressed this issue previously by using pharmacological inhibitors, chromatin modifiers and mutations ("indirect") affecting polymerase elongation. Hence, the main point of the authors is that they use a "direct mutation". I do not understand why this way of addressing this issue would be better (or "more valid") than those used previously.

We apologize for the confusion. We carefully checked our revised manuscript for the mentioned claim, but the statement "… provide material to study the effects of accelerated RNAPII transcription in a multi-cellular context for the first time …" or similar is not present. Perhaps reviewer #1 refers to a bullet point in our rebuttal letter that simplified the context of our discovery. It is correct that we provide the first mutation in the trigger loop in *Arabidopsis* with direct effects on RNAPII elongation. We appreciated and extensively cited the publications (Dolata et al., Herz et al.) that had previously approached this question by using pharmacological inhibitors, chromatin modifiers and mutations affecting polymerase elongation. We highlighted the overlap of our findings to the *ntr1* mutant in lines 254-256, but some of our findings differ from previous reports, perhaps because we use the novel plaNET-seq method that is now available to address questions concerning RNAPII elongation genome-wide and with single nucleotide resolution in plants.

We address this comment by toning down the claim of our novelty, particularly in line 305.

Secondly, and connected to this, the phenotype of the mutant (NRPB2Y732F on the nrpb2-2 background) is astonishing. I was expressing this before and I still do. I see this as a negative thing for establishing comparisons to whatever that looks like the wild type. I think there was a misinterpretation of my previous comment on this, when I said: "I don't see it simple to use this mutant line to compare anything with the "wild type". A plant looking like this mutant may have tons of transcriptional differences that might not be direct but a consequence of the extreme phenotype or even stress related." I meant that using such a strong phenotype to compare effects of polymerase speed is, from my point of view, wrong. The expression of NRPB2Y732F on the nrpb2-2 background shows an extremely dwarf and affected phenotype that would-most likely-provoke tons of differences, at any parameter tested, with a "wild type" plant (in this case the NRPB-FLAG on the nrpb2-2 background). I am sorry and apologize to the authors if I'm not getting their point from the manuscript or they response to the reviewers, but these main concerns are substantial from my perspective.

Any mutation in an organism likely confers direct and indirect effects. This might seem more intuitive for mutants that confer strong phenotypes, but even at the single cell level this is the case. However, even when phenotypes may be indirect they can also be quite specific. The phenotypes we describe here for RNAPII mutants are widespread yet still specific. Because RNAPII is required for expression of all genes, any analysis of a RNAPII mutant, regardless of gross developmental defect or not, will necessarily result from a combination of direct and indirect effects. We argue here that the phenotypes we observe are extremely interesting and point to general control of Pol II at 5' and 3' gene ends in *Arabidopsis*.

We apologize for the confusion. We have added an extra sentence in lines 352-354: "Nevertheless, some molecular effects we reported could represent indirect effects caused by differences in growth and development between NRPB2Y732F +/+ nrpb2-2 -/- and NRPB2WT +/+ nrpb2-2 -/-."

Referee #2:

I am satisfied with the authors' responses to my review.

We thank reviewer 2 for the positive evaluation of our revisions.

Referee #3:

The authors addressed my concerns and the paper appears ready to publish.

We thank reviewer 3 for endorsing our manuscript for publication.

Cross-comments by referee 3:

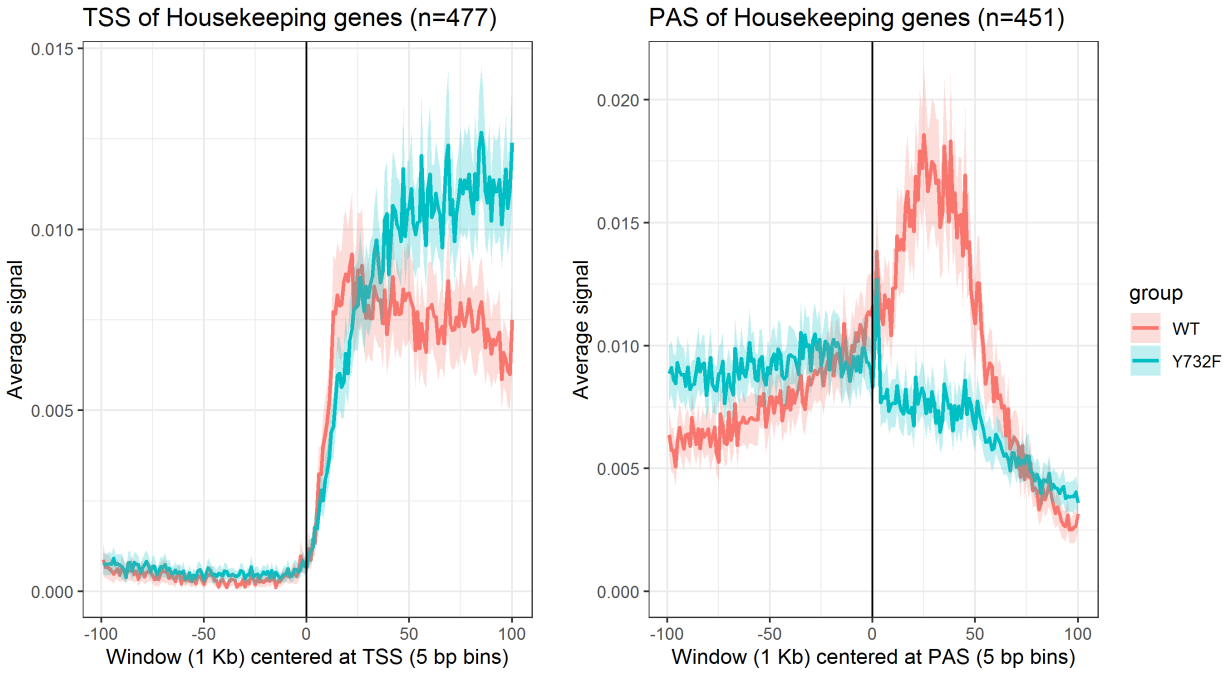
I disagree with the referees first point. Perhaps the referee has a point that the paper overstresses the novelty, but I think the authors should just tone down this in the text. It is still the first time any such study has been done in plants, and to be this seems novel enough.

We thank referee #3 for utilizing the cross-comment option. We have toned this down in the text, particularly in line 305.

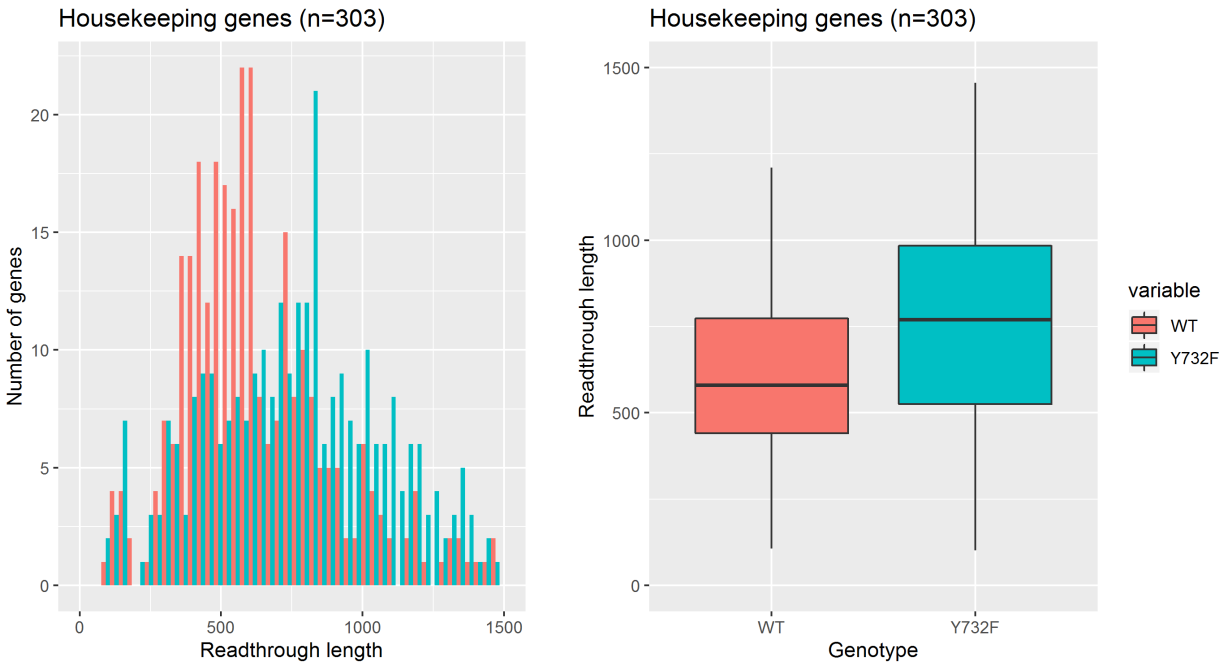
On the second point, I would think this issue is addressable. The plants are dwarfed, etc etc, and so it is hard to compare with wild type which has a normal morphology. So yes, some of the effects will be indirect. So what if the authors parse out different gene groups and see if they can reproduce the difference, for instance only at housekeeping genes that should be the same in tall vs dwarfed plants.

We thank referee 3 for this useful comment. We have validated some of our findings on the set of housekeeping genes obtained from Cheng et al., 2017 (PMID 27862469). This set contains 692 genes which were found to have the most stable expression among multiple RNA-Seq datasets. We found that the Y732F-associated patterns of RNAPII transcription described in our manuscript were fully reproduced on the limited set of housekeeping genes:

1) The defect of TSS- and PAS-associated RNAPII stalling remains clearly visible on metagene plots of plaNET-seq signal in WT vs Y732F genotypes:



2) The readthrough (RT) length is clearly increased in Y732F compared to WT in the housekeeping genes:



The median values of RT length are 592 bp and 797 bp in WT and Y732F, respectively. As expected, they are significantly different (Wilcoxon p-value = 1.68e-08).

Therefore, it is unlikely that potential indirect effects through differences in growth are fully responsible for our genome-wide findings about RNAPII transcription.

Accepted 30 January 2020

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

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Corresponding Author Name: Sebastian Marquardt
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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 χ^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?	Yes.
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.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA.
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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA.

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Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	NA.

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).	Yes.
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.	NA.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA.
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.	NA.

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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.	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	Yes.
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).	NA.
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).	NA.
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