NTR1 is required for transcription elongation check-points at alternative exons in Arabidopsis

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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.)

Editors: Alexander Kohlmaier and Anne Nielsen

1st Editorial Decision 14 March 2014

Thank you for submitting your manuscript (“spliceosome disassembly factor NTR1 is involved in transcriptional pausing at alternative exons in Arabidopsis.”) to The EMBO Journal. We have to apologize that that we did not contact you with a decision earlier: We have considered your manuscript carefully within our editorial team meeting, including discussion with our chief editor Dr. Pulverer. As described in detail below, parts of your manuscript raised significant interest while other aspects caused equal concern. Therefore, we asked for external advice in the form of in-depth consideration by an acknowledged expert in the field, which caused the delay. Together, unfortunately we must come to the conclusion that we cannot offer publication in The EMBO Journal.

We do realize that you present a functional characterization of the A. thal. homolog of NTR1. Beyond the previously described increased intron retention and exon skipping in atntr1 mutants, a preference for distal 5’ splice site selection is reported here in the atntr1 mutant. The manuscript develops and tests the hypothesis that a regulation of RNAP II elongation rate by AtNTR1 might underlie this phenomenon. While CpG DNA methylation changes in gene bodies were indeed observed in the atntr1 mutant, these did not correlate with altered splicing at affected targets. Yet, indirect interference with endonucleolytic cleavage of RNAP II via an engineered dominant-negative TFIIS mutant construct caused a mutant splicing phenotype that was largely opposite to
defects in the atntr1 mutant. Beyond this correlative evidence, decreased occupancy of RNAP II was observed at alternatively spliced junctions in the atntr1 mutant, at least at a subset of targets.

The relation between Pol II elongation and splicing/alternative splicing has been less explored in plants than in mammalian cells and yeast. Although we agree that the manuscript is, thus, conceptually interesting in principle, our external adviser as well as our editorial team felt that the main conclusions of the present manuscript were based on correlations. As such, in our combined view, the manuscript remained rather speculative and did not offer the sort of sufficiently major conceptual advance to warrant publication in the EMBO Journal.

In particular, the expert acknowledges that the manuscript shows that mutants of the NTR1 factor indeed have defects in alternative splicing of relevant genes. However, the link to elongation was elusive because it was solely based on Pol II ChIPs, where the landscape distribution of Pol II is investigated in mutants of the transcription elongation factor TFIIS and is compared to the NTR1 mutants. As such, the manuscript did show that the TFIIS and NTR1 mutants show opposite effects both in Pol II pausing and in alternative splicing patterns. At this state of development this sort of insight was rather descriptive though. Besides, the fact that the introns involved in the alternative splicing decisions are very short made it difficult to reconcile with a role of pausing and elongation. As you also acknowledged, the results would also be compatible with an effect of splicing on transcription, as reported by the Beggs laboratory. If so, although experimentally difficult in plants, inhibition of splicing by spliceostatin or similar drugs should abolish the effects of the NTR1 mutant on PolII distribution. At this point, whether the changes in RNAP II pausing were consequence or cause of splicing defects remains to be decisively determined.

Our combined reservations regarding the cause-effect nature of observations and thus the conclusiveness of this work leave me little choice but to conclude that we will not be able to offer publication of this study. I am sorry to be unable to communicate more encouraging news, but hope that our feedback was helpful.

Resubmission 31 July 2014

2nd Editorial Decision 04 September 2014

The original editor is out of office for the next two weeks, and I will, therefore, step in, as I have also handled your paper in the first phase of the review process. Thank you for submitting your manuscript "NTR1 is required for transcription elongation check-points at alternative exons in Arabidopsis" for our consideration. We have now received the comments of two expert referees. As you will see, both referees find the reported findings interesting in principle, and rate the novelty of your findings high. Both referees list, however, also a number of concerns as well as constructive suggestions for corrections (see attached below). Pending adequate addressing the specific concerns raised, we are therefore happy to consider a revised version of your manuscript for publication in The EMBO Journal.

I have, however, to focus here on one central point that crystallized as being crucial for the conclusiveness of the paper: Both referees concur, that direct experimental evidence for faster RNAP II elongation in the absence of AtNTR1 is still missing. Only this could conclusively substantiate that kinetic coupling occurs. While referee #1 does not insist on the direct measurement of the RNAP II elongation rate for the paper to be ultimately considered for publication, referee #2 thinks that only performing this assay could allow to conclusively answer the question whether kinetic coupling occurs.

I realize, as the referees do, that this issue may be difficult to tackle for technical reasons in your specific experimental system. Therefore, I would appreciate hearing back from you within the next
week, if possible, how you could envision responding to this specific concern. Could you please send to me by mail a detailed response to this concern, as well as a provisional point-to-point response to the other referees' comments, in order to be able to discuss the requirement of this specific elongation assay for a successful revision of this work.

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REFEREE COMMENTS

Referee #1:

This is nice report that shows a possible control by RNA polymerase II elongation of alternative splicing in plants. Such a control has been extensively studied in mammalian cells, but little was known about the existence of a coupling between transcription and splicing in plant cells. The paper goes farther by identifying the roles of the splicing factor NTR1 that seems to act by association to strong 5's splice sites in the nascent pre-mRNA promoting a localized decrease in Pol II elongation. The authors make use of a series of methods and tools that include Arabidopsis mutants, NTR1 and Pol II ChIPs, use of splicing inhibitors and alternative splicing assays of endogenous individual genes, panels of alternative splicing events and transfected minigenes. The main objection is that the authors consider the changes observed Pol II ChIP patterns as proof for a role of elongation. Ideally such a proof should be provided by experiments measuring elongation using, for example, the method developed by Singh and Padgett. However, because this would be laborious and time consuming, I will not request such an evidence but, instead, I strongly think that the authors should tone down their conclusions all over the manuscript by stating that the Pol II ChIP data are consistent with a role for elongation instead of being a proof. Provided they comply with this request and with the following comments, the paper will be suitable for publication in EMBO Journal as a timely and interesting contribution to the filed of pre-mRNA processing.

Major comments

Figure 2 I think this figure should be moved to Supplementary Material. The paper has many figures and this one interrupts the reasoning flow.

Figure 3B In the text referring to this figure, the authors should not take as a fact that phospho-Ser5 is associated to initiation of transcription and phospho-Ser2 to active transcription. There are plenty of examples in the literature in which this simplified assignment is questioned. Elongating Pol II has phospho-Ser2 and phospho-Ser5 as well.

Figure 4 I liked the fact that the authors use the mutant as a control. However, I do not think that this experiment allows the authors to conclude that NTR1 facilitates co-transcriptional splicing. This is a speculation that could be consistent with the result but not a conclusion from the result.

Figure 5

The use of the splicing inhibitor to rule out general effect of splicing inhibition is an excellent experiment. I would strongly recommend to redistribute panels between Fig. 5 and Suppl. Fig. 4, in order to improve the reading flow and to show the Pol II ChIP of the DOG1 gene, used as a model in the paper, in a main figure. My proposal is:

New Figure 5 should show: Top panel: bottom panel of present Suppl Fig 4. Middle panel: top panel of present Fig 5. Bottom panel: top panel of present Suppl Fig 4.

New Supplementary 4 should Figure show: Top panel: middle panel of present Fig 5. Middle panel: bottom panel of present Fig 5. Bottom panel: middle panel of present Suppl Fig 4.

Figure 6 The lettering of the "y" axis (Pol II ChIP) is missing. The term "native" (that refers to the wt 5'SS) is confusing. Please use "5' SS wt" of "5'SS strong" to name the two possible constructs. Furthermore, please state that the fact that NTR1 is required for an efficient Pol II pausing applies to the particular gene in the figure but not in general.

Figure 8 I think it can be easily moved to Supplementary Material without affecting the manuscript.

Minor comments

I would suggest to cite Dujardin et al., (2014) How slow RNA polymerase II elongation favors alternative exon skipping, Mol Cell. 54(4):683-90 in the introduction as a case in which slow elongation promotes exon skipping. In general the manuscript would greatly benefit from English Grammar revision. Supplementary figure S10 is missing?
Referee #2:

In this manuscript Jakub and colleagues investigate the role of the splicing factor, AtNTR1, in the coupling of splicing and transcription in Arabidopsis. The authors use the DOG1 (delay of germination) pre-mRNA as a model to study the connection between transcription and splicing. First, the authors show that AtNTR1 mutants display a number of phenotypes, including reduced seed dormancy, which is attributed to low DOG1 expression, whose mutants showed a similar phenotype. They went on to show that atnr1 mutants displayed enhanced exon skipping and preferential selection of 5' and 3' distal splice sites. This is attributed to enhanced elongation kinetics of RNA polymerase II in those mutants. Conceptually, this is in agreement with the kinetic model for alternative splicing regulation put forward by Kornblihtt and colleagues.

The authors went on to show that atNTR1 interacts with U6 and U1 snRNA (it is NOT snoRNA, as mentioned in the Figure legend). Next, based on similar phenotypes displayed by AtNTR1 and ILP1, the authors decide to probe for their interaction. They detected an interaction of AtNTR1 with ILP1 using Bimolecular Fluorescence complementation assay (BiFC) that was confirmed in a two-hybrid assay. The authors went on to show that ILP1 mutants also displayed similar splicing phenotypes, as those described for AtNTR1. ChIP analysis revealed the presence of AtNTR1 at target genes. The authors went on to show that the decrease in RNA pol II is not due to reduced splicing, as evidence by Herboxidiene.

Next, Jakub and colleagues show that strong alternative splice sites act as AtNTR1-dependent pause sites for transcriptional elongation. This was extended to an analysis of TFIIS mutants, where the observed changes in alternative splicing selection as well as in RNA pol II occupancy were in opposite direction to the ones observed in AtTNR1 mutants.

There are both positive and negative aspects of this manuscript. Overall, the authors present a large number of experiments, which are suggestive but do not really proof the role for AtNTR1 in kinetic coupling.

Critique

The authors based their model in i) the colocalization of AtNTR1 with the elongating form of RNA pol II; ii) AtNTR1 physical presence at target genes iii) polar effects in alternative splicing, some of which are very modest.

What is needed here is a direct proof that AtNTR1 has a direct role in RNA polymerase II processivity and that in its absence; RNA pol II has faster elongation. This should be analyzed not indirectly by ChIP, rather in more direct way looking at changes in RNA polymerase II elongation rate, as was done by Padgett and colleagues (PubMed PMID: 19820712). Without this, I am afraid; this work is mostly based on correlations and not entirely convincing. Along these lines, a few examples of alternative splicing could be investigated in steady-state transcription vs. DRB-treated plants (PubMed PMID: 21163941), the latter should slow down elongation rate and could potentially recapitulate the AtNTR1 mutant result.

Specific comments

- There seems to be a general problem with the definition of Distal and proximal splice sites. In mammalian systems, a Proximal 5' splice site is the one that is closer to the intron, whereas here, the authors seem to refer to Distal and proximal in just the opposite manner, with Distal being here the site closer to the intron. This has to be clarified (see Robin Reed and Krainer papers for nomenclature).

- The authors analyzed 144 alternative splicing events in atnr1-1 mutants. It is not obvious from the text how exactly these 144 events were selected.

- Where is the analysis for acceptor splice consensus sequence analysis mentioned on page 5?
- Fig. 2B is trivial and should be eliminated

- The data on Fig. 2D is not entirely convincing. Whereas, it is clear that atNTR1 has a role in splice site selection, the conclusions from this experiment are based on very minor subtle effects

- On Fig. 3 the authors conclude that the interaction of AtNTR1 with U6 snRNA is consistent with a role of AtTNR1 in U6 release. This is, at best, suggestive, but this interaction does not demonstrate that.

- The Kornblihtt lab has mainly shown examples of increased exon inclusion upon reduced elongation rate; however other work (including some from the Kornblihtt and Baralle labs) also reported instances when reduced Pol II elongation can actually result in increased exon skipping, for example, by facilitating the binding of negative-acting regulators to pre-mRNA silencer sequences that are proximal to alternative exons. This should be also taken into consideration.

Below we provide below a point by point response to the Reviewers.

Referee #1:

*I strongly think that the authors should tone down their conclusions all over the manuscript by stating that the Pol II ChIP data are consistent with a role for elongation instead of being a proof. Provided they comply with this request and with the following comments, the paper will be suitable for publication in EMBO Journal as a timely and interesting contribution to the field of pre-mRNA processing.*

We are very happy that our work is regarded “as a timely and interesting contribution to the field of pre-mRNA processing.” We agree with the Referee that although the experiments we present are consistent with NTR1 function in transcription elongation, they are not a direct proof. We have therefore changed the discussion and results description to reflect this point.

*Figure 2 I think this figure should be moved to Supplementary Material. The paper has many figures and this one interrupts the reasoning flow.*

We agree with the referee and have moved this Figure to Supplementary Materials

*Figure 3B In the text referring to this figure, the authors should not take as a fact that phospho-Ser5 is associated to initiation of transcription and phospho-Ser2 to active transcription. There are plenty of examples in the literature in which this simplified assignment is questioned. Elongating Pol II has phospho-Ser2 and phospho-Ser5 as well.*

We have changed the text to match this suggestion.

*Figure 4 I liked the fact that the authors use the mutant as a control. However, I do not think that this experiment allows the authors to conclude that NTR1 facilitates co-transcriptional splicing. This is a speculation that could be consistent with the result but not a conclusion from the result.*

We absolutely agree with the reviewer and have changed this statement to make it clear that this result is consistent with but does not allow to infer the role of NTR1 in co-transcriptional splicing.

*Figure 5 The use of the splicing inhibitor to rule out general effect of splicing inhibition is an excellent experiment. I would strongly recommend to redistribute panels between Fig. 5 and Suppl. Fig. 4, in order to improve the reading flow.*

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We are very happy that this experiment was regarded by the Reviewer as an “excellent experiment”. We thank the Reviewer for this insightful comment, we have made changes to the Figure 5 and Suppl. Fig. 4 in accordance with this suggestion.

Figure 6 The lettering of the "y" axis (Pol II ChIP) is missing. The term "native" (that refers to the wt 5'SS) is confusing. Please use "5'SS wt" of "5'SS strong" to name the two possible constructs. Furthermore, please state that the fact that NTR1 is required for an efficient Pol II pausing applies to the particular gene in the figure but not in general.

In accordance with the reviewers suggestion we have change the labelling of our “native” construct to “5’SS wt” and mutated to “5’SS strong” throughout the text. We agree that the PolII pausing by NTR1 in WT is evident on the transgene but less so on the endogenous targets. We therefore have amended the text to match this reservation. The labelling of “y” axis has been corrected.

Figure 8 I think it can be easily moved to Supplementary Material without affecting the manuscript.

We have followed the reviewers comment and moved the Figure 8 to Supplementary Materials.

I would suggest to cite Dujardin et al., (2014) Supplementary figure S10 is missing?

We are grateful to reviewers for this suggestion. We have updated the introduction to include the suggested reference.

We are sorry for the omission of Supplementary Figure 10. It has been added and merged with Supplementary Figure 9.

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Referee #2:

U1 snRNA (it is NOT snoRNA, as mentioned in the Figure legend).

We are grateful for pointing out this error and have corrected it.

What is needed here is a direct proof that AtNTR1 has a direct role in RNA polymerase II processivity and that in its absence; RNA pol II has faster elongation. This should be analyzed not indirectly by ChIP, rather in more direct way looking at changes in RNA polymerase II elongation rate, as was done by Padgett and colleagues (PubMed PMID: 19820712). Without this, I am afraid; this work is mostly based on correlations and not entirely convincing. Along these lines, a few examples of alternative splicing could be investigated in steady-state transcription vs. DRB-treated plants (PubMed PMID: 21163941), the latter should slow down elongation rate and could potentially recapitulate the AtNTR1 mutant result.

We are grateful for this excellent comment. We agree that this work would greatly benefit from a direct proof of PolII elongation rate as described by Padgett and colleagues. Unfortunately as mentioned by Referee 1 this assay is highly challenging due to several aspects:

- nature of material used by us - whole plants, compared to single cells used in Padgett et. al.
- makes the synchronised release from DRB difficult
- relatively small size of plant genes makes the detection of differences challenging
- localised effect of NTR1 on PolII occupancy- we detect only localized change in PolII occupancy on our targets in atntr1 making it even more difficult to detect using the Padgett et al assay

We are however very grateful for the suggestion of an alternative approach as described in the suggested reference “Global impact of RNA polymerase II elongation inhibition on alternative splicing regulation.” (PubMed PMID: 21163941). We have performed the suggested experiment and described the results in the first paragraph of this response.

- There seems to be a general problem with the definition of Distal and proximal splice sites
We are very grateful for pointing this error in our manuscript. Indeed the alternative splice sites have been traditionally labelled proximal and distal in relation to intron and not the transcription start site. We have therefore updated the manuscript and figures and changed the “proximal SS” to “upstream SS” and “distal SS” to “downstream SS” in accordance with the suggested references.

- The authors analyzed 144 alternative splicing events in atnr1-1 mutants. It is not obvious from the text how exactly these 144 events were selected.

The 144 splicing events are part of an alternative splicing panel originally designed to investigate splicing at 14 days old seedlings stage as described in (Simpson et al., 2007; Raczynska et al., 2009). As the TFIISmutated plants where analysed at 3 weeks old stage, we have focused our analysis on a robust set of 144 splicing events that can be analysed at this stage of development with high confidence. We are grateful for pointing out this omission and have added the appropriate description to the methods section. The figure legend has been updated to state the developmental stage used for the splicing analysis.

- Where is the analysis for acceptor splice consensus sequence analysis mentioned on page 5?

We apologise for this omission. This analysis has been added to Supplementary Figure S2.

- Fig. 2B is trivial and should be eliminated

We agree with referee and in accordance with the Referee 1 suggestion have moved the whole Figure 2 to Supplementary Materials and eliminated panel B.

- The data on Fig. 2D is not entirely convincing. Whereas, it is clear that atNTR1 has a role in splice site selection, the conclusions from this experiment are based on very minor subtle effects

We would like to stress that although the change in splice site selection in atnr1 on the transgene are relatively modest they are significant with p-value less then p<0.01 . In line with referees comment we have therefore updated the text to reflect the subtle splicing change observed.

- On Fig. 3 the authors conclude that the interaction of AtNTR11 with U6 snRNA is consistent with a role of AtTNR1 in U6 release. This is, at best, suggestive, but this interaction does not demonstrate that.

We agree with the referee and have changed the text to reflect this.

- The Kornblihtt lab has mainly shown examples of increased exon inclusion upon reduced elongation rate; however other work (including some from the Kornblihtt and Baralle labs) also reported instances when reduced Pol II elongation can actually result in increased exon skipping, for example, by facilitating the binding of negative-acting regulators to pre-mRNA silencer sequences that are proximal to alternative exons. This should be also taken into consideration.

We are grateful to both reviewers for pointing out to us this recent paper. In agreement with our observation this work show that not all splicing changes observed in slow PolII elongation mutants match with the prediction made based on kinetic coupling model. We have therefore updated the introduction and discussed this reference in revised manuscript in line with this suggestion.

3rd Editorial Decision 12 November 2014

Thank you for submitting your revised manuscript "NTR1 is required for transcription elongation check-points at alternative exons in Arabidopsis" to The EMBO Journal. The manuscript has now been seen again by the two referees. You will find their comments pasted below. I am pleased to inform you that the EMBO Journal is accepting your manuscript for publication.
REFEREE COMMENTS

Referee #1:

I am happy with the modifications introduced in the revised manuscript for which I recommend publication in the EMBO Journal.

Referee #2:

The authors have performed a thorough revision. I do agree with the explanation by the authors, which is backed by Reviewer 1, that it would be extremely challenging to use the assay described by the Padgett lab as a direct proof that AtNTR1 has a direct role in RNA pol II processivity. I am satisfied by the alternative approach chosen by the authors.

In summary, the revised manuscript adds more experimental evidence in support of the main conclusions of the paper. With the revisions provided, the manuscript is acceptable for publication in the EMBO J.