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A slow transcription rate causes embryonic lethality and perturbs kinetic coupling of neuronal genes

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Transaction Report:

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

1st Editorial Decision   7th Jan 2019

Your manuscript has now been seen by three referees and their comments are provided below. As you can see the referees find the analysis interesting, but they also find that further experiments are needed to support the key conclusions. Their comments are constructive and reasonable. Should you be able to address the concerns raised then I would like to invite you to submit a revised version. I should add that it is EMBO Journal policy to allow only a single round of major revision and that it is therefore important to resolve the key concerns at this stage.

Let me know if we need to discuss any experiments further.

REFEREE REPORTS:

Referee #1:

This manuscript investigates the in vivo connection and relevance of transcription speed and gene expression. This is a worthwhile extension of the many studies linking pol II speed and gene expression in cell lines and biochemical systems.

The authors convincingly show that ESCs that express slow polymerase are not competent for development. Therefore, the authors are limited to testing the role of transcription speed in a cultured model of development, rather than truly addressing the question of the molecular impact of slowing polymerase in vivo. (which would presumably require an inducible system later in development).

While not fully addressing the ultimate in vivo relevance, overall the data in this manuscript support the notion that polymerase speed is important for proper cellular development. However, in some places further analysis or clarity in the text would strengthen the manuscript.

1) The abstract is written to imply that neural development is specifically sensitive to elongation speed. However the manuscript ONLY looks at neural development. This should be clarified.

2) Are the genes that exhibit speed-dependent AS also those that show expression differences? The authors have a paragraph saying "no" to this question, but no data is shown.

3) Are the differences in AS in ESC, NPC and neurons due to differential gene expression? In other words are the genes that only show speed-dependent AS in neurons only expressed in neurons, or are there any genes that are expressed in all stages of development but sensitive to Pol II speed only in one condition and not another.

4) EV Fig 5 is missing labels, rendering it difficult to understand.

Referee #2:

Pre-mRNA splicing is an essential process that largely occurs co-transcriptionally and allows for regulation of gene expression via alternative splicing (AS). Although AS is primarily regulated by RNA binding proteins, various lines of evidence have indicated that altered RNA Pol II kinetics can influence AS patterns via kinetic coupling. Many of these experiments have used an RNA Pol II large subunit mutation that slows the elongation rate, usually in conjunction with an alpha-amanatin resistance mutation that allows selection of transfected cells that are dependent on the "slow" (or "fast") RNA Polymerase.
The Cáceres lab now reports an attempt to test the consequences of the "slow" polymerase mutation (R749H) in vivo using mouse models without the need for alpha-amanatin resistance. Two independent attempts to introduce R749H into mice failed to achieve germline transmission, even as heterozygotes, indicating strong selection against this mutation. This in itself is a significant observation.
The authors then proceeded to analyse the effects of R749H in mES cells during differentiation to neural precursors, neural stem cells and neurons in vitro. A defect was found in NSCs, but direct neuronal differentiation from NPCs was viable allowing transcriptome profiling of WT and slow/slow cells in ESCs, NPCs and neurons. Alterations in RNA abundance and AS were observed, with long neuronal genes particularly affected.

Overall, I find this to be an interesting manuscript reporting significant new findings on the coupling of transcription and splicing. However, there are some points in the text and figures that need attention.

Major comments

1. In view of the fact that all the subsequent mRNA-Seq data are based on the direct differentiation route to neurons via poly ornithine/laminin, it would be appropriate to include more data in Fig 4 on the characterization of these neurons in addition to the Tuj1 staining shown in Fig 4E. The data in EV3 suggest not only that Syn1 levels are much lower, but also that Map2 is higher and the overall cellular organization looks quite different.

2. p13 continued. "..thus, these results underscore the predominant role of kinetic coupling as differentiation progresses." Also on p17, last sentence and Discussion section.
An alternative explanation might be that some of the changes seen in NPCs and neurons are indirect consequences of changes initially caused in ESCs or NPCs (e.g. is expression of Rbfox and/or Nova affected - see below). This should be discussed, and in general the manuscript could be improved by taking a more critical approach to the kinetic coupling model.

3. P14-15. The statement that binding of RbFOX and NOVA downstream of exons increases their skipping is incorrect. Both protein families are generally associated with activation of splicing from downstream locations. The observation is that exons showing more skipping with slow polymerase

in neurons are associated with RbFOX and NOVA motifs in locations where they would usually activate. It would also be appropriate to draw attention in the text to the strong association of slow-Pol upregulated exons with NOVA sites in a repressive upstream location.

4. Related to the preceding point, were Nova and/or Rbfox proteins themselves regulated at the RNA abundance or AS levels? This should be stated in the text. If either are down-regulated, it would provide a neat explanation, independent of kinetic coupling, for altered AS of these groups of exons. Conversely, if Rbfox and Nova expression are not affected, it could indicate an interesting functional connection between Rbfox/Nova regulation and kinetic coupling.

5. P15. The observation of a small overlap between genes regulated at the splicing and RNA abundance levels is quite important. It would be useful to have Venn diagrams (or some other representation) showing the overlap of genes regulated at each level - perhaps separated by AS event type, given the possibility of NMD coupling with intron retention.

6. Figure 5A. The data on AS changes could be summarized more informatively to include information on the magnitude of splicing changes (delta PSI) as well as the raw numbers of up and down regulated events.

Minor points

7. Fig EV2C. Quantification of the correlation should be given.

8. Fig 3D. Given the bimodality of WT/WT in Fig 3C, it would be appropriate to show a violin plot rather than a boxplot.

9. Fig 4A. It would help to indicate on the Figure the stages at which different markers (Sox1, Pax6, Nestin, Tuj1, Map1, Syn1) are expressed.

10. The whole manuscript is predicated on the fact that R749H results in a "slow" RNA polymerase. The mutant was originally characterized before high resolution RNA Pol structures were available. It would be useful to include a couple of sentences - either in Introduction or Discussion - on the molecular consequences of R749H on Pol II function in the context of high resolution RNA Pol II structures. Do the structures explain the elongation defect well? Is it possible that the mutation has other effects on Pol II function, in addition to the elongation defect, that might contribute to the observed phenotypes? If this has been discussed elsewhere, a citation would be sufficient.

11. The section describing the ESC, NPC, NSC and neuronal culture needs some clarification in the text (p11-12), as well as in Figs 4 and EV3. Fig EV3 gives no indication of what cells are shown (by which route were they derived), and the histograms in Fig EV3B need labeling to show WT and slow. Since Tuj1 neurons were seen in the NSC cultures, the subsequent text (further down p12) on Tuj1 positive neurons needs to emphasise that they were obtained by the poly-ornithine/laminin route (But see major point 1).

12. Page 12, line 4. It was not immediately clear which data (publicly available or new data) was used for GO analyses. The mRNA-Seq experiment is not described until p13, but it does not seem to include aggregates. This needs clarification.

13. p13 last sentence "...extent of splicing changes was much more pronounced in NPCs...and neurons...in comparison to ESCs."
P14 line 2 "Importantly, the extent of AS events observed in the different stages of neuronal differentiation is comparable". Presumably the second sentence refers only to NPC vs neurons? Might be better to state this explictly otherwise the two sentences seem to contradict each other.

14. Fig6A. Better contrast is needed between CC, BP and MF (BP and MF were indistinguishable on my printed copy).

15. Fig 6B needs an indication of which comparisons are significantly different.

16. In other Figures there is no indication of the number of replicates e.g. Fig 5B, EV1c, EV 3b

Referee #3:

The authors of this paper attempt to elucidate the role of transcriptional elongation rate in regulating splicing fidelity. Based on previous studies, it is known that an optimal rate of transcriptional elongation is necessary for co-transcriptional splicing activity in both yeast and mammalian cells. The mechanism controlling transcriptional coupling to alternative splicing (AS) in vivo remains undefined and the authors sought to address two main objectives: i) determine the effects of altered transcriptional elongation rate on gene expression and AS during mammalian development and ii) identify the consequences of tissue/organism phenotype when AS is misregulated.

In order to answer these questions, the authors generated a slow RNA polymerase II (RNAPII) mutation in embryonic stem cells (ESCs). The first attempt at introducing a mutant RNAPII allele by homologous recombination was unsuccessful in producing germline transmission in mouse chimeras. To investigate the developmental effects of a slow RNAPII allele further, the investigators generated mutant RNAPII embryos using CRISPR/Cas9. While the slow RNAPII mutation was embryonic lethal in ESCs both heterozygous and homozygous for the allele, the authors were able to show that the mutation results in a statistically significant decreased transcriptional elongation rate in mouse ESCs by DRB treatment.

The authors next looked at the effects of altered transcription elongation rates on splicing during neural differentiation. The authors claimed that the slow mutation in RNAPII causes defective self-renewal of neural stem cells rather than issues with neuronal differentiation. They also found differences in AS in neural progenitor cells (NPCs) and neurons differentiated from slow/slow ESCs when compared to WT. This effect was particularly enhanced in long genes expressed during neuronal differentiation, and the investigators suggest this is the result of improper kinetic coupling. Taken together, the authors indicate that an appropriate transcriptional elongation rate is required for normal gene expression during development.

This manuscript sets out to address interesting and important questions, and does so through an impressive amount of work. The authors provide evidence that the slow RNAPII allele is embryonic lethal and results in decreased elongation rates in ESCs, their experimental evidence in support of kinetic coupling during neural differentiation is less strong. However, there are a number of critical weaknesses with the paper. In particular, the authors claim that transcriptional elongation rate influences alternative splicing, but fail to provide any statistics or details on experiments to reproduce AS changes in WT and slow/slow ESCs, NPCs, and neurons. In the results section, the authors also suggest that kinetic coupling is enhanced in neurons, but do not provide any comparison to other cell types, let alone ESCs.

The following suggestions may improve the effectiveness of the paper:

(1) One critical weakness with the current manuscript is that many details on the extent and quality of replication experiments are lacking. The authors should provide details on the number of replicates that were generated in both mRNA-seq, 4sU-seq, and other genomic experiments, what the extent to which experiments were concordant to the main conclusions.

While the general idea that RNAPII mutants have a slower elongation rate is convincing as presented, there are many details that could be altered if the experiment were replicated (for example, are there really a group of genes that increases elongation rates as claimed, or is this just due to statistical fluctuations?!).

More importantly, to what extent are alternative splicing changes reproducible between mRNA-seq replicates? The individual gene PCR/ bioanalyzer validation experiments move toward this, but appear to have been completed once, and there is no indication on whether/ how many genes were selected for validation but failed in this test. The authors should show replication.

(2) Report the direct effects of AS on gene expression during NSC maintenance.

(3) The authors claim that kinetic coupling is enhanced in neurons due to increased chromatin compaction, but there is no direct evidence of either increased chromatin compaction, or that it is causally related to kinetic changes. Please either provide some evidence (i.e. immunofluorescence,

ATAC-seq, DNase-seq) that the changes in nucleosome occupancy during neuronal differentiation affects AS levels in NPCs and neurons, or weaken this statement substantially.

(4) Are the expression levels and splicing activity of long genes in ESCs similarly affected by a slow RNAPII as neurons or is this result specific to neurodevelopment? ESCs should be added to Fig. 6 panel C.

(5) I think it might help set the stage for readers to present the section "The R749H mutation decreases the transcription elongation rate in mouse ESCs" as the second section of the paper, as it probably will not be 100% clear to readers that previous studies of slower elongation rates with this mutation in Drosophila will hold up in mice. It is completely up to the authors whether or not to implement this suggestion.

1st Revision - authors' response   11th Feb 2019

We are grateful to all the referees for their constructive and rigorous input, which has helped to strengthen our conclusions. The manuscript has been revised in response to the comments of all reviewers with the addition of some new data.

We are including below a "List of major changes' as well as a detailed Point-by-Point response to referees, where we have attempted to address each one of the comments raised by the reviewers. Finally, a 'marked' version of the Manuscript text has been included with the Supplementary files.

Paste in PbP.

2nd Editorial Decision   1st Mar 2019

Thank you for sending us the revised manuscript. Your revision has now been seen by the three referees and their comments are provided below.

As you can see, the referees appreciate the introduced revisions and support publication here. Please take a look at the minor point raised by referee #2 and respond.

Besides this, we just need the following editorial comments taken care of. You can submit the revised version using the link below. Let me know if you have any questions
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REFEREE REPORTS:

Referee #1:

The authors have addressed all of my concerns with additional data and modifications to the text.

Referee #2:

The authors have engaged constructively with reviewer comments. I have no further serious issues to raise.

Minor point. Figure 5B shows AS events validated by RT-PCR. No indications of statistical significance are given, but the error bars for Pbrm1 suggest that there is no significant change. Perhaps a better example could be shown?

Referee #3:

The authors have addressed my comments.

The authors performed all requested editorial changes.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ⬇

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Javier F. Caceres and Ian R. Adams
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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 χ2 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)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Available data was used, there was no prior expectation of effect size
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For CRISPR injections followed by embryo isolation sample size was decided based on Fisher's exact test. Breeding of 8 male chimaeras was performed to detect germline transmission.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples/animals were excluded
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.	No randomization was applied
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was applied
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.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, non-parametric methods were used as indicated in the figure legend in methods
Is there an estimate of variation within each group of data?	No
Is the variance similar between the groups that are being statistically compared?	Yes

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).	The following antibodies were used as descirbed in the Methods Section: 1. Purified anti-Tubulinb3 (TUBB3,Tuj1) (Biolegend Cat#801213, RRID: AB_2721322) , 2. Anti-MAP2, clone AP20 (Millipore Cat# MAB3418, RRID:AB_94856), 3. Anti-Synapsin I (Novus Cat# NB300-104, RRID:AB_10078308), 4. Anti-Nestin (Abcam Cat# ab24692, RRID:AB_448229), 5. anti-NeuN (Abcam Cat# ab177487, RRID:AB_2532109)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not additionally authenticated. Tested mycoplasma-free.

* 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.	Mus musculus, C57BL/6, Animals were bred and maintained under pathogen-free conditions at the Animal House at Western General Hospitl campus of University of Edinburgh according to approved animal welfare guidelines. Briefly, animals were kept under 12 hour dark/light cycle, fed ad libitum and water was available all the time.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal experiments were carried out under authority of UK Home Office Project Licence PPL 60/4424 after ethical approval by University of Edinburgh Welfare and Ethical Review Body
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.	Complied with the guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

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	Data deposited in GEO database. Accession numbers provided
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).	
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).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NO