EMBO reports

NONO enhances mRNA processing of super enhancer-associated GATA2 and HAND2 genes in neuroblastoma

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(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Fox,

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports, and I apologize for the unusual delay in getting back to you. We have now received the full set of referee reports as well as cross-comments from referee 3 that are pasted below. Unfortunately, referee 2 has not gotten back to us with comments on the other reports.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also all raise important concerns and it is clear that your study is a very borderline case. I decided to send all reports to you now in order to ask whether you think that you can address them. In particular, it is clear that the data supporting Nono phase separation must be strengthened, that it should be clarified how Nono impacts 5' processing of mRNAs, the claim of a preferential activity on super-enhancer genes must be toned down, and a stronger link between Nono phase separation and its gene regulatory role must be established.

If you like, we can also discuss the revision requirements in a video chat, just let me know and we can make an appointment.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (26th Jul 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

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1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

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7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at
<https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,

- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,

- the nature of the bars and error bars (s.d., s.e.m.),

- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

- Please also include scale bars in all microscopy images.

11) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised form of your manuscript when it is ready.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In this manuscript a few claims are made about the properties and functions of the RNA/DNA binding protein NONO. I found most of the claims to be not supported by the data, and/or not sufficiently interpretable.

Claim 1: NONO forms phase-separated condensates in vivo. The evidence provided for this claim is slim, amounting to (a) it is found in puncta, and (b) these puncta decrease when treated with 1,6-hexanediol. These are not really sufficient to confidently assign the puncta as phase-separated condensates. Typically, more rigorous lines of evidence would be presented as well, such as determining the ability for droplets to undergo fusion/fission, FRAP to determine dynamic rearrangement, etc. At present, therefore, the claims made in the title, abstract, and throughout the manuscript are not strongly supported. If these evidences are not provided, a more appropriate claim would simply be that "NONO puncta" are observed. Perhaps this makes for a "lower impact" finding, but it makes for a finding with stronger support.

Claim 2: NONO maintains proper processing at the 5' end of transcripts. The evidence for this claim as presented is confusing to the degree that I do not know how to assess it. For example, what is being displayed in histogram Fig 5C, or D? How can there be a negative number of exons differentially used? Moreover, the claim needs to be clarified: in what way are the exons differentially used? Are they skipped? Do the introns fail to be removed? Are exons alternatively spliced via mutually-exclusive splicing patterns? Or perhaps there is simply a 5' bias for these sequenced samples, and everything seems more abundant? It is impossible for me to tell from the data as presented.

Claim 3: NONO affects pre-mRNA processing of super enhancer genes. (a) I do not see an analysis showing that NONO preferentially affects genes associated with super enhancers. It seems the claim is simply that two of NONO's target genes happen to be associated with super enhancers? If so, the claim in the title (and elsewhere) is over-promising and misleading. (b) I do not see evidence that GATA2 or HAND2 undergo altered processing in the KD of NONO. Evidence is provided that their mRNA and protein levels change, but not that their processing per se is altered.

Referee #2:

By carrying out genome-wide and single cell analyses, the authors have proposed a new model of NONO's function. In this model, NONO binding to pre-mRNAs of nascent SE-related genes, promoting the formation of RNA-processing condensates, allowing efficient processing of pre-mRNAs, including important transcription factors HAND2 and GATA2. To support this notion, the authors have shown that NONO bound the 5' ends of nascent introns and that NONO depletion led to decreased expression of HAND2 and GATA2 at both the mRNA and protein levels, which likely resulted from inappropriate processing and splicing.

Together, this study provides a new insight into NONO in gene regulation, in addition to its well-known role in parapeckle formation and transcriptional regulation, supported by solid experimental data. I have a few questions for the authors to consider to strengthen the current study.

General concerns:
1. Can the authors speculate how NONO RNA binding at the 5' end is required for pre-mRNA processing?
2. The authors proposed that NONO phase separation is involved in efficient pre-mRNA processing/splicing, likely by coordinating with the nascent SE formation. If it were the case, would disruption of SE by chemicals disrupt NONO-formed mini-foci?

Specific concerns:
3. The authors proposed that NONO puncta are a type of condensates dependent on RNA and DNA in Fig 1. Can the authors provide an example of DNA (i.e. GATA2 gene identified by CHIP-qPCR in Fig S4A-S4B) in the proposed NONO condensates by cell imaging?
4. The puncta formed by YFP-NONO_ΔRRM1 appeared larger than those by YFP-NONO_WT (Fig 2C-2E). Is the formation of the larger puncta dependent on RNA/DNA?
5. Loading controls in Western Blotting assays (Fig 5A, 6C,6F) should be provided.

Referee #3:

The manuscript by Zhang et al. addresses the mechanisms of gene expression regulation in neuroblastoma by an abundant RBP, NONO, known to be dysregulated in this type of tumor. First, the authors investigated condensate formation by NONO and its RNA binding deficient mutant in vitro and in vivo. They next examined the RNA targets of NONO in neuroblastoma and their changes upon NONO depletion, finding that its ability to maintain a specific gene regulation program relies on its binding to 5' end of pre-mRNAs, and super enhancer associated genes in particular. I found the results presented here solid and novel and I do not have concerns regarding the robustness of the datasets. My major points for the author's attention are the following. The authors are keen on the premise that RNA binding is vital for the NONO-driven gene expression signature in neuroblastoma, making it a circular argument of the study (including specifically stating this in the title). Yet this fact appears quite obvious to me - RNA binding capacity of any RBP would be expected to play a major role in the gene regulatory properties of that protein. Secondly, I feel that in its present form, the findings on NONO phase separation properties and its gene regulatory activities are somewhat disconnected. I would encourage the authors to focus on enhancing the evidence on the link between the two.
Technical comments
1. Could authors comment on the choice of cell lines for this study? High-risk neuroblastoma cells were used, but compared to Hela cells - are low risk cell lines available?
2. It was found that 1,6HD treatment reduces NONO signal intensity. If it dissolves NONO-rich structures, then it would be expected to decrease the number of droplets but not the total signal. Puncta quantification rather than signal intensity should be used as a readout. Also, in this experiment, a halo is seen for 6% but not for other concentrations, why is this?
3. The effect RNA addition on NONO phase separation in vitro should be examined (including for dRRM1 protein which should not be sensitive to RNA presence/concentration). Related to this, the rationale of using a PS-ASO in in vitro experiments is not clear to me. This ASO is complementary to the NONO target NEAT1, in addition I could not find whether it was a DNA or RNA PS oligonucleotide.
4. RRM1 of NONO is a relatively large domain whose deletion will likely compromise the 3D protein structure. Have authors considered using point mutations in this domain rather than its full deletion - this domain is a canonical RRM where substitution of four conserved aromatic residues should be sufficient to abolish RNA binding.
5. The same group previously showed that the non-paraspeckle NEAT1 isoform localises to "microspeckes" (Li et al, 2017 RNA). Do NONO condensates characterised here colocalise with these structures?
6. NONO accumulation in 1-2 foci formed by HAND2 and GATA, likely transcription sites, was demonstrated. Since only two transcripts were studied, it cannot be firmly concluded that NONO nuclear foci in general "represent sites of NONO binding to a variety of lncRNA and pre-mRNA targets, particularly within the 5' part of pre-mRNAs regulated by super enhancers." and further in discussion, that "This 5' associated RNA processing activity is linked to NONO nuclear condensates that form at individual gene loci, including those of the super-enhancer regulated genes GATA2 and HAND2". These statements should be revised. Relatedly, it is offered that "In the absence of functional NONO-RNA condensates, GATA2 and HAND2 protein levels decrease, with evidence for stalled 5' RNA processing." but the link between presence of NONO in condensates and expression of these factors was not directly demonstrated, in addition it is not clear what is meant by 'functional' condensates' (i.e. are there RNA containing but dysfunctional ones?)
7. I felt that the main conclusion of the study (last sentence of Introduction) was formulated in the way that it does not reflect the results of this manuscript, instead sounding as "common knowledge": "Thus overall, NONO requires the coordinated integration of multilevel components of mechanistic processes and signals to enact its oncogenic program."
Minor
1. In the introduction: "Liquid-liquid phase separation is an emerging phenomenon explaining the dynamic association of molecules ...". I would argue that LLPS has developed into a fully-fledged and thoroughly studied phenomenon by now.
2. Does dRRM NONO overexpression disrupt paraspeckles - ie does it have a dominant negative effect?
3. In discussion: "We also showed that RNA binding, via RRM1, attenuates NONO phase separation.." It should be stated that this refers to in vitro.
4. In discussion: "However, NONO is different to FUS in that wildtype NONO is not diffuse, but instead forms many hundreds of smaller condensates, each likely representing a site of nascent transcription." For instance, in Passon et al PNAS 2012 study, NONO distribution looks quite diffuse. Would it depend on the antibody used and sample processing?
5. It is stated in the abstract that biophysical methods were used, which are those? I would class protein phase separation analysis as protein biochemistry. Genome wide / sequencing methods could be mentioned instead.

Cross-comments from referee 3:

Reviewer 1 - "Claim 1". I would agree that cellular data are less strong than in vitro data and that the dataset would benefit from further studies, in particular fusion/fission of droplets. However I see it as a lesser issue as compared to fleshing out the link between the phase separation and gene regulatory activity of NONO, as outlined in my review.
"Claim 2". This is outside of my area of expertise therefore I am not able to confidently comment on this.
"Claim 3". Authors did do an analysis of public CHIPseq data after identifying that 2 out of 10 top bound genes are SE regulated genes (Fig 3). Changes for GATA2 and HAND2 upon NONO knockdown were confirmed, however indeed the evidence for

altered processing of such genes on a global scale was not presented, just the fact of binding: "We found that transcripts from genes within SE regions had substantially greater NONO RNA binding when compared with expression-matched controls, suggesting a preferential RNA binding of NONO to SE-regulated target gene transcripts (Fig 3F)."

Response to Referees reports for Zhang et al

We are grateful to all three Reviewers for constructive comments and suggestions. We have now added additional data and analysis according to their comments. To assist re-review, original comments are also included.

Formatting key:

Referee comments are shown in Arial, regular

Our responses are shown in **Arial, Bold**

Sentences from the manuscript are shown in Red, Times New Roman

Referee #1:

Claim 1: NONO forms phase-separated condensates in vivo. The evidence provided for this claim is slim, amounting to (a) it is found in puncta, and (b) these puncta decrease when treated with 1,6-hexanediol. These are not really sufficient to confidently assign the puncta as phase-separated condensates. Typically, more rigorous lines of evidence would be presented as well, such as determining the ability for droplets to undergo fusion/fission, FRAP to determine dynamic rearrangement, etc. At present, therefore, the claims made in the title, abstract, and throughout the manuscript are not strongly supported. If these evidences are not provided, a more appropriate claim would simply be that "NONO puncta" are observed. Perhaps this makes for a "lower impact" finding, but it makes for a finding with stronger support.

(Related cross-comment from Referee 3: Reviewer 1 - "Claim 1". I would agree that cellular data are less strong than in vitro data and that the dataset would benefit from further studies, in particular fusion/fission of droplets. However I see it as a lesser issue as compared to fleshing out the link between the phase separation and gene regulatory activity of NONO, as outlined in my review.)

We have significant data *in vitro* showing that NONO can form droplets, that an RNA-binding mutant forms droplets more readily and that in vivo 1,6-HD can dissolve the tiny NONO puncta (Figure 1C-E, Figure 2). Nevertheless, we understand the need for additional manipulation of the NONO puncta inside cells to show liquid-like properties, such as FRAP, or fusion/fission of the tiny puncta. Unfortunately, we are not equipped here in Perth with microscopes capable of such *in vivo* high-resolution photodynamics or micro-manipulation experiments. Indeed, these puncta in the nucleus are much smaller than the usual paraspeckles we have studied in the past. We have therefore focused our efforts on experiments Referee 3 suggested to examine if a NONO mutant that cannot form droplets was capable of rescuing NONO knockdown in terms of GATA2 and HAND2 expression levels. We believe this provides greater strength to our claims that the ability of NONO to undergo phase separation is important for its functional activities in this context. Nevertheless, we have also re-worded various sections of the manuscript to tone down the claims relating to the *in vivo* puncta and phase separation.

Here is a re-worded sentence from the relevant section in the Results:
Together, these *in vitro* and *in vivo* observations suggest that the numerous non-paraspeckle NONO puncta are dependent on both RNA (distinct from NEAT1_2) and DNA for their structural integrity. Further, the puncta may be condensates, as they are sensitive to 1,6-hexanediol and NONO can form droplets *in vitro*.

Claim 2: NONO maintains proper processing at the 5' end of transcripts. The evidence for this claim as presented is confusing to the degree that I do not know how to assess it. For example, what is being displayed in histogram Fig 5C, or D?

We apologise for the confusion. We have now simplified Figure 5C. We normalised all transcripts to a length of 100, and separated this length into 100 bins for analysis. We have now split the data into two graphs. The top graph shows number of exons displaying positive LFC in NONO KD compared to control. The bottom graph shows number of exons displaying negative LFC in NONO KD compared to control. We think it makes it easier now to see the patterns emerging.
We also included a new graph in Figure 5C, where we look at each of the 1-100 bins, and measure the proportion of positive LFC exons in each bin. This shows us that only bins in the 5' part of the transcript (bins 1-13 for DEXseq, and 1-7 for DEseq), have a significant (ie >2 SD over the median) over-representation of positive LFC exons, over negative LFC ones.
We also chose to show the DEXseq data in the main Figure, and moved all the DEseq data to the supplement, to avoid Figure 5 becoming too crowded. However, we are happy to put both DEXseq and DEseq into Figure 5 if the Referees think that is better.

Here are the new graphs for Fig 5C:

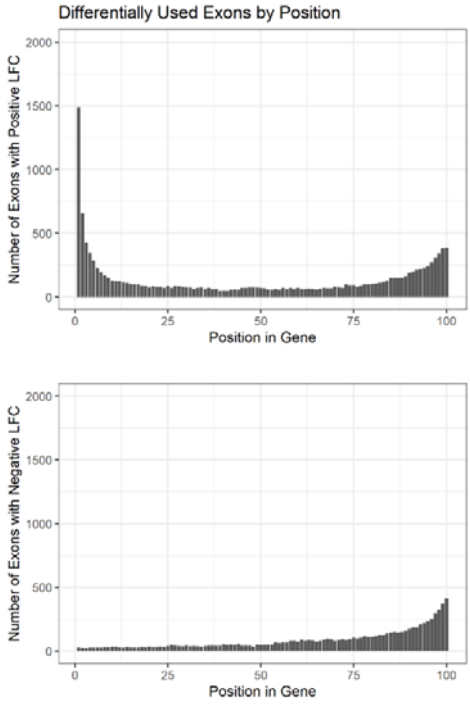
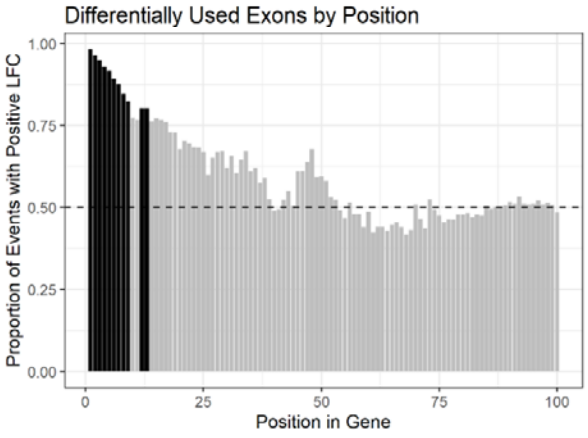
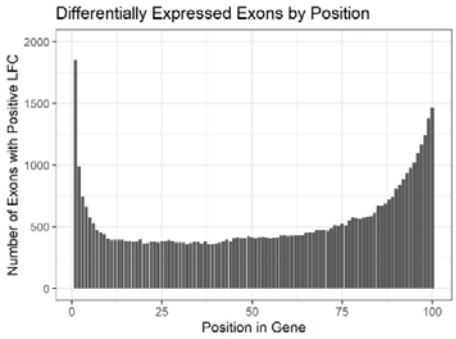
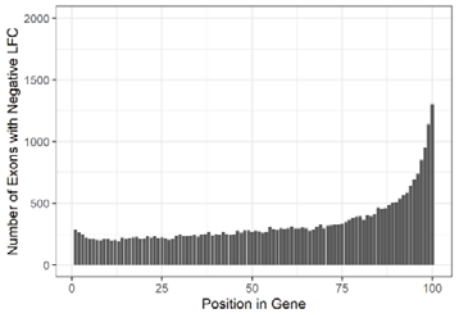
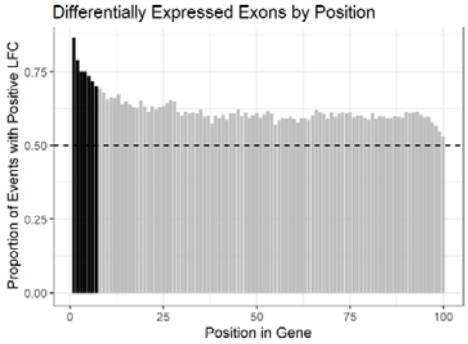


Fig EV3E:









We have also modified the text in the results section, and the Figure legend to make it clearer:

Here is the new text:

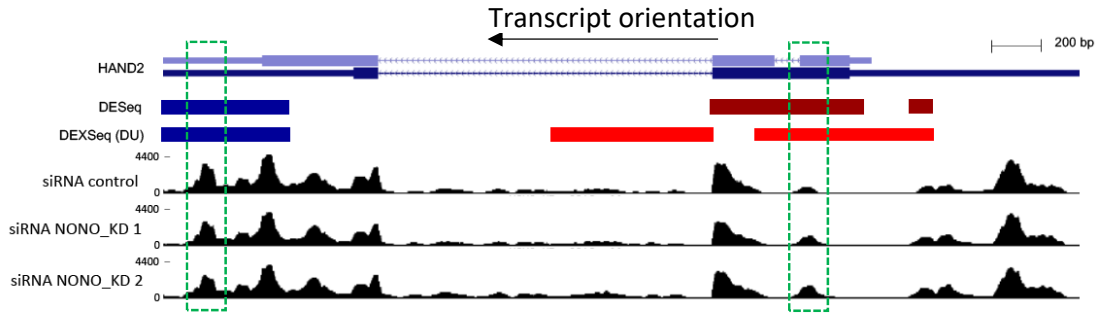
To look for a pattern of regulatory change with NONO KD, we divided each gene into 100 bins and looked at the position of each differentially expressed and differentially used exon/intron. Because this is standard RNA-seq data, the number of reads for introns was insufficient to give clear results. However, we saw many thousands of exons, at different positions in transcripts, showing either significant positive, or negative LFC in NONO KD compared to control (Fig5C, Fig EV3E). We noticed a pattern towards greater usage, or expression, of exons in the 5' parts of transcripts, for NONO KD (ie positive LFC), as opposed to negative LFC (compare Figure 5C, or Figure EV3E, top and bottom plots). To test if this pattern is significant, we calculated the proportion of positive LFC events for each bin position. We found, for bins at the 5' end of transcripts that positive LFC events were significantly more likely than negative LFC events. Exons within the first 13 bins had a significantly greater proportion of upregulated usage events (Fig. 5C, right) and exons within the first 7 bins had a greater proportion of significantly upregulated expression changes (Fig EV3E, right).

Figure 5D: In Figure 5D, we show individual gene outputs from DEXseq and DEseq, but only for the 2237 genes where there are exons in the first 1-13 bins that have a significant LFC event. Most of these genes show positive LFC at the 5' end (bins 1-13), but there are also a small number of genes with negative LFC at the 5' end. Each row is an individual gene/transcript (exons plus introns) where the length has been normalised to 100 bins. The relative changes for NONO KD over control are displayed for each of the 100 bins.

We now state:

There were 2237 genes containing exons that had significant expression and/or usage events (both positive LFC, and negative LFC) identified in Fig 5C.

For the Referees information we made a Figure of HAND2 transcript with the DEXseq and DEseq tracks highlighted (red is positive LFC, blue is negative LFC). Note this transcript is on the – strand and runs right to left. If you look carefully at certain regions, you can see by eye that there are more reads for NONO KD tracks at the 5' end than the 3' end of the transcript (summing the area under the curve, not just looking at the height of the peaks). Some examples are shown in green columns. We consider it is easier to see these patterns with the bioinformatic display of all genes, as in Fig5D, as opposed to individual genes. However, we can include this figure in the manuscript if the Referee considers it appropriate.



How can there be a negative number of exons differentially used?

We apologise. As we explain above, this is the number of exons for which there is a negative LFC in differential usage for NONO KD compared to control. It is not a negative number. Our new graph reflects this.

Moreover, the claim needs to be clarified: in what way are the exons differentially used?

We hope our new explanation makes this clearer now. For DEseq this is standard differential gene expression, but at the exon level instead of whole transcript level. In contrast, for DEXseq, this is a relative abundance for one exon/part of the transcript, compared to the abundance of the rest of the exons in that transcript. Some bins overlapping some exons are significantly more abundant in NONO KD compared to control and we class these as positive LFC. The opposite is true for negative LFC exons (less abundant for DEseq, or less of a proportion of the whole gene for DEXseq).

Are they skipped? Do the introns fail to be removed? Are exons alternatively spliced via mutually-exclusive splicing patterns?

We assume the referee is thinking here about whole exons that have a negative LFC in DEseq. The 'IsoformSwitchAnalyzR pipeline did not detect significant alternative splicing events, intron retention, or mutually exclusive splicing patterns (Figure EV3D). However, it is possible there is some intron retention, but specifically for the first intron. IsoformSwitchAnalyzR does not

differentiate between first intron retention and subsequent intron retention. Our RT-qPCR analysis of GATA2 and HAND2 shown in Fig 6D could be a result of intron retention, or of a delayed processing of the intron.

Or perhaps there is simply a 5' bias for these sequenced samples, and everything seems more abundant? It is impossible for me to tell from the data as presented.

We are confident there is no 5' bias for our RNA-seq libraries as the patterns we see only occur in a subset of total transcripts, and even then, there are examples of negative LFC bins at the 5' end as well. We hope the new Figures make this easier to see now.

Claim 3: NONO affects pre-mRNA processing of super enhancer genes. (a) I do not see an analysis showing that NONO preferentially affects genes associated with super enhancers. It seems the claim is simply that two of NONO's target genes happen to be associated with super enhancers? If so, the claim in the title (and elsewhere) is over-promising and misleading. (b) I do not see evidence that GATA2 or HAND2 undergo altered processing in the KD of NONO. Evidence is provided that their mRNA and protein levels change, but not that their processing per se is altered.

(a) As also mentioned by Referee 3, Figure 3F shows that NONO preferentially binds genes that are associated with super-enhancers.
Figure EV3G shows that NONO-bound RNAs were significantly more likely to be differentially expressed. However, we have not performed a global analysis of how all super-enhancer associated genes alter in expression with NONO knockdown. We have therefore altered our wording, where appropriate, to include the caveat that we have looked only at GATA2 and HAND2 as examples of SE-related genes that respond to NONO levels. We have also modified the title to better reflect this.

New sentences:

Taken together, these data indicate that NONO binds GATA2 and HAND2 pre-mRNAs and enhances their RNA processing and splicing close to the 5' end of transcripts. This NONO activity, driving optimal expression levels of these oncogenes, is dependent on RNA binding, as well as low-complexity domain mediated foci formation.

(b) I do not see evidence that GATA2 or HAND2 undergo altered processing in the KD of NONO. Evidence is provided that their mRNA and protein levels change, but not that their processing per se is altered.

There are four lines of evidence used to support our claim that NONO influences GATA2 and HAND2 RNA processing:

1. **Figure 6D shows NONO KD increases the relative proportion of intron-exon regions of GATA2 or HAND2 (RT-qPCR using intron-exon primer pairs), compared to regions within the exon (RT-qPCR using exon-exon primer pair). We call this '% of 5' pre-mRNA'.**
2. **Figure 5D shows with DEXseq that transcripts, including GATA2 and HAND2, have increased usage at the 5' end compared to the rest of the gene body**

3. **Figure 4A shows binding of NONO to the GATA2 and HAND2 pre-mRNA is stronger than NONO binding to the mRNA**
4. **Figure 4C shows NONO colocalising with transcription sites of GATA2 and HAND2**

We think we did not explain the way we got the data for Figure 6D very well, so we modified the wording to hopefully make it clearer.

In contrast, we found with RT-qPCR using exon-exon, and exon-intron primer pairs, that NONO KD induced a higher relative expression of GATA2 and HAND2 pre-mRNAs at the 5' end when compared to their mature transcripts (Fig 6D).

Referee #2:

..this study provides a new insight into NONO in gene regulation, in addition to its well-known role in paraspeckle formation and transcriptional regulation, supported by solid experimental data.

We thank the Referee for their support of the study and highlighting the interest to the field.

General concerns:
1. Can the authors speculate how NONO RNA binding at the 5' end is required for pre-mRNA processing?

The Discussion section contains quite a lot of speculation about the mechanism. Ideally, we need to do long-read nascent-RNA seq, or long read chromatin-associated nascent RNA-seq, however we lack the current capacity and resources to do so. We have spent quite some words in the Discussion on speculation relating to intron retention, alternative splicing, initiation and elongation. We also contrast co-transcriptional splicing and post-transcriptional splicing and put forward a possibility there. We have included some of these sentences here for the referee:

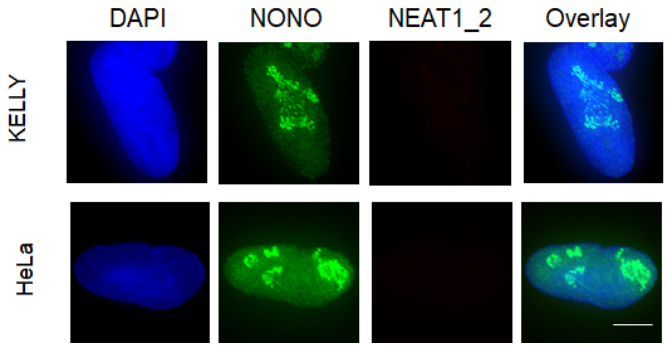
Another factor to consider is the importance of NONO condensates for intron removal in co-transcriptional splicing, as opposed to post-transcriptional splicing. Whilst earlier findings support a role for NONO in co-transcriptional splicing (through association both with nascent RNA and PolII CTD) (Kameoka et al., 2004, Emili et al., 2002), new evidence of the importance of 'nuclear anchoring' of partially processed, but fully transcribed, pre-mRNA transcripts at the gene locus is emerging (Girard et al., 2012, Popp and Maquat, 2013, Quinn and Chang, 2016). In this context, a chromatin-anchored nuclear pool of partially spliced, but polyadenylated RNA, may act in a regulatory manner as a reservoir for mature mRNA, upon splicing. Intriguingly, such a mechanism seems to be important in the neuronal gene regulation context (Yeom et al., 2021). Important future experiments would therefore include testing if NONO condensates act at the co- or post-transcriptional level, by repeating NONO KD RNA-seq, but with a polyA-restricted library. If the 5' usage bias is still apparent it suggests that pre-mRNAs, already decorated with polyA tails, depend on NONO for proper splicing, supporting post-transcriptional splicing.

2. The authors proposed that NONO phase separation is involved in efficient pre-mRNA processing/splicing, likely by coordinating with the nascent SE formation. If it were the case, would disruption of SE by chemicals disrupt NONO-formed mini-foci?

Thank you for the useful suggestion. We ordered two inhibitors, JQ1 and THZ1. We observed that JQ1 did indeed diminish NONO foci, specifically in KELLY, and not Hela cells, and have included this important new data in the manuscript (Fig EV2A-D).

However, THZ1 resulted in global transcription inhibition, loss of NEAT1_2 and complete loss of paraspeckles. THZ1 also resulted in the relocalisation of NONO to nucleoli as we have observed in prior studies when global Pol II transcription was inhibited with Actinomycin D, or DRB. Therefore, we did not

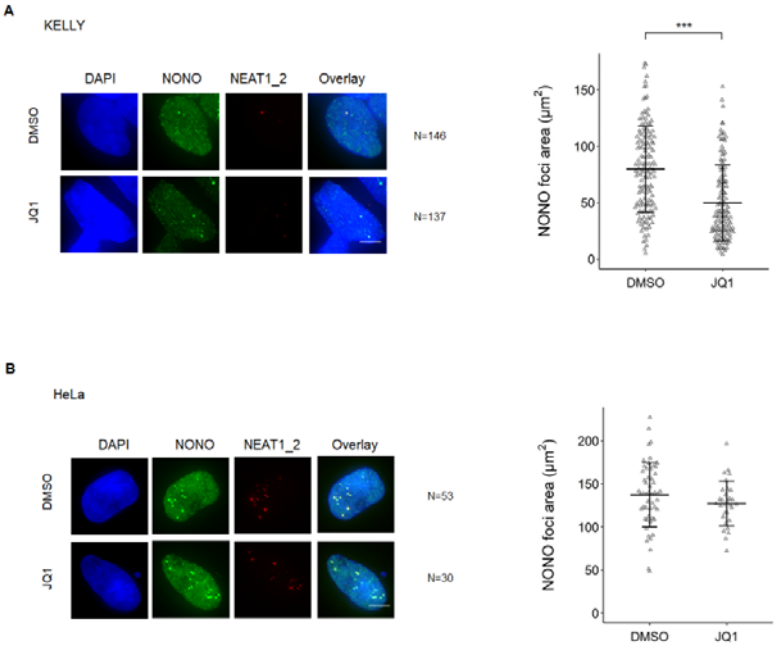
include the new THZ1 data in the manuscript. Nevertheless, we include this data here for the Referees information:



Fluorescence micrograph images of representative cells stained for NONO and NEAT1_2 in KELLY and HeLa cells treated with THZ1. THZ1 (MedChem Express, HY-80013) was dissolved in DMSO at 1 mM and further diluted with growth medium to a final concentration of 100 nM for 18 h incubation before imaging. DAPI (blue) stain indicates cell nuclei, NONO immunofluorescence (green) and NEAT1_2 RNA FISH (red). Scale bar: 5 µm.

We added new sentences and data to the manuscript:
We next sought to selectively inhibit SE-associated foci, using the BET inhibitor JQ1 that prevents the SE-assembling cofactor BRD4 from binding acetylated histones (Lovén et al., 2013). We observed that JQ1 treatment diminished NONO foci formation in KELLY (Fig EV2A-B) but not HeLa cells (Fig EV2C-D). Combined, these data suggest NONO associates with SE-associated genes in neuroblastoma cells.

We include new data for JQ1 for the Referees information here as well:



Specific concerns:
3. The authors proposed that NONO puncta are a type of condensates dependent on RNA and DNA in Fig 1. Can the authors provide an example of DNA (i.e. GATA2 gene identified by CHIP-qPCR in Fig S4A-S4B) in the proposed NONO condensates by cell imaging?

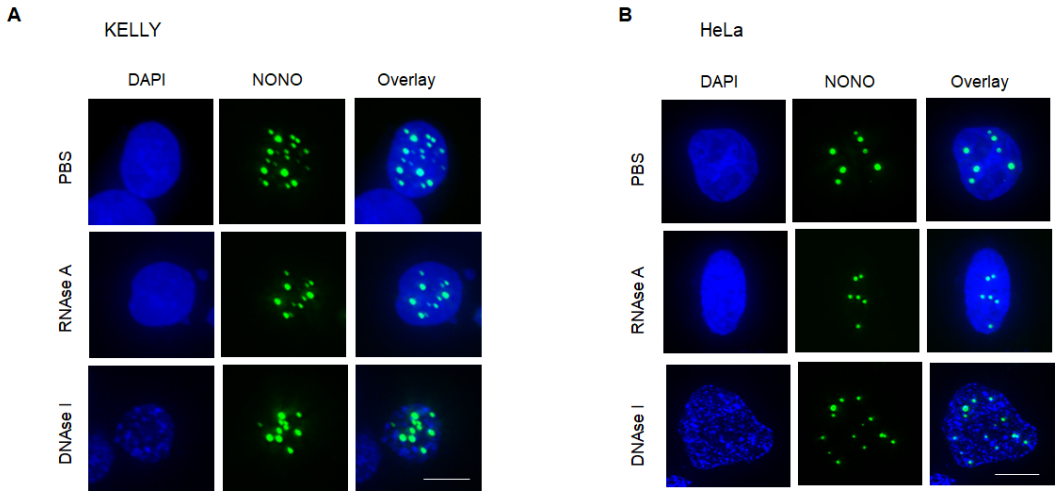
This is an excellent suggestion, however in the face of limited time, budget and manpower we decided not to attempt this experiment as we felt the aspect of the study related to NONO DNA binding was less critical compared to the RNA binding and phase separation. This experiment would have required ordering expensive probe sets to genomic loci and the costs and time for delivery to Australia were beyond our capability. We hope the Referee understands this decision.

4. The puncta formed by YFP-NONO_ΔRRM1 appeared larger than those by YFP-NONO_WT (Fig 2C-2E). Is the formation of the larger puncta dependent on RNA/DNA?

Thank you for this valuable suggestion. Indeed, we have now repeated the experiments expressing YFP-NONO_ΔRRM1, but this time included RNase and DNase digestion. In contrast to wildtype NONO we did not observe any sensitivity to these enzymes. Thus the spherical droplets likely consist mainly of protein that is phase separating in a way that would be normally modulated by its ability to bind nucleic acid.

We added this sentence to results:
These NONO_ΔRRM1 puncta were resistant to nuclease digestion, suggesting their formation is independent of RNA and DNA (Fig EV1A-B).

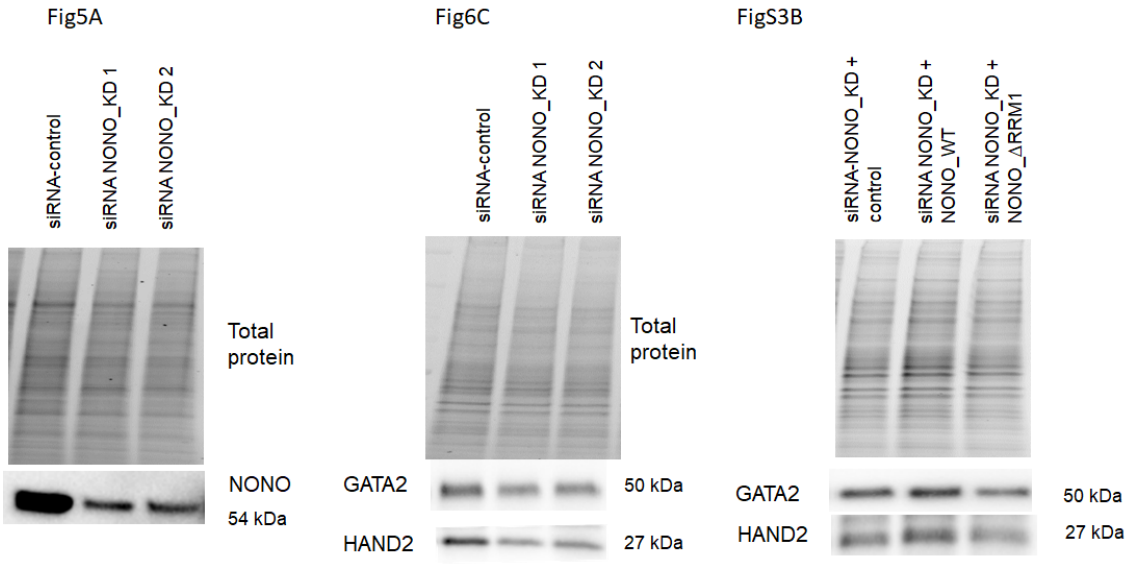
We include new data here as well:



5. Loading controls in Western Blotting assays (Fig 5A, 6C,6F) should be provided.

The corresponding total protein images obtained from Biorad are provided

below. This method generates a normalisation factor, using total protein signal for the lane, and then applies this factor to a quantitated measure of the band intensity. It is this normalised band intensity, combined for three biological replicates, that is presented in the graphs in Figure 5A, 6B and Appendix Figure S3B. As this method is a common way to present WB results and routinely used in our and other groups (Naveed et al., 2021), we do not consider they add much to the manuscript and suggest not to include them, but present them here for your information. Note these westerns below are each just one of three biological replicates.



Referee #3:

..I found the results presented here solid and novel and I do not have concerns regarding the robustness of the datasets.

We thank the Referee for their positive feedback.

My major points for the author's attention are the following. The authors are keen on the premise that RNA binding is vital for the NONO-driven gene expression signature in neuroblastoma, making it a circular argument of the study (including specifically stating this in the title). Yet this fact appears quite obvious to me - RNA binding capacity of any RBP would be expected to play a major role in the gene regulatory properties of that protein.

We agree that it is not surprising that RNA binding should play a major role in the activity of an RNA binding protein! However, having worked on this family of DBHS proteins for so long, we are acutely aware of their ability to interact in many different ways with many different partners (protein, DNA and RNA) and this wide array of interactions makes them very multifunctional. Indeed, we wrote a review on this topic: 'DBHS proteins SFPQ, NONO and PSPC1: a multipurpose molecular scaffold' (Knott et al., 2016). For this reason we believe it is critical for future drug development that particular activities such as RNA binding be highlighted as the key interaction for future targeting.

Secondly, I feel that in its present form, the findings on NONO phase separation properties and its gene regulatory activities are somewhat disconnected. I would encourage the authors to focus on enhancing the evidence on the link between the two.

We take the Referees point and have added further experiments to address this. We first created mutant GFP-NONO constructs that lacked either the N-terminal low complexity domain (LCD) (residues 1-52), the C-terminal LCD (residues 313-466), or both LCDs. We transfected these mutants into KELLY and HeLa cells to examine their localisation.

We have chosen not to include those preliminary experiments in the manuscript, but include them here for the Referees.

Figure for reviewers and associated text removed

We observed that the C-terminal LCD appeared to be the dominant feature driving puncta formation. As shown above, protein lacking the region spanning 313-466 is much less punctate than wildtype, or protein lacking the N-terminal LCD. However, we chose to continue with the double mutant, lacking both LCD (NONO_Δ1-52+Δ313-466). [...] Moreover, NONO_Δ1-52 +Δ313-466 is soluble and globular, indeed, we have solved the crystal structure of this protein, which would not be possible for a protein capable of droplet formation (Passon et al 2011 is the crystal structure of the NONO/PSPC1 heterodimer with the same residue start and end positions (Passon et al., 2011)).

We therefore repeated our NONO-knockdown and GATA2/HAND2 expression rescue experiment (Figure 6E) with inclusion of GFP-NONO_Δ1-52+Δ313-466.

We have included in the supplement the fact that the protein is much more diffuse throughout the nucleus than wildtype NONO (Figure EV5A-B).

We found, as with the mutant that cannot bind RNA, the new construct that does not phase separate *in vitro*, and is more diffuse, was also not able to rescue GATA2 and HAND2 expression (Figure 6E). We believe this new data supports the claim that phase separation is involved in this activity of NONO.

We include the new data here as well:

Expanded View figure 5

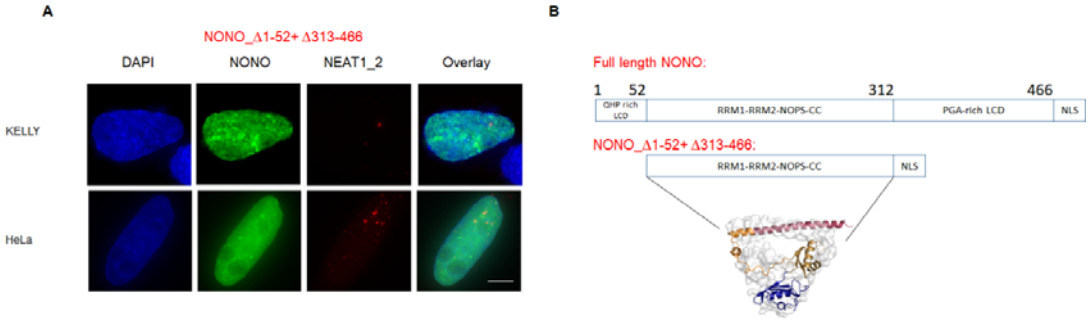
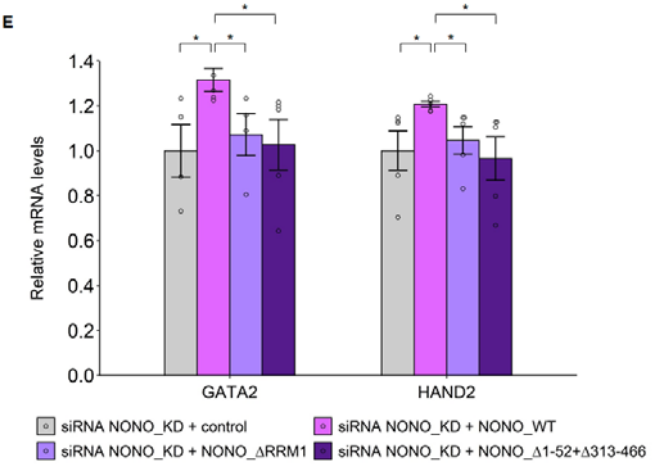


Figure 6



Technical comments

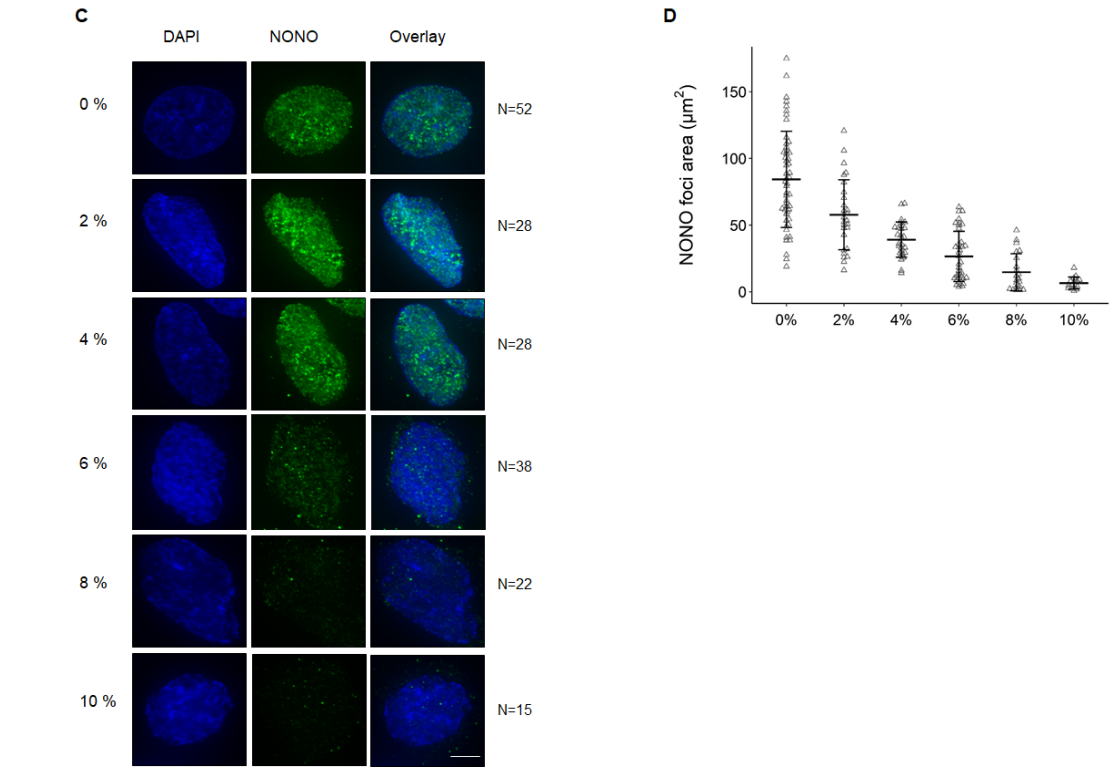
1. Could authors comment on the choice of cell lines for this study? High-risk neuroblastoma cells were used, but compared to Hela cells – are low risk cell lines available?

There are low-risk neuroblastoma cell lines available, however we carried out our RNA-seq and PAR-CLIP in the high risk lines and therefore chose to carry out the companion experiments in these same lines for the sake of continuity.

2. It was found that 1,6HD treatment reduces NONO signal intensity. If it dissolves NONO-rich structures, then it would be expected to decrease the number of droplets but not the total signal. Puncta quantification rather than signal intensity should be used as a readout. Also, in this experiment, a halo is seen for 6% but not for other concentrations, why is this?

Thank you for this suggestion. We have reanalysed NONO foci area as puncta quantification for 1,6HD treatment in KELLY and HeLa cells. We have also replaced the representative images at 6% in KELLY cells with those without an imaging artefact.

Figure 1



3. The effect RNA addition on NONO phase separation in vitro should be examined (including for dRRM1 protein which should not be sensitive to RNA presence/concentration). Related to this, the rationale of using a PS-ASO in in vitro experiments is not clear to me. This ASO is complementary to the NONO target NEAT1, in addition I could not find whether it was a DNA or RNA PS oligonucleotide.

We apologise for the confusion. Having worked for many years with these proteins *in vitro*, there is no simple universal RNA substrate to use in such studies. Moreover, we lack capacity to *in vitro* transcribe long complex RNA targets such as NEAT1. Instead, we have arrived at using the 2'-O-methyl-PS-ASO as a simple model, easily obtained, for generic nucleic acid that DBHS proteins bind with high affinity (Knott et al., 2021). Other groups have also

showed tight binding of DBHS proteins to these modified ASO (Shen et al., 2014). We also see binding of DBHS proteins to such ASO inside cells (Flynn et al., 2022). We have therefore altered the text of the manuscript to better reflect the nature of the PS-ASO to make it clearer why we have used it for the *in vitro* studies. Actually, PS-ASOs have phosphorothioate instead of phosphate inter-nucleotide linkages where a sulphur atom is substituted for one of the non-bridging oxygen atoms. Perhaps as PS-ASO are chemically modified at the 2' position, the ASO is neither a true DNA, nor an RNA.

Added sentence:
We next incubated recombinant NONO with a nucleic acid substrate, a 2'-O-methyl phosphorothioate antisense oligonucleotide (PS-ASO) against NEAT1 (Vickers et al., 2019). PS-ASOs with this chemistry are bound with high affinity by DBHS proteins, in an RRM1 dependent manner (Knott et al., 2021).

4. RRM1 of NONO is a relatively large domain whose deletion will likely compromise the 3D protein structure. Have authors considered using point mutations in this domain rather than its full deletion - this domain is a canonical RRM where substitution of four conserved aromatic residues should be sufficient to abolish RNA binding.

We have prior structural studies showing that deletion of RRM1 does not compromise the dimerization and structural stability of the protein (Knott et al., 2021). We solved the structure of the related protein, SFPQ, lacking RRM1 (Lee et al., 2015). In addition, we show in Appendix Figure S2F that NONO_ΔRRM1 is capable of co-immunoprecipitating its dimerization partner SFPQ, also suggesting it is structurally competent. Although we agree that the use of four aromatic-to-alanine point mutants in RRM1 would also abolish canonical RNA binding. Indeed, we used a PSPC1 mutant of this nature in some of our early papers (Fox et al., 2005) although have since found that recombinant PSPC1 with F-A mutations is insoluble (unpublished observations). Another reason we decided to use the RRM1 deletion is that RRM1 also engages in non-canonical RNA binding (Knott et al., 2021). We wanted to make sure that we completely ablated RRM1-driven RNA binding, both canonical and non-canonical, in making the mutant. Indeed, when we tested some point mutants designed to abrogate this non-canonical binding we disturbed dimerization ability (Fig EV1C). In summary, we are confident the deletion of the domain is the most comprehensive way to ablate RNA binding without disturbing the overall structural stability.

We added this sentence:
Prior experiments showed NONO_ΔRRM1 is structurally stable and can readily dimerise (Knott *et al*, 2021).

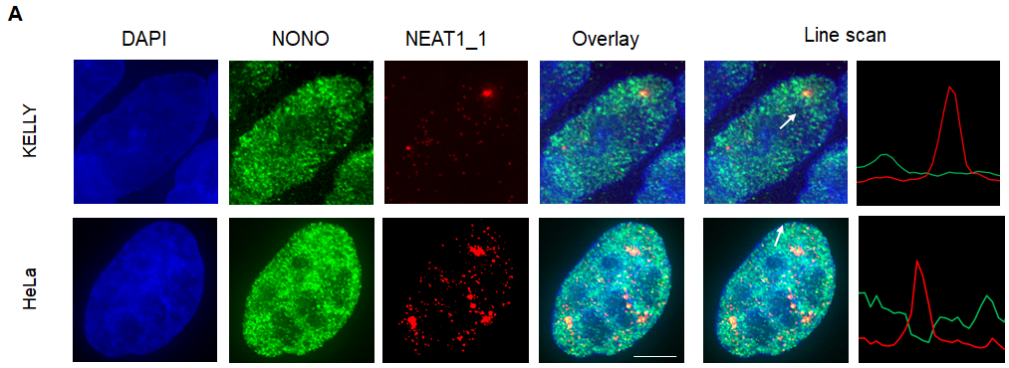
5. The same group previously showed that the non-paraspeckle NEAT1 isoform localises to "microspeckes" (Li et al, 2017 RNA). Do NONO condensates characterised here colocalise with these structures?

Thank you for the suggestion. We have now tested this and found no colocalization between NEAT1_1 labelled microspeckles and NONO foci. We

also did not see colocalization of NONO with NEAT1_1 microspeckles in U2OS cells in the Li et al paper (Li et al., 2017).

Here is the new sentence in the manuscript:
We also checked if these small non-paraspeckle NONO foci corresponded to 'microspeckles', individual non-paraspeckle NEAT1_1 RNA foci, however we did not observe co-localisation of NONO with NEAT1_1 in KELLY and HeLa cells, consistent with previous observations (Li et al., 2017) (Appendix Figure S1A).

Here is the data:



6. NONO accumulation in 1-2 foci formed by HAND2 and GATA, likely transcription sites, was demonstrated. Since only two transcripts were studied, it cannot be firmly concluded that NONO nuclear foci in general "represent sites of NONO binding to a variety of lncRNA and pre-mRNA targets, particularly within the 5' part of pre-mRNAs regulated by super enhancers." and further in discussion, that "This 5' associated RNA processing activity is linked to NONO nuclear condensates that form at individual gene loci, including those of the super-enhancer regulated genes GATA2 and HAND2". These statements should be revised. Relatedly, it is offered that "In the absence of functional NONO-RNA condensates, GATA2 and HAND2 protein levels decrease, with evidence for stalled 5' RNA processing." but the link between presence of NONO in condensates and expression of these factors was not directly demonstrated, in addition it is not clear what is meant by 'functional' condensates' (i.e. are there RNA containing but dysfunctional ones?)

We have now revised these statements according to the Referees suggestion. We feel it is reasonable to draw on the genome-wide results in speculation that additional foci are likely to represent NONO binding to the targets we identified with PAR-CLIP. We have therefore moved this sentence to the discussion.

Here are the new sentences:

RESULTS:
Thus, we showed that some of the nuclear NONO foci represent NONO bound to GATA2 and HAND2 transcripts, examples of SE-regulated transcripts.

DISCUSSION:

NONO also forms numerous non-paraspeckle nuclear foci, some of which co-localise with the super-enhancer regulated GATA2 and HAND2 transcription sites...
The genome-wide PAR-CLIP findings allow us to speculate that additional NONO foci may be sites of binding to other lncRNA and pre-mRNA targets, particularly within the 5' part of pre-mRNAs regulated by super enhancers.

The new sentences in the first paragraph of the discussion are:
In the absence of NONO, GATA2 and HAND2 protein levels decrease, with evidence for stalled 5' RNA processing. Neither the NONO RNA-binding mutant, or a NONO mutant lacking low complexity domains is able to rescue GATA2 and HAND2 levels. Therefore, we propose a model whereby NONO binds to, and coats the 5' ends of GATA2 and HAND2 transcripts, forming gene-body splicing-associated condensates to enhance gene expression and support an oncogenic program.

7. I felt that the main conclusion of the study (last sentence of Introduction) was formulated in the way that it does not reflect the results of this manuscript, instead sounding as "common knowledge": "Thus overall, NONO requires the coordinated integration of multilevel components of mechanistic processes and signals to enact its oncogenic program."

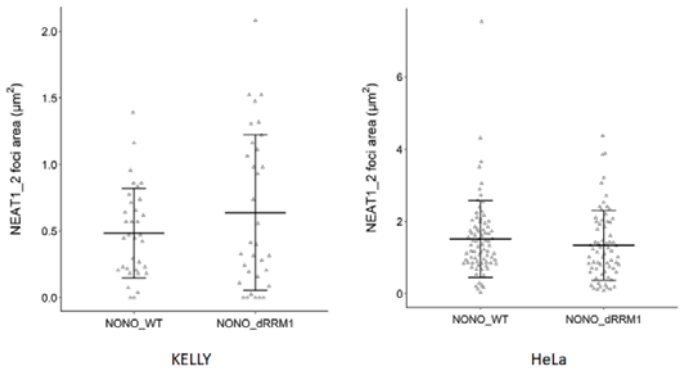
We have now deleted this sentence from the final paragraph of the Introduction.

Minor
1. In the introduction: "Liquid-liquid phase separation is an emerging phenomenon explaining the dynamic association of molecules ...". I would argue that LLPS has developed into a fully-fledged and thoroughly studied phenomenon by now.

We have amended the sentence in the Introduction.

2. Does dRRM NONO overexpression disrupt paraspeckles - ie does it have a dominant negative effect?

Overexpression of NONO_ΔRRM1 has no dominant negative effect on paraspeckles determined by NEAT1_2 foci area per nucleus. Since our study is not really focused on paraspeckles, we have not included this data in the manuscript, however if the Referee would like it included, we are happy to do so.



3. In discussion: "We also showed that RNA binding, via RRM1, attenuates NONO phase separation.." It should be stated that this refers to in vitro.

We have amended this sentence as follows:

We also showed that RNA binding, via RRM1, attenuates NONO localisation *in vivo* and phase separation *in vitro*. A mutant with impaired RNA binding ability more readily phase separates *in vitro,* as well as forming large, nuclease resistant, spherical droplets in the nuclei of cells.

4. In discussion: "However, NONO is different to FUS in that wildtype NONO is not diffuse, but instead forms many hundreds of smaller condensates, each likely representing a site of nascent transcription." For instance, in Passon et al PNAS 2012 study, NONO distribution looks quite diffuse. Would it depend on the antibody used and sample processing?

Indeed, there is some role for imaging modalities in revealing nucleoplasmic puncta, instead of showing diffuse signal. For our 2012 PNAS study we used HeLa cells, as well as an older widefield fluorescence microscope that lacked deconvolution capability (Passon et al., 2012). In contrast, we now use as standard the deltavision fluorescence microscope with deconvolution. The deconvolution allows us to reveal more foci above a background diffuse signal. However, even given this difference in image processing, when we first imaged endogenous NONO in neuroblastoma cell lines, we were immediately struck by the more clearly defined nucleoplasmic NONO puncta. In contrast, in HeLa cells the nucleoplasmic NONO puncta are much less distinct, even imaged with the deltavision and subject to deconvolution. It is indeed this difference that inspired our entire study, where we postulated that there was something special in neuroblastoma that was resulting in more clear NONO non-paraspeckle puncta. Our new data that the JQ1 treatment is only effective in diminishing NONO puncta in neuroblastoma KELLY, but not HeLa cells, would support this notion.

5. It is stated in the abstract that biophysical methods were used, which are those? I would class protein phase separation analysis as protein biochemistry. Genome wide / sequencing methods could be mentioned instead.

We have amended the sentence, and indeed, we have altered the abstract substantially to better reflect our findings.

Cross-comments from referee 3:

Reviewer 1 - "Claim 1". I would agree that cellular data are less strong than in vitro data and that the dataset would benefit from further studies, in particular fusion/fission of droplets. However I see it as a lesser issue as compared to fleshing out the link between the phase separation and gene regulatory activity of NONO, as outlined in my review.

Please see response to Referee 1, point 1, above.

"Claim 2". This is outside of my area of expertise therefore I am not able to confidently comment on this.
"Claim 3". Authors did do an analysis of public CHIPseq data after identifying that 2 out of 10 top bound genes are SE regulated genes (Fig 3). Changes for GATA2 and HAND2 upon NONO knockdown were confirmed, however indeed the evidence for altered processing of such genes on a global scale was not presented, just the fact of binding: "We found that transcripts from genes within SE regions had substantially greater NONO RNA binding when compared with expression-matched controls, suggesting a preferential RNA binding of NONO to SE-regulated target gene transcripts (Fig 3F)."

Please see response to Referee 1, point 3, above

Reference:

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YEOM, K. H., PAN, Z., LIN, C. H., LIM, H. Y., XIAO, W., XING, Y. & BLACK, D. L. 2021. Tracking pre-mRNA maturation across subcellular compartments identifies developmental gene regulation through intron retention and nuclear anchoring. *Genome Res,* 31**,** 1106-1119.

Dear Archa,

Thank you for your patience while your revised manuscript was peer-reviewed at EMBO reports. We have now received the referee comments and cross-comments that are pasted below.

As you will see, referee 1 does not support the publication of your study here, and referee 3 is asking for some more experimental data, and I would like to know whether you can provide these? Regarding referee 1's comments, referee 2 thinks that these can be thoroughly discussed in the manuscript text. I would thus like to invite you to address these remaining concerns. Please let me know in case you would like to discuss these revisions further.

A few editorial requests will also need to be addressed:

- Please update the conflict of interest statement to "Disclosure and Competing Interest Statement"

- Please remove the author credits from the ms file. We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions, if you wish. See also guide to authors https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines.

- The current reference format lists more than 10 authors, the names are in uppercase and the year is not bracketed. Please correct the reference format to the EMBO reports (Harvard) style.

- Fig EV3G callout is missing, please add.

- Page numbers are missing from the table of content of the Appendix file, please add.

- The SYNOPSIS IMAGE you sent is not in the correct format, please send us an image file that is exactly 550 pixels wide and 200-600 pixels high.

- I attach to this email a related ms file with comments by our data editors. Please address all comments in the final ms file.

I would like to suggest that you re-write the following sentence of the abstract:
"NONO preferentially regulates super enhancer-associated genes, including HAND2 and GATA2."
May be delete "preferentially" or tone down the statement, given referee 3's comments.

I look forward to seeing a final version of your manuscript when it is ready.

Best wishes,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors have addressed my 1st major concern by toning down their claims about phase separation. My 2nd and 3rd major concerns, however, remain concerning.

#3 - The manuscript still claims, without supporting evidence that I can see, that NONO preferentially regulates super-enhancer associated genes. CLIP data indicates that NONO is crosslinked with (somewhat) greater frequency to super-enhancer associated genes, but I don't see any data that this leads to preferential NONO-mediated *regulation* of super-enhancer associated genes.

#2 - The claims regarding 5' exon accumulation are even more baffling to me now than they were before. The authors suggest "a potential deficiency in processing at the 5' end of transcripts in the absence of NONO." But that is a very vague conclusion - what possible biological mechanism could explain such a phenomenon? Potential mechanisms such as alternative transcription start sites or splicing failure seem to be excluded by existing evidence. After seeing the example provided in the case of HAND2,

my suspicion that we are observing exonucleolytic cleavage is increased. That's the only mechanism I can think of that could explain this phenomenon. Whether this cleavage is a technical artifact of library preparation, or a biological effect of NONO's activity, I don't know. But it would significantly change the conclusions of the manuscript either way.

Referee #2:

The revised manuscript has addressed my previous concerns and I support its publication at EMBO Reports.

Referee #3:

The authors have addressed most of my comments.
I do feel however that it is quite important to address point 3 (3. The effect RNA addition on NONO phase separation in vitro should be examined, including for dRRM1 protein which should not be sensitive to RNA presence/concentration)- since the argument on RNA dependency of condensates does appear throughout the study.
The authors reply that : 'there is no simple universal RNA substrate to use in such studies. Moreover, we lack capacity to in vitro transcribe long complex RNA targets such as NEAT1.' However total RNA purified from cells or mix of synthetic short RNA oligos can be used for this type of study - so should not be too long/onerous/expensive to do.

Cross-comments from referee 2:

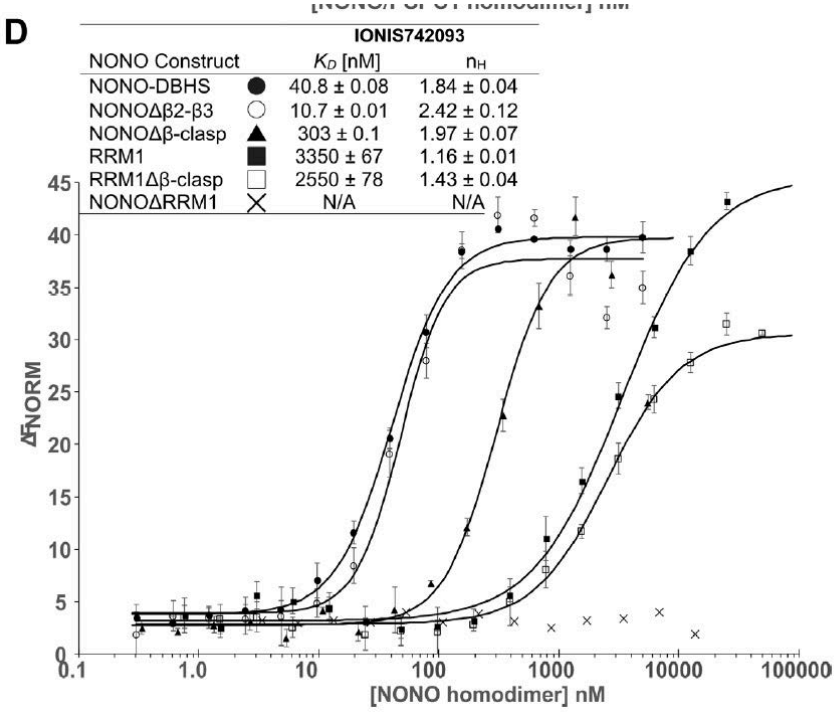
#3. I see the potential regulation of NONO on super-enhancers, because NONO binds to such regions. However, it appears too strong to conclude the "regulation" indeed happens there. In this manner, I suggest the authors to soften the related claims.
#2. This manuscript was submitted as a descriptive paper, as we all agreed. That being said, it will be quite difficult for the authors to dive into the related mechanisms. I wonder if you could ask the authors to have a thorough discussion on what the Referee #1 raised?

Dear Esther,

Thank you for this email and my apologies for the delay replying as I was travelling last week.

In regards to referee 1: I understand the concerns, and I agree with referee 2 that the discussion is the best place for speculating about the mechanism. I will insert additional sentences to (a) refute the concern about technical processing of the library and (b) include a speculated potential role for exonucleases in the mechanism to explain the observed results from the RNA-seq.

In regards to referee 3: It could be possible to do the experiments, as requested. However, this is a large undertaking and represents an entirely new project. The strength of the current data in Figure 2H is that it draws upon prior published data for the NONO-antisense oligo interaction where we measured the affinity of both wildtype NONO, and the mutant lacking RRM1, using microscale thermophoresis (Knott et al NAR 2021<https://academic.oup.com/nar/article/50/1/522/6460791>). These data from Knott et al are shown here:



To repeat Figure 2H using total cellular RNA as the substrate would mean introducing several unknowns into the experimental design that would each require significant orthogonal methods to resolve. For example, the complex total RNA mixture may only contain a small fraction of high affinity NONO substrate, and before doing the droplet assays, first the MST would need to be repeated with fluorescently labelled protein, rather than fluorescent-labelled antisense RNA, introducing even more variation. Thus this represents a considerable undertaking.

Further, repeating Figure 2H with total RNA is highly likely to give identical results to what is already shown (ie that NONO deltaRRM1 droplets do not alter in the presence of RNA). There are additional data in the paper to support the conclusion that the NONO-deltaRRM1 construct cannot bind RNA: Figure 2B shows with RNA-FISH that NONO-deltaRRM1 does not co-localise with NEAT1, and Figure 4B shows that it does not bind to pre-mRNA with RNA Immunoprecipitation assays.

In summary, given the large amount of work involving orthogonal methods required for the new experiments, and the additional data we already have in the paper, using a variety of methods, that show the same finding (ie inability of the delta-RRM1 construct to bind RNA), we respectfully request that Figure 2H be accepted as it is.

Please let me know your thoughts.

All the best,
Archa Fox

The authors addressed the remaining editorial issues.

Dr. Archa Fox
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School of Human Sciences and Molecular Sciences
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Crawley, Western Australia 6009
Australia

Dear Archa,

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.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Best wishes,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

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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?
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  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
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Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	

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For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	

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