

Peer Review Information

Journal: Nature Cell Biology

Manuscript Title: Shell protein composition specified by lncRNA NEAT1 domains dictates the formation of paraspeckles as distinct membraneless organelles

Corresponding author name(s): Professor Tetsuro Hirose

Editorial Notes:

Reviewer Comments & Decisions:

Decision Letter, initial version:
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*Please delete the link to your author homepage if you wish to forward this email to co-authors.

Dear Professor Hirose,

Thank you for submitting your manuscript, "Shell protein composition encoded by NEAT1 domains dictates the formation of paraspeckles as distinct membraneless organelles", to Nature Cell Biology, and thank you for your patience with the process. The manuscript has now been seen by 3 referees, who are experts in nuclear bodies, membraneless organelles (Referee #1); nuclear RNA compartments (Referee #2); and quantitative RNA biology, nuclear speckles (Referee #3). As you will see from their comments (attached below), they found this work of potential interest but have raised substantial concerns, which in our view would need to be addressed with considerable revisions before we can consider publication in Nature Cell Biology.

Nature Cell Biology editors discuss the referee reports in detail within the editorial team, including the chief editor, to identify key referee points that should be addressed with priority, and requests that are overruled as being beyond the scope of the current study. To guide the scope of the revisions, I have listed these points below. Our standard revision period is six months; we are committed to providing a fair and constructive peer-review process, so please feel free to contact me if you would like to discuss any of the referee comments further or anticipate any issue or delay addressing the reviews.

I should stress that the referees' concerns point to a premature dataset and concerns regarding the characterizations of the bodies and the mechanisms ensuring separation or incorporation would need to be addressed with experiments and data, and reconsideration of the study for this journal and re-engagement of referees would depend on strength of these revisions. In particular, in our view, it would be essential to:

-- provide additional characterizations of the paraspeckles and mini-paraspeckles, and bodies obtained with the mutants:

Rev#1 "What is the average size difference between the fully functional paraspeckle nucleated by full-length NEAT1_2 and mini-paraspeckles formed by mini-NEAT1? Schemes in Extended Data Figure 1a suggest that wild-type paraspeckles and mini-paraspeckles are equal in size and similar in the overall shell-core sub-organization. However, mini-paraspeckles in multiple figures look less compact and widespread. They also look more segregated into smaller subdomains. Are they structurally equal? Or is there a flux/gradient of molecules of nuclear speckles penetrating incorporated mini-paraspeckles visible at super-resolution microscopy? The presented images need to be more convincing. This fact is a critical issue in super-resolution microscopy when threshold levels of each channel are selected and presented. It is highly possible that the continuous exchange of molecules across mini-paraspeckles inside nuclear speckles displays a complex non-uniform structure with multiple liquid-like partially miscible phases. It will make mini-paraspeckles less structurally defined.

The size criticality can play an important role in the segregation process between paraspeckles and nuclear speckles. Nuclear speckles are significantly larger and range in size from one to several micrometers in diameter. In contrast, paraspeckles are smaller, about 0.5–1.0 micrometers in size. Furthermore, mini-NEAT1 and MALAT1 of nuclear speckles intermingle extensively in fused nuclear speckles/mini-paraspeckles (Extended Data Fig 1). If mini-paraspeckles inside nuclear speckles are less compact than wild-type distinct paraspeckles, their organization level might be quite different than proposed in Figure 6."

Rev#3 point #8

-- deeper investigations of the mechanisms maintaining the nuclear bodies separate or mediating incorporation of paraspeckles into nuclear speckles:

Rev#1 "The authors proposed that "mini-paraspeckles are formed outside nuclear speckles and then incorporated into them." What is the targeting mechanism? A possible explanation here is that this phenomenon is transcription-driven, either in paraspeckles or nuclear speckles, likely both. The authors should clearly explain the mechanistic details and physiological meanings of this fusion. There is a lack of sure confidence in the discussion. For example, individual nucleoli frequently fuse in highly proliferating cells due to their size expansion and gene repositioning. Do mini-paraspeckles grow during this fusion process? Are they targeted to nuclear speckles due to their expansion driven by continuous transcription of mini-NEAT mutant RNA?"

"The authors indicated that U2 snRNP subcomplex proteins preferentially interact with the 5'-end of NEAT1. It has been established that U2 snRNP has a role in splicing and stimulating transcriptional elongation through interactions with the elongation factors. The authors should investigate the transcription activity (perhaps by super-resolution RNA FISH using oligo(dT) probes at the periphery of

paraspeckles and mini-paraspeckles. It is possible that it might be actively involved in the process of association and/or coalescence with nuclear speckles."

"The U2 snRNP, a central component of the spliceosomes, is very highly abundant across the nucleus. Therefore, the proposed mechanism of sequestration of the U2 snRNPs (and possibly other splicing factors) in co-transcriptionally formed paraspeckles antagonizing the association of U2 snRNP in splicing for regulatory purposes requires more investigation."

Experimentally addressing Rev#2's question "9. Line 208. "The mini-PSs are incorporated into NSs, but not into other nuclear bodies". Have the authors quantified incorporation of PSs into other nuclear bodies."

Rev#3 points #1, 2abcd

-- controls and additional methodological details should be provided as requested by the revs; please also address the questions about data interpretation from Rev#2

Rev#1 "Most assays in the manuscript were done using the over-expression systems, including the artificial tethering system targeting selected proteins of interest on their specific mini-NEAT1 RNA target. This fact may allow a non-physiological increase in the local nuclear concentration of selected paraspeckle factors on NEAT1, which can have a condensation effect. Such a strategy has generated more concerns about assaying nuclear condensates and phase separation. It is imperative and relevant for this work because the authors mentioned the importance of reductions of recruitment of specific paraspeckle proteins in the segregation process and defects. Could some of the seen condensation effects (e.g., in Figure 3, 4b) be due to the over-expression of paraspeckle interacting partners?"

"In terms of controls of ASO treatment, depletion of U13 small nucleolar RNA functioning in pre-ribosomal biogenesis is not adequate to control to the U2 snRNA. The authors should deplete U1 snRNA since it does not directly interact with U2 snRNA but have the same impact on pre-mRNA splicing."

Rev#2 points #1, 2, 3, 4, 5, 6, 7, 10

-- Finally please pay close attention to our guidelines on statistical and methodological reporting (listed below) as failure to do so may delay the reconsideration of the revised manuscript. In particular please provide:

- a Supplementary Figure including unprocessed images of all gels/blots in the form of a multi-page pdf file. Please ensure that blots/gels are labeled and the sections presented in the figures are clearly indicated.

- a Supplementary Table including all numerical source data in Excel format, with data for different figures provided as different sheets within a single Excel file. The file should include source data giving rise to graphical representations and statistical descriptions in the paper and for all instances where the figures present representative experiments of multiple independent repeats, the source data of all

repeats should be provided.

We would be happy to consider a revised manuscript that would satisfactorily address these points, unless a similar paper is published elsewhere, or is accepted for publication in Nature Cell Biology in the meantime.

When revising the manuscript please:

- ensure that it conforms to our format instructions and publication policies (see below and <https://www.nature.com/nature/for-authors>).
- provide a point-by-point rebuttal to the full referee reports verbatim, as provided at the end of this letter.
- provide the completed Reporting Summary (found here <https://www.nature.com/documents/nr-reporting-summary.pdf>). This is essential for reconsideration of the manuscript will be available to editors and referees in the event of peer review. For more information see <http://www.nature.com/authors/policies/availability.html> or contact me.

When submitting the revised version of your manuscript, please pay close attention to our [href="https://www.nature.com/nature-portfolio/editorial-policies/image-integrity">Digital Image Integrity Guidelines](https://www.nature.com/nature-portfolio/editorial-policies/image-integrity). and to the following points below:

- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
- that control panels for gels and western blots are appropriately described as loading on sample processing controls
- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

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This journal strongly supports public availability of data. Please place the data used in your paper into a public data repository, or alternatively, present the data as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public

repository is mandatory - more information on our data deposition policies and available repositories appears below.

Please submit the revised manuscript files and the point-by-point rebuttal to the referee comments using this link:

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We hope that you will find our referees' comments and editorial guidance helpful. Please do not hesitate to contact me if there is anything you would like to discuss. Thank you for considering our journal for your work.

Best wishes,

Melina

Melina Casadio, PhD
Senior Editor, Nature Cell Biology
ORCID ID: <https://orcid.org/0000-0003-2389-2243>

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

In this manuscript, the authors investigated the biological question of how a nuclear RNA-scaffolded membraneless organelle (MLO), paraspeckles, maintain their structural independence within the cell nucleus through phase separation. It has been established that paraspeckles are located adjacent to another prominent MLO, nuclear speckles, which store splicing factors and regulate pre-mRNA splicing at their periphery. However, it needs to be made clear how paraspeckles do not merge with nuclear speckles. Authors identified the deletion mutants of scaffolding lncRNA NEAT1 responsible for the nucleation of paraspeckles that nucleate a form of a version of paraspeckles capable of fusing with nuclear speckles. Then they dissected the direct interactions between NEAT1 and some paraspeckle proteins that likely contribute to the segregation process. They hypothesize that components of the U2 snRNP subcomplex are involved in the internalization of paraspeckles into nuclear speckles.

The manuscript is well-structured and informative. Overall, the experiments were generally carefully performed. However, in places, this reviewer has questions about appropriate controls and interpretation of the results using artificial non-physiological tools to study phase separation, etc. (please see below). The results are well described, but the manuscript lacks a clear overview of a fundamentally novel mechanism responsible for structural segregation between two adjacent MLOs and defects.

Below are specific comments:

What is the average size difference between the fully functional paraspeckle nucleated by full-length NEAT1_2 and mini-paraspeckles formed by mini-NEAT1? Schemes in Extended Data Figure 1a suggest that wild-type paraspeckles and mini-paraspeckles are equal in size and similar in the overall shell-core sub-organization. However, mini-paraspeckles in multiple figures look less compact and widespread. They also look more segregated into smaller subdomains. Are they structurally equal? Or is there a flux/gradient of molecules of nuclear speckles penetrating incorporated mini-paraspeckles visible at super-resolution microscopy? The presented images need to be more convincing. This fact is a critical issue in super-resolution microscopy when threshold levels of each channel are selected and presented. It is highly possible that the continuous exchange of molecules across mini-paraspeckles inside nuclear speckles displays a complex non-uniform structure with multiple liquid-like partially miscible phases. It will make mini-paraspeckles less structurally defined.

The size criticality can play an important role in the segregation process between paraspeckles and nuclear speckles. Nuclear speckles are significantly larger and range in size from one to several micrometers in diameter. In contrast, paraspeckles are smaller, about 0.5–1.0 micrometers in size. Furthermore, mini-NEAT1 and MALAT1 of nuclear speckles intermingle extensively in fused nuclear speckles/mini-paraspeckles (Extended Data Fig 1). If mini-paraspeckles inside nuclear speckles are less compact than wild-type distinct paraspeckles, their organization level might be quite different than proposed in Figure 6.

The authors proposed that “mini-paraspeckles are formed outside nuclear speckles and then incorporated into them.” What is the targeting mechanism? A possible explanation here is that this phenomenon is transcription-driven, either in paraspeckles or nuclear speckles, likely both. The authors should clearly explain the mechanistic details and physiological meanings of this fusion. There is a lack of sure confidence in the discussion. For example, individual nucleoli frequently fuse in highly proliferating cells due to their size expansion and gene repositioning. Do mini-paraspeckles grow during this fusion process? Are they targeted to nuclear speckles due to their expansion driven by continuous transcription of mini-NEAT mutant RNA?

Most assays in the manuscript were done using the over-expression systems, including the artificial tethering system targeting selected proteins of interest on their specific mini-NEAT1 RNA target. This fact may allow a non-physiological increase in the local nuclear concentration of selected paraspeckle factors on NEAT1, which can have a condensation effect. Such a strategy has generated more concerns about assaying nuclear condensates and phase separation. It is imperative and relevant for this work because the authors mentioned the importance of reductions of recruitment of specific paraspeckle proteins in the segregation process and defects. Could some of the seen condensation effects (e.g., in Figure 3, 4b) be due to the over-expression of paraspeckle interacting partners?

The authors indicated that U2 snRNP subcomplex proteins preferentially interact with the 5'-end of NEAT1. It has been established that U2 snRNP has a role in splicing and stimulating transcriptional elongation through interactions with the elongation factors. The authors should investigate the transcription activity (perhaps by super-resolution RNA FISH using oligo(dT) probes at the periphery of paraspeckles and mini-paraspeckles. It is possible that it might be actively involved in the process of association and/or coalescence with nuclear speckles.

In terms of controls of ASO treatment, depletion of U13 small nucleolar RNA functioning in pre-

ribosomal biogenesis is not adequate to control to the U2 snRNA. The authors should deplete U1 snRNA since it does not directly interact with U2 snRNA but have the same impact on pre-mRNA splicing.

The U2 snRNP, a central component of the spliceosomes, is very highly abundant across the nucleus. Therefore, the proposed mechanism of sequestration of the U2 snRNPs (and possibly other splicing factors) in co-transcriptionally formed paraspeckles antagonizing the association of U2 snRNP in splicing for regulatory purposes requires more investigation.

Reviewer #2:

Remarks to the Author:

Paraspeckles (PSs) are microscopic structures assembled around the long noncoding RNA (lncRNA) NEAT1 in the nucleus and maintaining unique structural identity despite the lack of a membrane boundary. PSs have been originally defined based on their proximity to the nuclear speckles (NSs), somewhat larger bodies recruiting diverse splicing factors and nuclear RNAs. What prevents coalescence of the two two ribonucleoprotein compartments or engulfment of one by the other has been unknown. The current study addresses this exciting question using a series of NEAT1 mutants produced by CRISPR/Cas9 engineering, super-resolution imaging, and MS2 tethering and immunoprecipitation assays. The authors show that the NEAT1 sequences occupying the outer shell of PSs recruit a characteristic set of RNA-binding proteins, including SFPQ, hnRNPF, and BRG1, which in turn prevent the occlusion of the PSs by the NSs. Evidence is provided that the key outer shell protein SFPQ achieves this effect by antagonising the recruitment of NS-enriched spliceosomal components to NEAT1. I think that these results are novel and of high quality and that the study should appeal to the wide readership interested in RNA-protein interactions, membraneless organelles, and the mechanisms and biological functions of phase separation. However, I also have several concerns, which should be addressed in the revision.

Specific comments

1. I could not find a description of the procedure used to classify PSs as segregated or incorporated. Was it based on co-localization? Occlusion of PSs by NS pixels? In any case, this key procedure must be clearly described in the Results or/and Methods. If the classification was done manually, the authors should also show a few typical examples of segregated and incorporated PSs in the supplement.
2. Related to the point above, the authors show stacked bar plots to report the fractions of segregated or incorporated PSs throughout the manuscript. However, no statistical tests are provided. Such tests are especially important in cases where the effects are relatively subtle (e.g., Fig. 6c,e,g). The authors should estimate the significance of their key comparisons by Fisher's exact or chi2 tests.
3. All new CRISPR/Cas9 mutants and knock-ins generated in this study must be validated by PCR genotyping and the results shown in the supplement.
4. Fig 1, Fig S1 and lines 95-105. The authors must rule out that reduced co-localization between NEAT1 and SRRM2 at the beginning of the release period is not due to a major rearrangement of the NS system in response to DRB. This can be done for example by showing a field of cells stained for SRRM2 +/-DRB. To further strengthen the argument that older PSs are more often incorporated

compared to newly assembled ones, the authors should compare NS incorporation of the NEAT1tail and NEAT1_5' signals in Fig S1i. In addition, the current version of Fig S1i is not ideal since it uses NEAT1_5'+3' for WT and NEAT1_5' for mini-NEAT1.

5. Fig. S2c. The authors do not discuss the apparent discrepancy between the relatively strong incorporation of the D1-8k/16.6-20.2k mutant and the enrichment of SFPQ binding sites in the 20.2-22.6k region in Fig. S7f. This caveat must be addressed - at least in the Discussion. It may be also helpful to check if the incorporation of D1-8k/16.6-20.2k is significantly different from mini-NEAT1 in Fig.S2C.

6. Fig.4d and Fig.S5f. The IP experiments must be repeated at least three times, quantified, and compared by t-test or a similar statistical procedure.

7. line 163 "BRG1 tethering did not facilitate recruitment of SFPQ and HNRNPF". Fig S4e shows that MCP-BRG1 recruits HNRNPF and that this effect is significant. Fig S4d does not compare between MCP-BRG1 and MCP-GFPNLS, but the median of MCP-BRG1 exceeds that of MCP-GFPNLS. The authors should provide a more nuanced description of the data in the text.

8. Fig. S5g-h and lines 198-204. "Although most of the SFPQ mutants exhibited a compromised ability to achieve PS assembly, etc". I find this confusing. Up until this point in the manuscript, the assumption was that mini-NEAT1 and mini-NEAT1/6×MS2@8.2kb are fully proficient in PS assembly. This point must be clarified.

9. Line 208. "The mini-PSs are incorporated into NSs, but not into other nuclear bodies". Have the authors quantified incorporation of PSs into other nuclear bodies. If they have not, this claim must be deleted.

10. Are the effects shown in Fig. S7e statistically significant? Also, the authors should explain in the text why they think U2 ASO appears to dampens NEAT1.

Minor points

The word "encoded" in the title may mislead some readers into thinking about protein-coding capacity of NEAT1. "Shell protein composition specified by NEAT1 domains..." might be a safer choice.

Fig 1g. the label should say "DRB 4 hours and release", as in Fig 1h.

Line 87. Please mention in the text that SRRM2 was used as an NS marker in Fig 1b-c to clarify the SON/MALAT1 statement on lines 88-89.

Line 90. SRM and SIM are mentioned interchangeably in the text and the Figures. If the authors did not use SRM methods other than SIM, why not consistently use just one acronym - e.g., SIM.

Line 93. The conclusion "These findings together show that the mini-PSs form organized, immiscible structures within NSs and thus lack the ability to exist as distinct MLOs" sends mixed messages. There must be a more nuanced way to say that although mini-PSs are organized and immiscible, they are occluded by NSs.

Reviewer #3:

Remarks to the Author:

Summary:

This is a highly original and well conducted study addressing the immiscibility of nuclear biomolecular condensates by focusing on paraspeckles and the scaffolding non-coding RNA NEAT1. Figures are nicely organized and clearly demonstrate their findings. I would like to thank the authors for making this manuscript easy to read and understand.

Authors build their work on an earlier study on NEAT1_2 RNA domains, where a construct that makes mini- paraspeckles (mini-PSs) becomes incorporated into nuclear speckles (NS). They carefully dissect out mislocalized PS proteins (PSPs) in mini-PS and focus on the three proteins, SFPQ, HNRNPF and BRG1, that induce segregation of mini-PS. SFPQ turns out to be hierarchically more important for this process. Authors use mutational analysis of the key protein SFPQ and identify separable functions of SFPQ in PS segregation (from NS) and assembly. Furthermore, shell localizing SFPQ is found to be the key to segregate PS from NS. This atypical incorporation of PS into NS is suggested to be caused by the failure to escape from the canonical mRNA maturation pathways, particularly splicing, indicated by the affiliation of the mini-PS with U2 snRNP.

In Figure 6, authors provide a compelling cartoon drawing to provide a mechanistic view of their data. The proposed mechanism of PS and NS segregation requires a few more experimental evidence. Moreover, the outcome of mini-PS incorporation into NS is not investigated or discussed. Therefore, ideally this manuscript needs to be revised in order to be suitable for publication at NCB. Suggestions to the authors are below.

Suggested improvements:

1. The incorporation of mini-PS into NS might not lead to obvious cellular phenotypes as in the form of viability, however potential disturbance to the function of NS in RNA processing should to be addressed to make the story complete. An RNA-seq experiment followed by computational analysis would give a genome-wide view, however this could be challenging for the groups who lack the expertise, therefore RT-PCR experiments on certain constructs could be used as an alternative if this happens to be the case. For example:

A recent correlative study from the group of Gil Ast, suggests the role of gene architecture in splicing unit determination modes (exon definition vs. intron definition). According to this model some, but not all, splicing events should be NS-dependent. Using the mini-gene constructs described in Fig.2 of Tammer et al. Mol. Cell 2022

(<https://www.sciencedirect.com/science/article/abs/pii/S109727652200106X>), the authors could find answers to the impact of incorporated PS on NS function.

Furthermore, SF3B1 is important for branch point sequence (BPS) selection and the incorporation of mini-PS into NS could lead to a titration effect and alter BPS selection of transcripts in general. The authors could use gene examples shown in Fig.2 of Alsafadi et al. Nat. Comm. 2016 (<https://www.nature.com/articles/ncomms10615>) in order to show how the selection might be unaffected/affected.

2. There are a few sections in the manuscript that would require deeper investigation or discussion. These refer to:

a) mislocalization of a subset of PSPs: what differentiates the PSPs which are strongly reduced in PS from those aren't affected? The protein domains they contain, repeat regions (i.e. G-rich, PLD), or their affinities towards each other (a molecular grammar), or the amount of total protein found in PS under unperturbed conditions?

b) artificial tethering experiments: TDP-43 in Fig.3b shows cytoplasmic localization as well as nuclear pool. Could this affect the segregation success of mini-PS from NS?

c) the different mechanism of mini-PS segregation defect rescue by SFPQ & HNRNPF from BRG1: are they sequentially recruited on the RNA? Or is this driven by protein-protein interactions? Could the authors perform a sequential IP experiment to find out about the hierarchies to refine this different mechanism? Perhaps in-vitro droplet formation assays with truncated proteins would also be useful to determine the importance of protein-protein interactions in this process.

d) SFPQ Q-to-G mutant construct has a diffused localization in the cytoplasm and nucleus (Fig.4b), could this alter its success in rescuing the segregation of the PS?

Minor points:

1. ED Fig.4 colored legend is shared between panels c and d, however it is closest to panel b and is a bit confusing. A re-arrangement of the panels could solve this.

2. There is a typo on the Mass Spec list of "hNEAT1 8-9.2 kb RNA pulldown" where the column is labeled as unique peptode -> should be peptide.

GUIDELINES FOR MANUSCRIPT SUBMISSION TO NATURE CELL BIOLOGY

READABILITY OF MANUSCRIPTS – Nature Cell Biology is read by cell biologists from diverse backgrounds, many of whom are not native English speakers. Authors should aim to communicate their findings clearly, explaining technical jargon that might be unfamiliar to non-specialists, and avoiding non-standard abbreviations. Titles and abstracts should concisely communicate the main findings of the study, and the background, rationale, results and conclusions should be clearly explained in the manuscript in a manner accessible to a broad cell biology audience. Nature Cell Biology uses British spelling.

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AUTHOR AFFILIATIONS – should be denoted with numerical superscripts (not symbols) preceding the names. Full addresses should be included, with US states in full and providing zip/post codes. The corresponding author is denoted by: "Correspondence should be addressed to [initials]."

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ACKNOWLEDGEMENTS – should be kept brief. Professional titles and affiliations are unnecessary. Grant numbers can be listed.

AUTHOR CONTRIBUTIONS – must be included after the Acknowledgements, detailing the contributions of each author to the paper (e.g. experimental work, project planning, data analysis etc.). Each author should be listed by his/her initials.

FINANCIAL AND NON-FINANCIAL COMPETING INTERESTS – the authors must include one of three declarations: (1) that they have no financial and non-financial competing interests; (2) that they have financial and non-financial competing interests; or (3) that they decline to respond, after the Author Contributions section. This statement will be published with the article, and in cases where financial and non-financial competing interests are declared, these will be itemized in a web supplement to the article. For further details please see <https://www.nature.com/licenceforms/nrg/competing-interests.pdf>.

REFERENCES – are limited to a total of 70 for Articles, Resources, Technical Reports; and 40 for Letters. This includes references in the main text and Methods combined. References must be numbered sequentially as they appear in the main text, tables and figure legends and Methods and must follow the precise style of Nature Cell Biology references. References only cited in the Methods should be numbered consecutively following the last reference cited in the main text. References only associated with Supplementary Information (e.g. in supplementary legends) do not count toward the total reference limit and do not need to be cited in numerical continuity with references in the main text. Only published papers can be cited, and each publication cited should be included in the numbered reference list, which should include the manuscript titles. Footnotes are not permitted.

METHODS – Nature Cell Biology publishes methods online. The methods section should be provided as a separate Word document, which will be copyedited and appended to the manuscript PDF, and incorporated within the HTML format of the paper.

Methods should be written concisely, but should contain all elements necessary to allow interpretation and replication of the results. As a guideline, Methods sections typically do not exceed 3,000 words.

The Methods should be divided into subsections listing reagents and techniques. When citing previous methods, accurate references should be provided and any alterations should be noted. Information must be provided about: antibody dilutions, company names, catalogue numbers and clone numbers for monoclonal antibodies; sequences of RNAi and cDNA probes/primers or company names and catalogue numbers if reagents are commercial; cell line names, sources and information on cell line identity and authentication. Animal studies and experiments involving human subjects must be reported in detail, identifying the committees approving the protocols. For studies involving human subjects/samples, a statement must be included confirming that informed consent was obtained. Statistical analyses and information on the reproducibility of experimental results should be provided in a section titled "Statistics and Reproducibility".

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- Accession codes for primary datasets (generated during the study under consideration and designated as "primary accessions") and secondary datasets (published datasets reanalysed during the study under consideration, designated as "referenced accessions"). For primary accessions data should be made public to coincide with publication of the manuscript. A list of data types for which submission to community-endorsed public repositories is mandated (including sequence, structure, microarray, deep sequencing data) can be found here <http://www.nature.com/authors/policies/availability.html#data>.
- Unique identifiers (accession codes, DOIs or other unique persistent identifier) and hyperlinks for datasets deposited in an approved repository, but for which data deposition is not mandated (see here for details <http://www.nature.com/sdata/data-policies/repositories>).
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- If a dataset has a Digital Object Identifier (DOI) as its unique identifier, we strongly encourage including this in the Reference list and citing the dataset in the Methods.

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FIGURES – Colour figure publication costs \$600 for the first, and \$300 for each subsequent colour figure. All panels of a multi-panel figure must be logically connected and arranged as they would

appear in the final version. Unnecessary figures and figure panels should be avoided (e.g. data presented in small tables could be stated briefly in the text instead).

All imaging data should be accompanied by scale bars, which should be defined in the legend. Cropped images of gels/blots are acceptable, but need to be accompanied by size markers, and to retain visible background signal within the linear range (i.e. should not be saturated). The boundaries of panels with low background have to be demarked with black lines. Splicing of panels should only be considered if unavoidable, and must be clearly marked on the figure, and noted in the legend with a statement on whether the samples were obtained and processed simultaneously. Quantitative comparisons between samples on different gels/blots are discouraged; if this is unavoidable, it should only be performed for samples derived from the same experiment with gels/blots were processed in parallel, which needs to be stated in the legend.

Figures should be provided at approximately the size that they are to be printed at (single column is 86 mm, double column is 170 mm) and should not exceed an A4 page (8.5 x 11"). Reduction to the scale that will be used on the page is not necessary, but multi-panel figures should be sized so that the whole figure can be reduced by the same amount at the smallest size at which essential details in each panel are visible. In the interest of our colour-blind readers we ask that you avoid using red and green for contrast in figures. Replacing red with magenta and green with turquoise are two possible colour-safe alternatives. Lines with widths of less than 1 point should be avoided. Sans serif typefaces, such as Helvetica (preferred) or Arial should be used. All text that forms part of a figure should be rewritable and removable.

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- We accept PowerPoint (.PPT) files if they are fully editable. However, please refrain from adding PowerPoint graphical effects to objects, as this results in them outputting poor quality raster art. Text used for PowerPoint figures should be Helvetica (preferred) or Arial.
- We do not recommend using Adobe Photoshop for designing figures, but we can accept Photoshop generated (.PSD or .TIFF) files only if each element included in the figure (text, labels, pictures, graphs, arrows and scale bars) are on separate layers. All text should be editable in 'type layers' and line-art such as graphs and other simple schematics should be preserved and embedded within 'vector smart objects' - not flattened raster/bitmap graphics.
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Regardless of format, all figures must be vector graphic compatible files, not supplied in a flattened raster/bitmap graphics format, but should be fully editable, allowing us to highlight/copy/paste all text and move individual parts of the figures (i.e. arrows, lines, x and y axes, graphs, tick marks, scale bars etc.). The only parts of the figure that should be in pixel raster/bitmap format are photographic

images or 3D rendered graphics/complex technical illustrations.

All placed images (i.e. a photo incorporated into a figure) should be on a separate layer and independent from any superimposed scale bars or text. Individual photographic images must be a minimum of 300+ DPI (at actual size) or kept constant from the original picture acquisition and not decreased in resolution post image acquisition. All colour artwork should be RGB format.

FIGURE LEGENDS – must not exceed 350 words for each figure to allow fit on a single printed NCB page together with the figure. They must include a brief title for the whole figure, and short descriptions of each panel with definitions of the symbols used, but without detailing methodology.

TABLES – main tables should be provided as individual Word files, together with a brief title and legend. For supplementary tables see below.

SUPPLEMENTARY INFORMATION – Supplementary information is material directly relevant to the conclusion of a paper, but which cannot be included in the printed version in order to keep the manuscript concise and accessible to the general reader. Supplementary information is an integral part of a Nature Cell Biology publication and should be prepared and presented with as much care as the main display item, but it must not include non-essential data or text, which may be removed at the editor's discretion. All supplementary material is fully peer-reviewed and published online as part of the HTML version of the manuscript. Supplementary Figures and Supplementary Notes are appended at the end of the main PDF of the published manuscript.

Supplementary items should relate to a main text figure, wherever possible, and should be mentioned sequentially in the main manuscript, designated as Supplementary Figure, Table, Video, or Note, and numbered continuously (e.g. Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2 etc.).

Unprocessed scans of all key data generated through electrophoretic separation techniques need to be presented in a supplementary figure that should be labelled and numbered as the final supplementary figure, and should be mentioned in every relevant figure legend. This figure does not count towards the total number of figures and is the only figure that can be displayed over multiple pages, but should be provided as a single file, in PDF or TIFF format. Data in this figure can be displayed in a relatively informal style, but size markers and the figures panels corresponding to the presented data must be indicated.

The total number of Supplementary Figures (not including the “unprocessed scans” Supplementary Figure) should not exceed the number of main display items (figures and/or tables (see our Guide to Authors and March 2012 editorial <http://www.nature.com/ncb/authors/submit/index.html#suppinfo>; <http://www.nature.com/ncb/journal/v14/n3/index.html#ed>). No restrictions apply to Supplementary Tables or Videos, but we advise authors to be selective in including supplemental data.

Each Supplementary Figure should be provided as a single page and as an individual file in one of our accepted figure formats and should be presented according to our figure guidelines (see above). Supplementary Tables should be provided as individual Excel files. Supplementary Videos should be provided as .avi or .mov files up to 50 MB in size. Supplementary Figures, Tables and Videos must be

accompanied by a separate Word document including titles and legends.

GUIDELINES FOR EXPERIMENTAL AND STATISTICAL REPORTING

REPORTING REQUIREMENTS – We are trying to improve the quality of methods and statistics reporting in our papers. To that end, we are now asking authors to complete a reporting summary that collects information on experimental design and reagents. The Reporting Summary can be found here <https://www.nature.com/documents/nr-reporting-summary.pdf> If you would like to reference the guidance text as you complete the template, please access these flattened versions at <http://www.nature.com/authors/policies/availability.html>.

STATISTICS – Wherever statistics have been derived the legend needs to provide the n number (i.e. the sample size used to derive statistics) as a precise value (not a range), and define what this value represents. Error bars need to be defined in the legends (e.g. SD, SEM) together with a measure of centre (e.g. mean, median). Box plots need to be defined in terms of minima, maxima, centre, and percentiles. Ranges are more appropriate than standard errors for small data sets. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test used needs to be stated in the legend. Statistics such as error bars must not be derived from $n < 3$. For sample sizes of $n < 5$ please plot the individual data points rather than providing bar graphs. Deriving statistics from technical replicate samples, rather than biological replicates is strongly discouraged. Wherever statistical significance has been derived, precise p values need to be provided and the statistical test stated in the legend.

Information on how many times each experiment was repeated independently with similar results needs to be provided in the legends and/or Methods for all experiments, and in particular wherever representative experiments are shown.

We strongly recommend the presentation of source data for graphical and statistical analyses as a separate Supplementary Table, and request that source data for all independent repeats are provided when representative experiments of multiple independent repeats, or averages of two independent experiments are presented. This supplementary table should be in Excel format, with data for different figures provided as different sheets within a single Excel file. It should be labelled and numbered as one of the supplementary tables, titled "Statistics Source Data", and mentioned in all relevant figure legends.

----- Please don't hesitate to contact NCB@nature.com should you have queries about any of the above requirements -----

Author Rebuttal to Initial comments
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Responses to the reviewers' comments

The authors would like to thank the reviewers for their thoughtful and constructive comments. All comments were very helpful, and we believe that our manuscript has been improved significantly after making associated revisions. Our point-by-point responses to the comments are provided below in black (reviewers' comments are shown in gray).

Reviewer

#1:

Remarks to the Author:

In this manuscript, the authors investigated the biological question of how a nuclear RNA-scaffolded membraneless organelle (MLO), paraspeckles, maintain their structural independence within the cell nucleus through phase separation. It has been established that paraspeckles are located adjacent to another prominent MLO, nuclear speckles, which store splicing factors and regulate pre-mRNA splicing at their periphery. However, it needs to be made clear how paraspeckles do not merge with nuclear speckles. Authors identified the deletion mutants of scaffolding lncRNA NEAT1 responsible for the nucleation of paraspeckles that nucleate a form of a version of paraspeckles capable of fusing with nuclear speckles. Then they dissected the direct interactions between NEAT1 and some paraspeckle proteins that likely contribute to the segregation process. They hypothesize that components of the U2 snRNP subcomplex are involved in the internalization of paraspeckles into nuclear speckles.

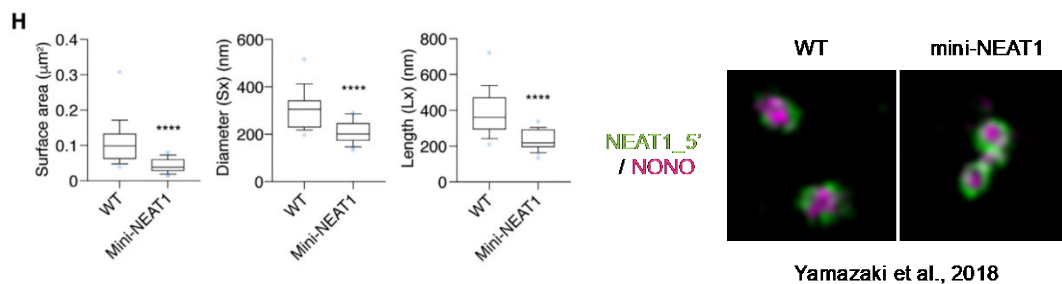
The manuscript is well-structured and informative. Overall, the experiments were generally carefully performed. However, in places, this reviewer has questions about appropriate controls and interpretation of the results using artificial non-physiological tools to study phase separation, etc. (please see below). The results are well described, but the manuscript lacks a clear overview of a fundamentally novel mechanism responsible for structural segregation between two adjacent MLOs and defects.

Below are specific comments:

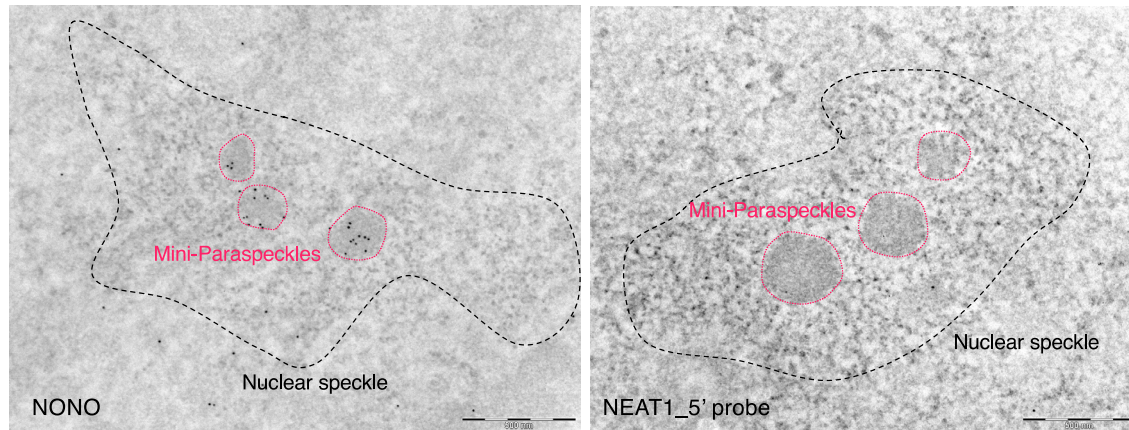
What is the average size difference between the fully functional paraspeckle nucleated by full-length NEAT1_2 and mini-paraspeckles formed by mini-NEAT1? Schemes in Extended Data Figure 1a suggest that wild-type paraspeckles and mini-paraspeckles are equal in size and similar in the overall shell-core sub-organization. However, mini-paraspeckles in multiple figures look less compact and widespread. They also look more segregated into smaller subdomains. Are they structurally equal? Or is there a flux/gradient of molecules of nuclear speckles penetrating incorporated mini-paraspeckles visible at super-resolution microscopy? The presented images need to be more convincing. This fact is a critical issue in super-resolution microscopy when threshold levels of each channel are selected and presented. It is highly possible that the continuous exchange of molecules across mini-paraspeckles inside nuclear speckles displays a complex non-uniform structure with multiple liquid-like partially miscible phases. It will make mini-paraspeckles less structurally defined.

The size criticality can play an important role in the segregation process between paraspeckles and nuclear speckles. Nuclear speckles are significantly larger and range in size from one to several micrometers in diameter. In contrast, paraspeckles are smaller, about 0.5–1.0 micrometers in size. Furthermore, mini-NEAT1 and MALAT1 of nuclear speckles intermingle extensively in fused nuclear speckles/mini-paraspeckles (Extended Data Fig 1). If mini-paraspeckles inside nuclear speckles are less compact than wild-type distinct paraspeckles, their organization level might be quite different than proposed in Figure 6.

Thank you for your comments and we apologize for the confusion. Our previous study showed that mini-PSs are smaller than WT-PS [surface area: mini-PS (43%) vs. WT-PS (100%)] [see the graphs below, Figure 4H in Yamazaki et al. Mol Cell (2018) 70, 1038]. In addition, the same study showed that the mini-PSs have the core-shell structure (see the images below). Therefore, we modified the schematic in Extended Data Fig. 1a and Extended Data Fig. 8 and scaled the actual sizes of the mini-PS and WT-PS.



We observed the mini-PSs by super-resolution microscopy (SRM) and electron microscopy (EM). EM analyses indicated that the electron density and ultrastructure of the PSs were quite comparable in NEAT1 WT and mini-NEAT1 (see EM images in Fig. 1f and Extended Data Fig. 1i). From these findings, we cannot recognize that mini-PSs are less compact than WT-PSs. EM observations also showed that multiple mini-PSs were often present in a nuclear speckle (see attached EM images and Extended Data Fig. 1i). These mini-PSs were independent of each other, not fused or connected. In our SRM images, mini-PSs often appeared to be connected MLOs due to the resolution being lower than that of EM, but this was not the case.



To clearly show the shapes and structures of mini-PSs, we have replaced some SRM images of mini-PSs in the revised manuscript (Fig. 5b and Extended Data Fig. 2c) and added additional EM data to the revised manuscript (Extended Data Fig. 1i). We have also added descriptions to clarify this point in the manuscript as follows.

Page 5, lines 89-94: Using a super-resolution microscope (SRM), the internalization of mini-PSs into NSs was observed²³ (Fig. 1d,e), confirming initial electron microscope (EM) observations indicating that almost all of the immunogold-labeled mini-PSs, **which have electron-dense structures similar to WT-PSs and are smaller than WT-PSs as previously reported²³**, were found within the ultrastructurally defined NSs (Fig. 1f **and Extended data Fig. 1i**).

The authors proposed that “mini-paraspeckles are formed outside nuclear speckles and then incorporated into them.” What is the targeting mechanism? A possible explanation here is that this phenomenon is transcription-driven, either in paraspeckles or nuclear speckles, likely both. The authors should clearly explain the mechanistic details and physiological meanings of this fusion. There is a lack of sure confidence in the discussion. For example, individual nucleoli frequently fuse in highly proliferating cells due to their size expansion and gene repositioning. Do mini-paraspeckles grow during this fusion process? Are they targeted to nuclear speckles due to their expansion driven by continuous transcription of mini-NEAT mutant RNA?

We appreciate the opportunity to provide further clarification on this point. We have shown that shell-localized U2 snRNPs facilitate the incorporation of mini-PSs into nuclear speckles. Because

U2 snRNPs are recruited to nascent RNAs [e.g., Schmidt et al., JCB (2011), 193(5), 819; Brody et al., PLoS Biol (2011), 9(1): e1000573], it is conceivable that these U2 snRNP proteins are co-transcriptionally recruited to mini-NEAT1 lncRNAs and the mini-NEAT1 lncRNAs form mini-PSs containing U2 snRNP proteins (Extended Data Fig. 7e). Because U2 snRNP components localize to the shell of NS and interact with other NS proteins and RNAs, these protein-protein and/or protein-RNA interactions occurring at the mini-PS-NS interface would contribute to the incorporation of mini-PSs into NSs.

With respect to the transcription-driven targeting mechanism, NEAT1_2 expression levels of NEAT1 WT and mini-NEAT1 are comparable, and mini-PSs are much smaller than WT-PSs [Yamazaki et al., Mol Cell (2018) 70, 1038]. Thus, there is no evidence for transcription-driven oversizing. Furthermore, the transcriptional activity is unlikely to play an important role in the incorporation of mini-PSs into the NSs because MG132 treatment, which increases NEAT1 transcription (~8-fold), did not affect the proportion of mini-PSs incorporated into NSs (Fig. 1c and Extended Data Fig. 1c). In some EM sections, two or even three mini-PSs, each of which was small in size and not fused, were present within a nuclear speckle (see above EM images). Although the nucleolus is formed by typical liquid-liquid phase separation (LLPS) and nucleoli fuse with each other [Lafontaine et al., Nat Rev Mol Cell Biol (2021) 22, 165], the paraspeckles basically do not fuse because they are formed as block copolymer micelles by micellization, a different mechanism than LLPS, and thus each paraspeckle repels the other [Yamazaki et al., EMBO J (2021) 40: e107270].

Regarding the physiological meaning of this incorporation, we investigated this point in various ways, but unfortunately, to date, we have not found any physiological, pathological, or stress conditions where WT-PSs are incorporated into NSs. Therefore, we think that this is an important issue for future research.

As suggested, we have discussed and clarified this issue in the discussion as follows.

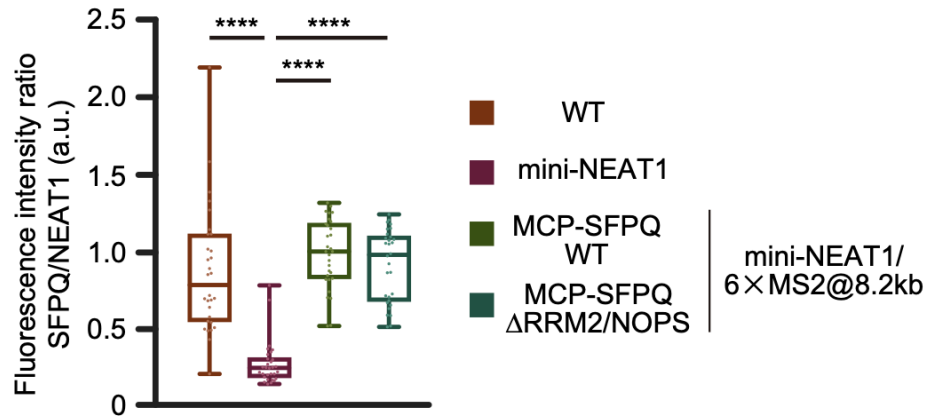
Page 13, lines 352-357: Because U2 snRNPs are recruited to nascent RNAs^{55,56}, it is conceivable that these U2 snRNP proteins are co-transcriptionally recruited to mini-NEAT1 lncRNAs and that the mini-NEAT1 forms mini-PSs containing U2 snRNPs (Extended Data Fig. 7e). Because U2 snRNP components interact with other NS proteins and RNAs, these protein-protein and/or protein-RNA interactions occurring at the interface between the mini-PS and the NS would contribute to the incorporation of mini-PSs into NSs.

Most assays in the manuscript were done using the over-expression systems, including the artificial tethering system targeting selected proteins of interest on their specific mini-NEAT1 RNA target. This fact may allow a non-physiological increase in the local nuclear concentration of selected paraspeckle factors on NEAT1, which can have a condensation effect. Such a strategy has generated more concerns about assaying nuclear condensates and phase separation. It is imperative and relevant for this work because the authors mentioned the importance of reductions of recruitment of specific paraspeckle proteins in the segregation process and defects. Could some of the seen condensation effects (e.g., in Figure 3, 4b) be due to the over-expression of paraspeckle interacting partners?

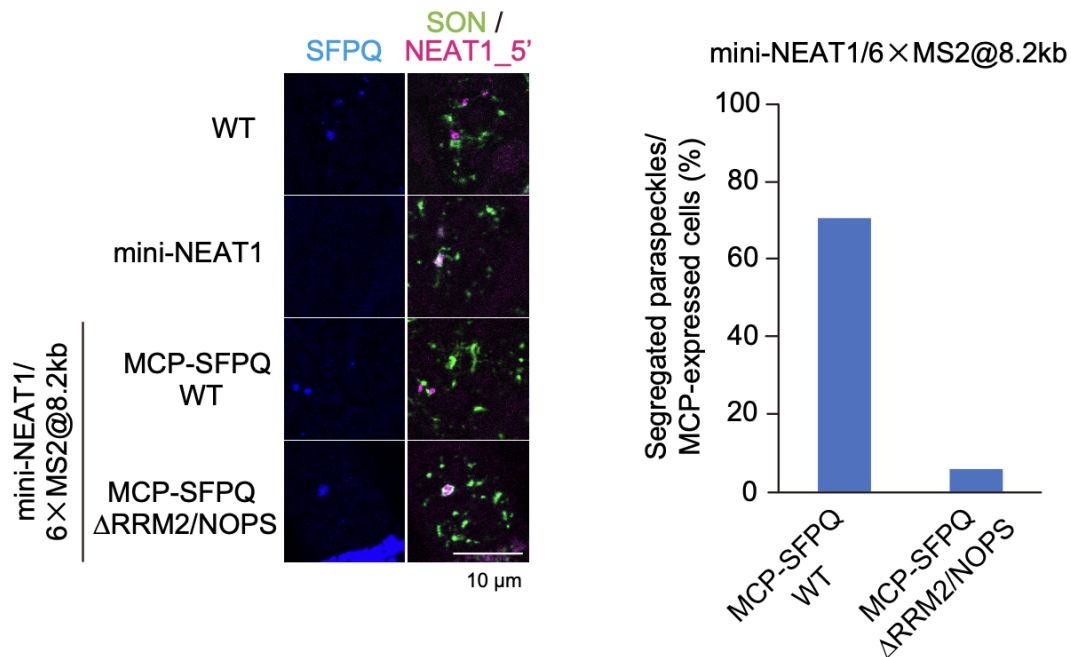
We thank the reviewer for these insightful comments. As indicated, the MS2 tethering experiment may have increased the local protein concentration to non-physiological levels, which could have condensation effects. Therefore, we analyzed cells in which the amount of MCP-SFPQ recruited to mini-PS is comparable to the endogenous amount of SFPQ in PSs. To do this, we selected cells with low expression of the MCP-SFPQ protein and examined the effect of the SFPQ tethering on the mini-PS segregation. The SFPQ recruitment to the WT-PSs or mini-PSs was quantified using IF with an SFPQ antibody, which detects both endogenous SFPQ and exogenous MCP-SFPQ. The total amount of SFPQ recruited to the mini-PSs in the tethering experiment was comparable to the endogenous SFPQ level to PSs (see graph below). Even under these conditions, the results were similar to the original results: MCP-SFPQ WT rescued the segregation defects, but MCP-SFPQ Δ RRM2/NOPS did not (see the images and graph below). These results suggest that PS segregation from NSs was unlikely to be induced by the increased concentration of SFPQ and physiological concentrations of SFPQ are sufficient to induce it.

We have added these data in Extended Data Fig. 5f-h and described this point in the results section as follows.

Page 8, lines 207-214: In these MS2 tethering experiments, the tethering might increase the local protein concentration to non-physiological levels in PSs. To test whether the recruitment of physiological levels of SFPQ to PSs is sufficient for the mini-PS segregation, we selected and analyzed the cells in which comparable amounts of SFPQ were localized to mini-PSs as in WT-PSs (Extended Data Fig. 5f). Even under these conditions, MCP-SFPQ WT rescued segregation defects, but MCP-SFPQ Δ RRM2/NOPS did not (Extended Data Fig. 5g,h). These results suggest that physiological concentrations of SFPQ are sufficient to induce PS segregation from NSs.



Data were compared using Kruskal-Wallis ANOVA and post hoc Dunn multiple comparison test.
 **** $P < 0.0001$

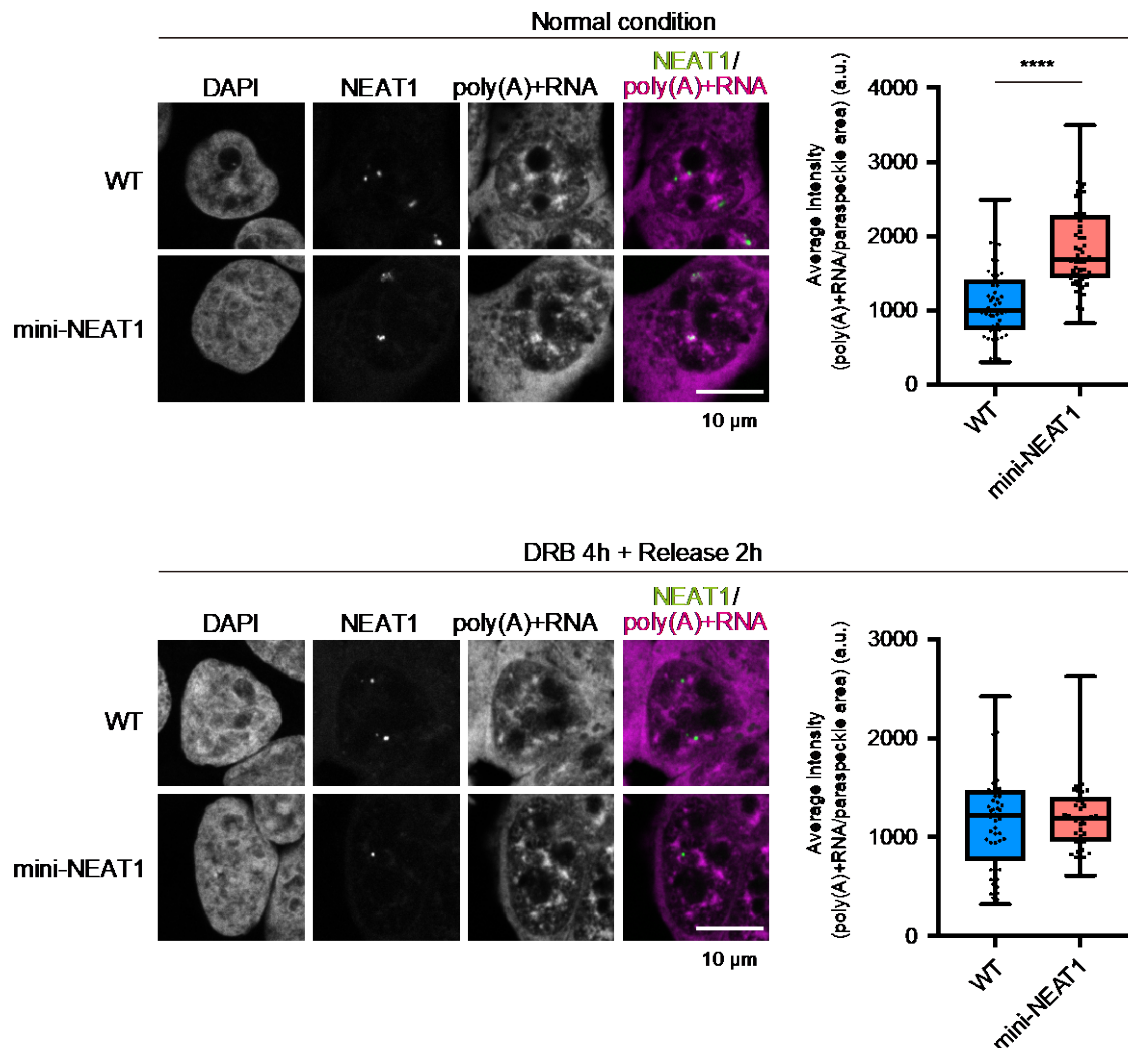


The authors indicated that U2 snRNP subcomplex proteins preferentially interact with the 5'-end of NEAT1. It has been established that U2 snRNP has a role in splicing and stimulating transcriptional elongation through interactions with the elongation factors. The authors should investigate the transcription activity (perhaps by super-resolution RNA FISH using oligo(dT)

probes at the periphery of paraspeckles and mini-paraspeckles. It is possible that it might be actively involved in the process of association and/or coalescence with nuclear speckles.

We appreciate these helpful suggestions. As the reviewer suggested, we performed oligo(dT) RNA-FISH in HAP1 WT and mini-NEAT1 cells and observed that mini-NEAT1 RNAs colocalized with poly(A)⁺ RNAs under normal conditions (see the below image and graph). However, because a population of poly(A)⁺ RNAs is usually resident in nuclear speckles [e.g., Carter et al., JCB (1991) 115, 1191], it was difficult to distinguish that these colocalized poly(A)⁺ RNA signals are derived from the resident RNAs in NSs surrounding the incorporated mini-PSs or transcriptional activity around the paraspeckles. Therefore, we performed the same experiment at the timepoint before the newly formed mini-PS was incorporated into the NS (DRB 4h and Release 2h, Fig. 1g,h). The results showed no significant difference in the amount of poly(A)⁺ RNA localized in mini-PSs compared to WT-PSs (see the below image and graph). In addition, we observed no change in the proportion of mini-PS incorporation into the NS with or without MG132 treatment, which increases NEAT1 transcription (Fig. 1c and Extended Data Fig. 1c). These observations provide evidence that transcriptional activity near the paraspeckles is not a major determinant of the incorporation of mini-PSs into NSs.

(left)



Confocal observation of the PSs and poly(A)+ RNA in HAP1 WT and mini-NEAT1 cells. PSs and poly(A)+ RNA were visualized by RNA-FISH using NEAT1 probes (green) and oligo-(dT) probes (magenta). Nuclei were stained with DAPI. Scale bar, 10 μ m. (right) Quantification of fluorescence intensity ratio [poly(A)+ RNA/NEAT1] in the HAP1 WT (blue) and mini-NEAT1 (pink) cells (all samples: $n = 50$). **** $P < 0.0001$. Data were compared using Mann-Whitney U-test. If the statistical test showed no significant difference ($P > 0.05$), it is not specified in the figure.

In terms of controls of ASO treatment, depletion of U13 small nucleolar RNA functioning in pre-ribosomal biogenesis is not adequate to control to the U2 snRNA. The authors should deplete U1

snRNA since it does not directly interact with U2 snRNA but have the same impact on pre-mRNA splicing.

We thank the reviewer for this suggestion. As suggested by the reviewer, we considered more appropriate controls for the U2 knockdown experiment. Regarding U1 ASO as a negative control, we thought that U1 ASO might not be a good control for the following reasons. First, U1 snRNAs are localized to WT-PSs [Visa et al., *European Journal of Cell Biology* (1993) 60, 308], and thus the knockdown of U1 snRNA may have a side effect on the PSs. Second, U1 snRNA has spliceosome-independent functions: telescripting, which suppresses premature 3'-end cleavage and polyadenylation [Kaida et al., *Nature* (2010) 468, 664; Berg et al., *Cell* (2012) 150, 53], and retention of noncoding RNAs on chromatin [Yin et al., *Nature* (2020) 580, 147]. Third, U1 snRNA and U2 snRNA are functionally linked, and inhibition of one may strongly affect the other [Yoshimoto et al., *Cell Chem Biol* (2021) 28, 1356]. Therefore, in addition to ASOs against U13 snoRNA, we used ASOs against 7SK snRNA, which is an abundant NS-localizing RNA less likely to affect U2 snRNA function, as negative controls. Knockdown of these snRNAs could not induce the segregation of mini-PSs from NSs (Fig. 6f,g and Extended Data Fig. 7f).

We have included these data in Fig. 6f,g and Extended Data Fig. 7e and described these data in the results section as follows.

Page 11, lines 288-290: Consistently, U2 KD resulted in segregation of the mini-PS from NSs, **whereas several control ASOs (ASOs against GFP, U13 snoRNA, 7SK snRNA) did not** (Fig. 6f,g and Extended Data Fig. 7f).

The U2 snRNP, a central component of the spliceosomes, is very highly abundant across the nucleus. Therefore, the proposed mechanism of sequestration of the U2 snRNPs (and possibly other splicing factors) in co-transcriptionally formed paraspeckles antagonizing the association of U2 snRNP in splicing for regulatory purposes requires more investigation.

We apologize for our unclear description. Our proposed model does not involve the sequestration of U2 snRNP, but rather involves the binding of SFPQ to NEAT1 lncRNA antagonizing the association of U2 snRNPs to NEAT1. To clarify this point, we changed this sentence as follows.

Page 13, lines 337-340: From these findings, we propose that the co-transcriptional formation of NEAT1_2 RNPs antagonizes the association of splicing factors (e.g., U2 snRNP) with NEAT1 lncRNAs to construct PSs as distinct nuclear MLOs (Fig. 6h).

Reviewer

#2:

Remarks to the Author:
 Paraspeckles (PSs) are microscopic structures assembled around the long noncoding RNA (lncRNA) NEAT1 in the nucleus and maintaining unique structural identity despite the lack of a membrane boundary. PSs have been originally defined based on their proximity to the nuclear speckles (NSs), somewhat larger bodies recruiting diverse splicing factors and nuclear RNAs. What prevents coalescence of the two ribonucleoprotein compartments or engulfment of one by the other has been unknown. The current study addresses this exciting question using a series of NEAT1 mutants produced by CRISPR/Cas9 engineering, super-resolution imaging, and MS2 tethering and immunoprecipitation assays. The authors show that the NEAT1 sequences occupying the outer shell of PSs recruit a characteristic set of RNA-binding proteins, including SFPQ, hnRNPF, and BRG1, which in turn prevent the occlusion of the PSs by the NSs. Evidence is provided that the key outer shell protein SFPQ achieves this effect by antagonising the recruitment of NS-enriched spliceosomal components to NEAT1. I think that these results are novel and of high quality and that the study should appeal to the wide readership interested in RNA-protein interactions, membraneless organelles, and the mechanisms and biological functions of phase separation. However, I also have several concerns, which should be addressed in the revision.

Specific comments

1. I could not find a description of the procedure used to classify PSs as segregated or incorporated. Was it based on co-localization? Occlusion of PSs by NS pixels? In any case, this key procedure must be clearly described in the Results or/and Methods. If the classification was done manually, the authors should also show a few typical examples of segregated and incorporated PSs in the supplement.

We appreciate the reviewer's suggestions. We manually performed the classification in fluorescence microscopy images and EM images. Therefore, as suggested, we have shown some typical examples of segregated and incorporated PSs in the fluorescence microscopy images in Extended Data Fig. 1b.

2. Related to the point above, the authors show stacked bar plots to report the fractions of segregated or incorporated PSs throughout the manuscript. However, no statistical tests are provided. Such tests are especially important in cases where the effects are relatively subtle (e.g., Fig. 6c,e,g). The authors should estimate the significance of their key comparisons by Fisher's exact or chi2 tests.

We thank the reviewer for the careful review of the manuscript. As suggested, we have added the results of the statistical tests using Fisher's exact tests (Fig, 6c, e, g and Extended Data Fig. 2d and 7c).

3. All new CRISPR/Cas9 mutants and knock-ins generated in this study must be validated by PCR genotyping and the results shown in the supplement.

We appreciate this helpful suggestion. As suggested, we have added the results of genotyping by PCR (Extended Data Fig. 2b, 4a, 7a). We have also confirmed deletions or insertions by Sanger sequencing analysis (see Material and Method). More detailed information on the identified deletions or insertions can be found in Supplementary Table 2.

4. Fig 1, Fig S1 and lines 95-105. The authors must rule out that reduced co-localization between NEAT1 and SRRM2 at the beginning of the release period is not due to a major rearrangement of the NS system in response to DRB. This can be done for example by showing a field of cells stained for SRRM2 +/-DRB. To further strengthen the argument that older PSs are more often incorporated compared to newly assembled ones, the authors should compare NS incorporation of the NEAT1tail and NEAT1_5' signals in Fig S1i. In addition, the current version of Fig S1i is not ideal since it uses NEAT1_5'+3' for WT and NEAT1_5' for mini-NEAT1.

Thank you very much for your suggestion. It is very helpful to organize the points. First, our argument is not that old mini-PSs are incorporated into NSs, but that mini-PSs are formed outside (not inside) the NSs before being incorporated into them. As shown in Fig. 1g, the NEAT1 transcription site marked by the NEAT1 tail probe is localized outside of the NSs in mini-NEAT1

cells under normal culture conditions (not DRB-treated conditions), in which most of the mini-PSs are present within the NSs. These findings suggest that mini-NEAT1 transcription and thus mini-PS assembly occur outside of NSs. In addition, our experiments with DRB showed that mini-PSs are completely diminished after 4 h of DRB treatment, and after washing out DRB, mini-PSs start to form outside of the NSs (Fig. 1h). Importantly, after several hours, the newly formed mini-PSs enter NSs (Fig. 1h). Thus, even when we created a starting point where mini-PSs are present outside the NSs, the mini-PSs would eventually enter them.

As the reviewer indicated, NEAT1 and SRRM2 are scarcely co-localized at the beginning of the period of DRB release. However, regardless of whether the rearrangement of the NSs affects incorporation into them or not, it does not affect our argument that mini-PSs are formed outside NSs and are then incorporated into them.

To make these points clearer, we have changed the text in results as follows.

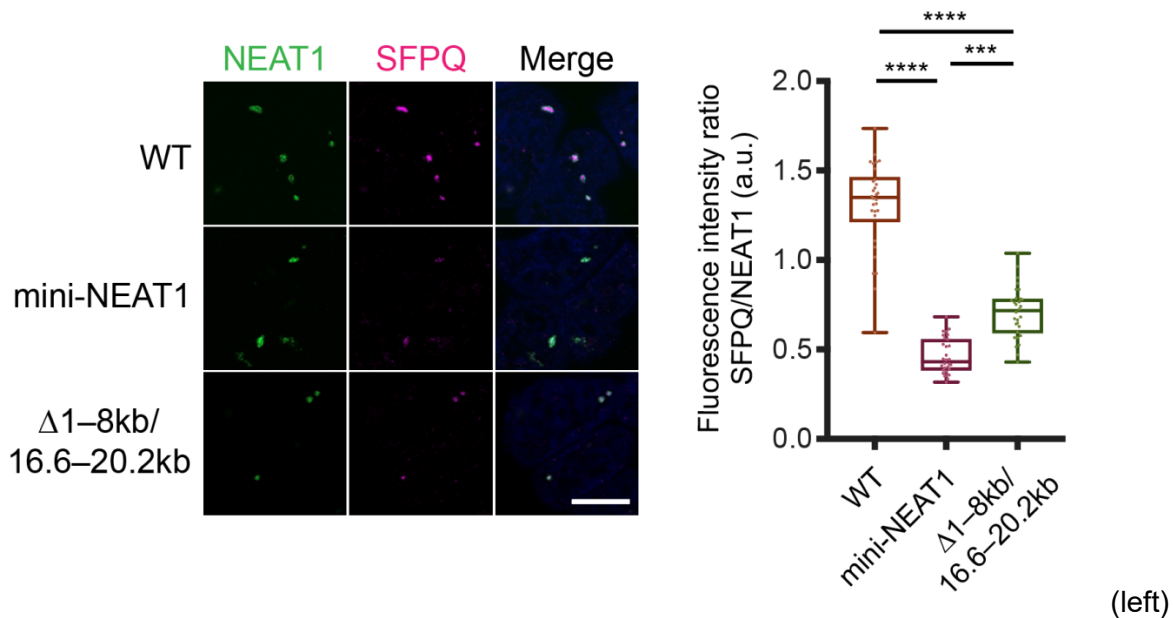
Pages 5-6, lines 101-116: Although mini-PSs were clearly present within NSs, it was unclear whether de novo synthesized mini-PSs formed inside or outside of NSs. To investigate this, we first examined whether the transcription sites of NEAT1, where PSs are formed, are inside or outside of NSs. We visualized the transcription sites by detecting nascent NEAT1 transcripts using NEAT1 tail probes, which detect unprocessed NEAT1 downstream regions, as used in a previous study³³ (Fig. 1g). The NEAT1 transcription sites were located outside and adjacent to NSs in both HAP1 WT and mini-NEAT1 cells (Fig. 1g), suggesting that mini-PSs are formed outside NSs. Furthermore, we observed de novo PS formation over time after release from a reversible Pol II inhibitor, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), which inhibits PS formation^{23,25,35}. After 4 h of DRB treatment, PSs were completely diminished. At 1–2 h after release, PSs started to re-form in both WT and mini-NEAT1 cell lines, and most of them were present adjacent to NSs (Fig. 1h,i). At 4–8 h after release, the localization of mini-PSs within NSs gradually increased (Fig. 1 h,i). Thus, even when we created a starting point where mini-PSs are present outside the NSs, mini-PSs eventually enter them. These findings suggest that mini-PSs form outside NSs and are then incorporated into them.

In addition, to clearly visualize the shells of WT-PSs and mini-PSs, we used NEAT1_5'+3' probes for WT and NEAT1_5' probes for mini-NEAT1, as in the previous study [Yamazaki et al., Mol Cell (2018) 70, 1038], because both 5' and 3' ends of NEAT1_2 are localized in the shell of WT-PSs and only 5' ends are localized in the shell of mini-PSs. Therefore, we believe that these are the best data available.

5. Fig. S2c. The authors do not discuss the apparent discrepancy between the relatively strong incorporation of the D1-8k/16.6-20.2k mutant and the enrichment of SFPQ binding sites in the 20.2-22.6k region in Fig. S7f. This caveat must be addressed - at least in the Discussion. It may be also helpful to check if the incorporation of D1-8k/16.6-20.2k is significantly different from mini-NEAT1 in Fig.S2C.

We thank the reviewer for raising an important point. As suggested, we have performed statistical analyses on the data shown in Extended Data Fig. 2d. (The statistical analysis data have been added to the figure and the figure legend.) The analyses showed that the proportion of Δ 1-8 kb/16.6-20.2 kb cells with segregated PSs is significantly higher than that of mini-NEAT1 cells. This is consistent with the SFPQ binding sites in the NEAT1 20.2-22.6 kb region in the SFPQ eCLIP data (Extended Data Fig. 7g).

We further experimentally investigated the importance of the SFPQ binding sites for SFPQ recruitment to PSs. We then examined the SFPQ recruitment to the PSs in WT, mini-NEAT1, and Δ 1-8 kb/16.6-20.2 kb mutant cells, which contain an additional 20.2-22.6 kb region compared to the mini-NEAT1 mutant (Extended Data Fig. 2a,c,d). The SFPQ recruitment to PSs was slightly increased compared with that in the mini-NEAT1 mutant cells (see attached data below). These results suggest that the NEAT1 20.2-22.6 kb region can recruit a small amount of SFPQ, but this makes a minor contribution to PS segregation (Extended Data Fig. 2d). Therefore, we believe that the eCLIP data are basically consistent with our experimental data.



Confocal observation of the PSs and SFPQ in HAP1 WT and the NEAT1 deletion mutant cells treated with MG132 (5 μM for 6 h). PSs and SFPQ were visualized by RNA-FISH using NEAT1_5' probes (green) and IF (magenta). Nuclei were stained with DAPI. Scale bar, 10 μm . (right) Quantification of fluorescence intensity ratio (SFPQ/NEAT1) (all samples: $n = 30$).

6. Fig.4d and Fig.S5f. The IP experiments must be repeated at least three times, quantified, and compared by t-test or a similar statistical procedure.

As suggested, we have quantified the results of the IP experiments and compared them by using Kruskal-Wallis ANOVA and post hoc Dunn's multiple comparison test (Fig. 4e and Extended Data Fig. 5j). In addition, on the basis of these results, we have changed the text in the results section as follows.

Page 9, lines 215-217: As expected, SFPQ $\Delta\text{RRM2/NOPS}$ and ΔCC completely lost the interactions with SFPQ, NONO, and PSPC1^{36,37} (Fig. 4d,e and Extended Data Fig. 5i,j)^{36,37}. In contrast, SFPQ ΔPLD interacted with SFPQ, NONO, and PSPC1 (Fig. 4d,e and Extended Data Fig. 5i,j).

7. line 163 "BRG1 tethering did not facilitate recruitment of SFPQ and HNRNPF". Fig S4e shows that MCP-BRG1 recruits HNRNPF and that this effect is significant. Fig S4d does not compare between MCP-BRG1 and MCP-GFPNLS, but the median of MCP-BRG1 exceeds that of MCP-GFPNLS. The authors should provide a more nuanced description of the data in the text.

We thank the reviewer for the careful review. If the statistical tests did not show significant differences, we do not specify that in the figure. Thus, we have added this information to the figure legends in all relevant sections of this paper. As you indicated, we cannot rule out the possibility that the tethering of BRG1 has an impact on SFPQ and HNRNPF recruitment. Therefore, we changed the text to a more nuanced wording.

Page 8, lines 175-184: **Meanwhile, the tethering of SFPQ did not facilitate HNRNPF recruitment (Extended Data Fig. 4f), suggesting a hierarchical relationship between SFPQ and HNRNPF in the recruitment. In addition, the tethering of either SFPQ or HNRNPF did not facilitate BRG1 recruitment (Extended Data Fig. 4g), although the tethering of BRG1 marginally facilitated HNRNPF recruitment and showed a trend toward increased SFPQ recruitment (Extended Data Fig. 4e,f). Collectively, these findings suggest that the mechanism by which mini-PS segregation defects are rescued by SFPQ or HNRNPF tethering appears to differ from that by BRG1, and that the tethering of HNRNPF likely rescues the segregation defect at least partly through SFPQ recruitment.**

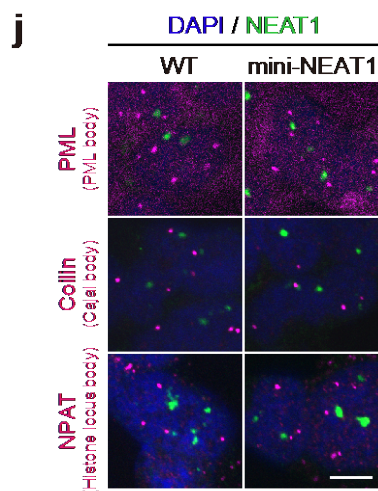
8. Fig. S5g-h and lines 198-204. "Although most of the SFPQ mutants exhibited a compromised ability to achieve PS assembly, etc". I find this confusing. Up until this point in the manuscript, the assumption was that mini-NEAT1 and mini-NEAT1/6×MS2@8.2kb are fully proficient in PS assembly. This point must be clarified.

We apologize for the confusing description. In Extended Data Fig. 5g–h, we examined the ability of SFPQ mutants in paraspeckle assembly, not segregation, using the paraspeckle assembly-deficient NEAT1 mutant cell lines (m13-16.6k mutant cell line) [Yamazaki et al., Mol Cell (2018) 70, 1038]. We tethered MS2-fused SFPQ WT or mutant to the PSs in the m13-16.6k mutant cells, in which PS assembly is compromised, as reported previously [Yamazaki et al., Mol Cell (2018) 70, 1038]. To clarify this point, we have changed the text as follows.

Page 9, lines 232-239: We next investigated whether this rescue activity is related to the ability to induce PS assembly by SFPQ²³. To examine the ability to perform assembly, we used an SFPQ tethering experiment in NEAT1 m13-16.6 kb mutant cells, in which the PS assembly is defective²³. Although most of the SFPQ mutants exhibited a compromised ability to achieve PS assembly, the SFPQ Q to G mutant, which cannot rescue the segregation defect, retained the PS assembly ability (Extended Data Fig. 5k,l). Thus, these two abilities are separable, and the ability to induce the assembly is not sufficient to rescue the segregation defect.

9. Line 208. "The mini-PSs are incorporated into NSs, but not into other nuclear bodies". Have the authors quantified incorporation of PSs into other nuclear bodies. If they have not, this claim must be deleted.

Thank you for your suggestion. As described in Figure 1c, ~90% of mini-PSs localize within the nuclear speckles. Similarly, most mini-PSs were clearly observed within nuclear speckles by EM, as shown in Fig. 1f and Extended Data Fig. 1i, which we added in this revision. Under EM, we can observe the mini-PSs and other nuclear bodies (e.g., nucleolus, nuclear speckles, Cajal bodies, PML bodies) at the same time and none of the mini-PSs is incorporated into the other nuclear bodies. Moreover, we confirmed by IF that the mini-PSs did not colocalize with PML bodies, Cajal bodies, or histone locus bodies (Extended Data Fig. 1j). From these findings, we have confidence that mini-PSs are incorporated into nuclear speckles, but not other nuclear bodies.



To clarify this point, we have added the following text.

Page 5, lines 94-98: We did not observe any colocalization of mini-PSs with nuclear bodies visible under EM. In addition, no colocalization of mini-PSs with several nuclear bodies was found by confocal microscopy (Extended Data Fig. 1j), suggesting that mini-PSs are specifically incorporated into NSs, but not into the other nuclear bodies.

10. Are the effects shown in Fig. S7e statistically significant? Also, the authors should explain in the text why they think U2 ASO appears to dampen NEAT1.

We appreciate the reviewer's suggestions. According to another reviewer's (#1) suggestion, we performed U2 snRNA knockdown with additional controls during this revision. The data are shown in Extended Data Fig. 7f. The NEAT1_2 expression level showed a trend toward reduction, but the difference was not significant in our statistical analysis. Thus, we decided not to include this point in the manuscript.

Minor points
The word "encoded" in the title may mislead some readers into thinking about protein-coding capacity of NEAT1. "Shell protein composition specified by NEAT1 domains..." might be a safer choice.

Thank you for your suggestion. We have considered this point and changed the title to "Shell protein composition specified by NEAT1 domains dictates the formation of paraspeckles as distinct membraneless organelles."

We have also changed similar descriptions in the Abstract and Introduction as follows.

Abstract line 15: Shell localization of these proteins in the paraspeckles, which is determined by NEAT1_2 lncRNA domains, is required for this segregation process.

Introduction (line 72): Our study revealed that specific shell-localizing proteins **specified** by NEAT1_2 RNA domains determine the independence of PSs from NSs, providing insights into the general molecular mechanisms governing the independence of MLOs.

Fig 1g. the label should say "DRB 4 hours and release", as in Fig 1h.

Thank you for your comment. We have changed the label in Fig. 1h accordingly.

Line 87. Please mention in the text that SRRM2 was used as an NS marker in Fig 1b-c to clarify the SON/MALAT1 statement on lines 88-89.

Thank you for your helpful recommendation. As suggested, we have added text to the results section as described below.

Page 5, lines 84-87: NEAT1 RNA-FISH and immunofluorescence (IF) of **SRRM2 as a marker for NS** revealed that about 90% of mini-PSs colocalized with NSs, while WT-PSs did not colocalize with NSs as expected (Fig. 1b,c, Extended Data Fig. 1b, c and Supplementary Video 1,2).

Line 90. SRM and SIM are mentioned interchangeably in the text and the Figures. If the authors did not use SRM methods other than SIM, why not consistently use just one acronym - e.g., SIM.

We apologize for our unclear use of the terms "SRM" and "SIM." The images taken by the ZEISS ELYRA PS.1 are referred to as SIM (structured illumination microscopy) images. The images taken by LSM900 with Airyscan2 are referred to as SRM (super-resolution microscopy) images. We have added text to the Materials and Methods section explaining this.

Page 17, lines 457-460: Confocal microscopy and **structured illumination** microscopy (**referred to as SIM**) analyses were performed as previously described²³. Super-resolution microscopy analyses were performed using LSM900 with Airyscan2 with a 63× objective lens (**referred to as SRM**).

Line 93. The conclusion "These findings together show that the mini-PSs form organized, immiscible structures within NSs and thus lack the ability to exist as distinct MLOs" sends mixed messages. There must be a more nuanced way to say that although mini-PSs are organized and immiscible, they are occluded by NSs.

Thank you for your kind suggestion. We changed this sentence as follows.

Page 5, lines 98-100: These findings together show that, **although the mini-PSs have a core-shell organization and are immiscible, they are occluded by NSs** and thus lack the ability to exist as distinct MLOs.

Reviewer

#3:

Remarks to the Author:

Summary:

This is a highly original and well conducted study addressing the immiscibility of nuclear biomolecular condensates by focusing on paraspeckles and the scaffolding non-coding RNA NEAT1. Figures are nicely organized and clearly demonstrate their findings. I would like to thank the authors for making this manuscript easy to read and understand.

Authors build their work on an earlier study on NEAT1_2 RNA domains, where a construct that makes mini-paraspeckles (mini-PSs) becomes incorporated into nuclear speckles (NS). They carefully dissect out mislocalized PS proteins (PSPs) in mini-PS and focus on the three proteins, SFPQ, HNRNPF and BRG1, that induce segregation of mini-PS. SFPQ turns out to be hierarchically more important for this process. Authors use mutational analysis of the key protein SFPQ and identify separable functions of SFPQ in PS segregation (from NS) and assembly. Furthermore, shell localizing SFPQ is found to be the key to segregate PS from NS. This atypical incorporation of PS into NS is suggested to be caused by the failure to escape from the canonical mRNA maturation pathways, particularly splicing, indicated by the affiliation of the mini-PS with U2 snRNP.

In Figure 6, authors provide a compelling cartoon drawing to provide a mechanistic view of their data. The proposed mechanism of PS and NS segregation requires a few more experimental

evidence. Moreover, the outcome of mini-PS incorporation into NS is not investigated or discussed. Therefore, ideally this manuscript needs to be revised in order to be suitable for publication at NCB. Suggestions to the authors are below.

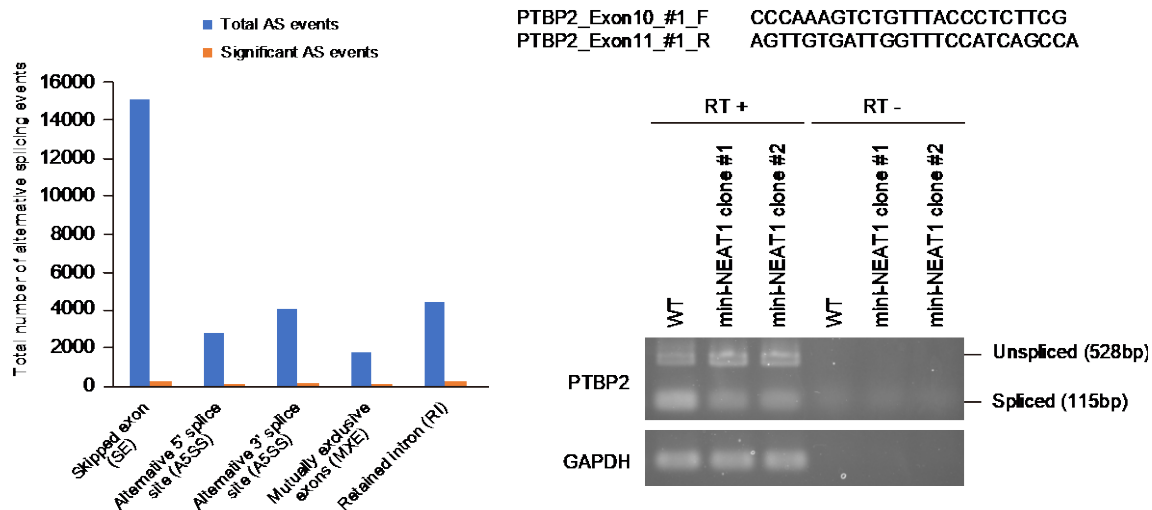
Suggested improvements:

1. The incorporation of mini-PS into NS might not lead to obvious cellular phenotypes as in the form of viability, however potential disturbance to the function of NS in RNA processing should be addressed to make the story complete. An RNA-seq experiment followed by computational analysis would give a genome-wide view, however this could be challenging for the groups who lack the expertise, therefore RT-PCR experiments on certain constructs could be used as an alternative if this happens to be the case. For example:

A recent correlative study from the group of Gil Ast, suggests the role of gene architecture in splicing unit determination modes (exon definition vs. intron definition). According to this model some, but not all, splicing events should be NS-dependent. Using the mini-gene constructs described in Fig.2 of Tammer et al. Mol. Cell 2022 (<https://www.sciencedirect.com/science/article/abs/pii/S109727652200106X>), the authors could find answers to the impact of incorporated PS on NS function.

Furthermore, SF3B1 is important for branch point sequence (BPS) selection and the incorporation of mini-PS into NS could lead to a titration effect and alter BPS selection of transcripts in general. The authors could use gene examples shown in Fig.2 of Alsafadi et al. Nat. Comm. 2016 (<https://www.nature.com/articles/ncomms10615>) in order to show how the selection might be unaffected/affected.

We thank the reviewer for these excellent comments. As suggested, we performed RNA-seq using HAP1 WT and mini-NEAT1 cells followed by genome-wide analyses of pre-mRNA alternative splicing (AS) patterns using rMATS [Shen et al., PNAS (2014) 111, E5593; <https://rnaseq-mats.sourceforge.net/>]. The results showed that a small number of AS events were altered in HAP1 mini-NEAT1 cells (see attached graph). To validate this result, we selected 11 genes that showed significantly altered splicing events and performed semi-quantitative RT-PCR on them. However, we could not detect any clear differences except for the intron retention of PTBP2 (see attached figure). It is also important to note that we cannot definitively discuss the effect on PTBP2 splicing because we cannot distinguish whether it is due to the PS incorporation into NSs or the partial deletion of the NEAT1 RNA region, which could give an independent effect. Therefore, we have decided not to include these data in the manuscript. Future research to elucidate the biological significance of the separate existence of PSs and NSs is important.



(left) Graph showing the number of alternative splicing events corresponding to five major event types. (right) Semi-quantitative RT-PCR validation of the PTBP2 intron retention in HAP1 WT and mini-NEAT1 deletion mutant. GAPDH was used as a loading control.

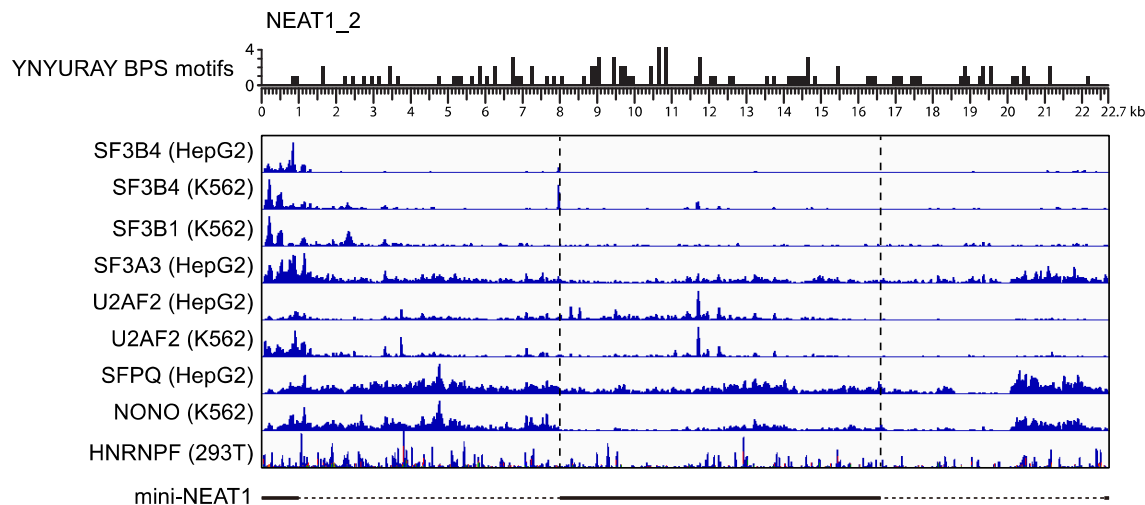
2. There are a few sections in the manuscript that would require deeper investigation or discussion. These refer to:

a) mislocalization of a subset of PSPs: what differentiates the PSPs which are strongly reduced in PS from those aren't affected? The protein domains they contain, repeat regions (i.e. G-rich, PLD), or their affinities towards each other (a molecular grammar), or the amount of total protein found in PS under unperturbed conditions?

We appreciate the opportunity to clarify this point. As suggested by the reviewer, the mechanisms via protein-protein interactions may be applicable to explain the recruitment of PSPs to WT and mutant PSs. However, although we searched for protein domains of mislocalized PSPs (SFPQ, TDP-43, HNRNPH1, HNRNPF, BRG1) and PSPs whose recruitment was not affected (NONO, FUS, PSPC1, RBM14, HNRNPA1, HNRNPH3), we did not find clear discriminatory features between them. Instead, previous studies demonstrated the importance of PSP-binding sites on NEAT1_2 in the recruitment of PSPs to PSs. For example, TDP-43 binds to UG-repeat regions of NEAT1_2, and deletion of these binding sites from NEAT1_2 abolishes the recruitment of TDP-43 to paraspeckles [Modic et al., Mol Cell (2019) 74, 951-965]. It is also reported that deletion of the middle domain of NEAT1_2, which contains multiple NONO binding sites, causes defects in paraspeckle assembly, which are likely due to reduced recruitment of NONO proteins essential for paraspeckle assembly [Yamazaki et al., Mol Cell (2018) 70, 1038-1053]. Therefore, it is

conceivable that the mislocalization of a subset of PSPs is caused by the deletion of their binding sites on NEAT1_2. Consistent with this notion, the available CLIP-seq data for SFPQ and HNRNPF show that a major fraction of their binding sites is located in the regions deleted in mini-NEAT1 (see the image below), while our reanalysis of BRG1 CLIP-seq [Cardenas et al., Nat Commun (2018) 9, 1009] did not detect significant interactions between BRG1 and NEAT1 lncRNA. Our analyses during this revision (see replies to the comments below) showed that HNRNPF and SFPQ are recruited to PSs through the same NEAT1 regions (Extended Data Fig. 4i,j). In addition, consistent with our data, a previous bioinformatic study on SFPQ recruitment showed that HNRNPF-binding sequences are enriched in the SFPQ binding sites [Iida et al., iScience (2020) 23, 101325 and personal communication with Dr. Kei Iida], suggesting a role for HNRNPF in SFPQ recruitment to RNAs. Therefore, at least part of the mislocalization can be explained by the direct recruitment by NEAT1 RNA domains.

b) artificial tethering experiments: TDP-43 in Fig.3b shows cytoplasmic localization as well as nuclear pool. Could this affect the segregation success of mini-PS from NS?

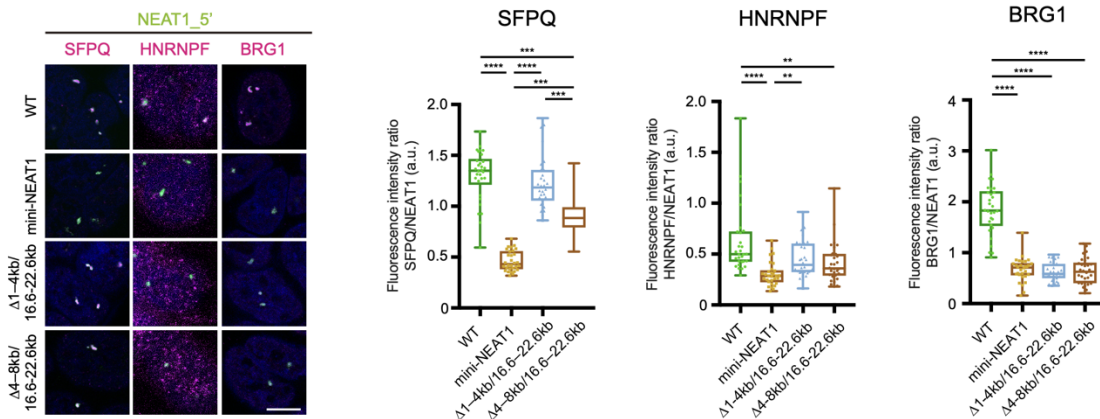


We thank the reviewer for the careful review of the manuscript. MCP-TDP-43 was also localized in the cytoplasm in some cells, but in many cells, it was predominantly localized in the nucleus. We have therefore changed the images to be more representative (Fig. 3b).

c) the different mechanism of mini-PS segregation defect rescue by SFPQ & HNRNPF from BRG1: are they sequentially recruited on the RNA? Or is this driven by protein-protein interactions? Could the authors perform a sequential IP experiment to find out about the hierarchies to refine this different mechanism? Perhaps in-vitro droplet formation assays with truncated proteins would also be useful to determine the importance of protein-protein interactions in this process.

Thank you for raising an important point. To address this point, we examined the in vivo recruitment of SFPQ, HNRNPF, and BRG1 to PSs in additional HAP1 NEAT1 deletion cell lines, instead of using sequential IP or in vitro droplet assays. We used NEAT1 Δ 4-8k/16.6-22.6k and Δ 1-4k/16.6-22.6k cell lines (Extended Data Fig. 2a), in which paraspeckles are segregated from nuclear speckles. The recruitment of SFPQ and HNRNPF to paraspeckles was partially or completely rescued by these two domains, 1-4 kb and 4-8 kb of NEAT1, respectively. Meanwhile, the recruitment of BRG1 to PSs was not rescued at all in these deletion cell lines. These findings suggest that the recruitment of SFPQ/HNRNPF and BRG1 is determined by distinct NEAT1 RNA domains.

Therefore, we have added these data as Extended Data Figure 4i,j and described these data in



Data were compared using Kruskal-Wallis ANOVA and post hoc Dunn multiple comparison test.

**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$

If the statistical tests did not show significant differences, we did not specify that in the figure.

the results as follows.

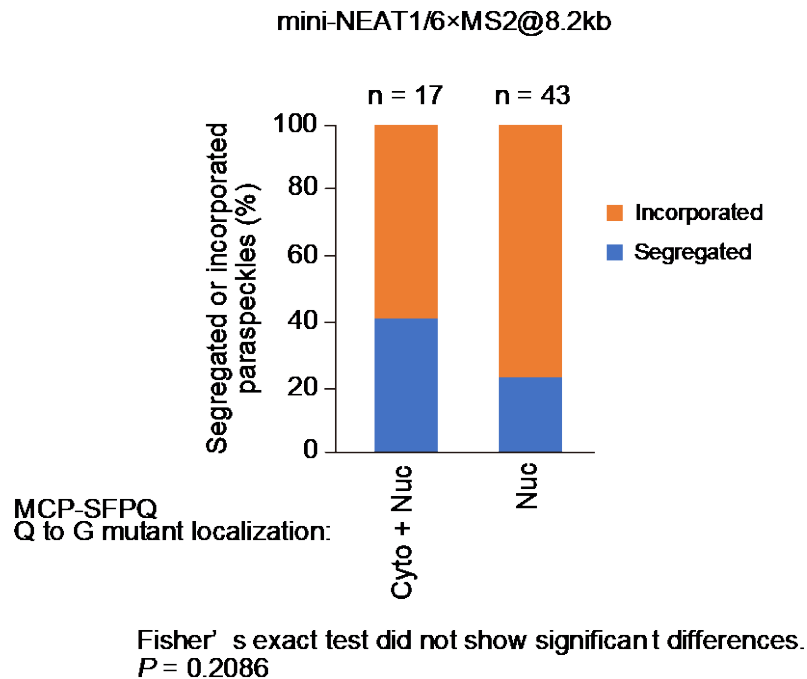
Page 8, lines 185-194: To gain insight into this recruitment mechanism, we examined how these proteins are recruited to PSs by NEAT1 RNA regions. We used NEAT1 $\Delta 4$ –8 kb/16.6–22.6 kb and $\Delta 1$ –4 kb/16.6–22.6 kb cell lines, which have additional NEAT1 regions compared to mini-NEAT1 and in which paraspeckles are segregated from nuclear speckles (Extended Data Fig. 2a,c,d). Recruitment of SFPQ and HNRNPF to PSs is restored in these cell lines compared with that in the mini-NEAT1 cell line, although the recruitment of BRG1 to PSs is not restored at all in these cell lines (Extended Data Fig. 4i,j). These findings are consistent with the above mechanism and suggest that the recruitment of SFPQ/HNRNPF and BRG1 is determined by different NEAT1 RNA domains.

Consistent with our data, a previous study investigated the mechanism of recruitment of SFPQ to target RNA sequences globally [Iida et al., iScience (2020) 23, 101325]. In that study, bioinformatics was used to search for proteins whose consensus sequences are enriched around the SFPQ binding sites. HNRNPF was then identified as the most enriched protein (personal communication with Dr. Kei Iida). Therefore, we described this point in the discussion as follows.

Page 12, lines 314-318: A bioinformatics study showed that HNRNPF binding sequences are enriched around the SFPQ binding sites and that HNRNPF plays a role in SFPQ recruitment to RNAs⁴³, which is consistent with our data (Extended Data Fig. 4e,i,j). Further studies on this multifunctional SFPQ and its recruitment mechanism will contribute to the understanding of the molecular basis of the role of the PS shell.

d) SFPQ Q-to-G mutant construct has a diffused localization in the cytoplasm and nucleus (Fig.4b), could this alter its success in rescuing the segregation of the PS?

We thank the reviewer for the careful review. We carefully examined the localization of the SFPQ Q to G mutant. In some cells, the MCP-SFPQ Q to G mutant proteins were localized not only in the nucleus but also in the cytoplasm. Therefore, we divided cells into two categories: those in which the MCP-SFPQ Q to G mutant localized predominantly to the nucleus and those in which it localized to both the cytoplasm and the nucleus. The results showed that the differences in localization were not associated with a major difference in rescue efficiency (see graph below).



Minor

points:

1. ED Fig.4 colored legend is shared between panels c and d, however it is closest to panel b and is a bit confusing. A re-arrangement of the panels could solve this.

Thank you for your careful review. As suggested, we have rearranged the panels (Extended Data Fig. 4).

2. There is a typo on the Mass Spec list of "hNEAT1 8-9.2 kb RNA pulldown" where the column is labeled as unique peptode -> should be peptide.

Thank you for indicating this typo. We have corrected it (Supplementary Table 1).

Decision Letter, first revision:

Author Rebuttal, first revision:

Our ref: NCB-A49906A

22nd June 2023

Dear Dr. Hirose,

Thank you for submitting your revised manuscript "Shell protein composition specified by NEAT1 domains dictates the formation of paraspeckles as distinct membraneless organelles" (NCB-A49906A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Cell Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

Minor revisions:

Please include a sentence to mention that there were no significant changes in alternative splicing upon rMATS analysis (as per reviewer#3 point 1).

Include a discussion acknowledging the limitations of the manual classification method.

We're happy to overrule Reviewer#3's point about training a model to distinguish between nuclear speckles and paraspeckles.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTeX)-- we can not proceed with PDFs at this stage.

We would encourage you to use the full A4 space in main Figure 2, and to that effect, more data can be moved from Extended Data Figures into main figure 2. No peer-reviewed data should be removed in the process.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Cell Biology Please do not hesitate to contact me if you have any questions.

Sincerely,

Sabrya Carim, PhD
(she/her/hers)
Associate Editor, Nature Cell Biology
Nature Portfolio

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<https://orcid.org/0000-0001-9485-1938>

Reviewer #1 (Remarks to the Author):

I want to thank the authors for the extensive revision of the manuscript. In my opinion, the manuscript in its revised form is suitable for publication in NCB now.

Reviewer #2 (Remarks to the Author):

The authors did an excellent job revising the manuscript and I support its publication in NCB.

Reviewer #3 (Remarks to the Author):

I would like to start by thanking the authors for addressing my comments and providing clarifications. The revised version of the manuscript presents a strong case for MLO immiscibility within the nucleus, particularly the one that is dictated by the shell composition of PS. I have only a few minor suggestions left for the authors:

1. The RNA-seq performed in HAP1 cells with WT or mini-NEAT1 indicated no significant changes in alternative splicing upon rMATS analysis. These findings are nevertheless interesting to mention, as it suggests U2-related spliceosomal protein association with NEAT1 does not necessarily alter normal splicing patterns. Splicing researchers would recognize this study with this finding, therefore I suggest to include a sentence in the text, but there is no need to show the data.

2. I only recognized with the newly added description into the materials and methods section that the authors followed a manual classification for the quantification of PS incorporation into NS. The manual classification of PSs as segregated or incorporated could be misleading. Therefore, I would like to suggest the authors to train a model using e.g. Ilastik in order to distinguish PS and NS, then use CellProfiler. This approach would help the presentation of results in a less biased manner. I understand this could be a bit tricky, but the manual classification should be followed only if automated options failed.

Decision Letter, final checks:

Our ref: NCB-A49906A

20th July 2023

Dear Dr. Hirose,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Cell Biology manuscript, "Shell protein composition specified by NEAT1 domains dictates the formation of paraspeckles as distinct membraneless organelles" (NCB-A49906A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-

up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: <https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication> for details).

In recognition of the time and expertise our reviewers provide to Nature Cell Biology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Shell protein composition specified by NEAT1 domains dictates the formation of paraspeckles as distinct membraneless organelles". For those reviewers who give their assent, we will be publishing their names alongside the published article.

Nature Cell Biology offers a Transparent Peer Review option for new original research manuscripts submitted after December 1st, 2019. As part of this initiative, we encourage our authors to support increased transparency into the peer review process by agreeing to have the reviewer comments, author rebuttal letters, and editorial decision letters published as a Supplementary item. When you submit your final files please clearly state in your cover letter whether or not you would like to participate in this initiative. Please note that failure to state your preference will result in delays in accepting your manuscript for publication.

Cover suggestions

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If you have any further questions, please feel free to contact me.

Best regards,

Kendra Donahue
Staff
Nature Cell Biology

On behalf of

Sabrya Carim, PhD
(she/her/hers)
Associate Editor, Nature Cell Biology
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Reviewer #1:

Remarks to the Author:

I want to thank the authors for the extensive revision of the manuscript. In my opinion, the manuscript in its revised form is suitable for publication in NCB now.

Reviewer #2:

Remarks to the Author:

The authors did an excellent job revising the manuscript and I support its publication in NCB.

Reviewer #3:

Remarks to the Author:

I would like to start by thanking the authors for addressing my comments and providing clarifications. The revised version of the manuscript presents a strong case for MLO immiscibility within the nucleus, particularly the one that is dictated by the shell composition of PS. I have only a few minor suggestions left for the authors:

1. The RNA-seq performed in HAP1 cells with WT or mini-NEAT1 indicated no significant changes in alternative splicing upon rMATS analysis. These findings are nevertheless interesting to mention, as it suggests U2-related spliceosomal protein association with NEAT1 does not necessarily alter normal splicing patterns. Splicing researchers would recognize this study with this finding, therefore I suggest to include a sentence in the text, but there is no need to show the data.

2. I only recognized with the newly added description into the materials and methods section that the authors followed a manual classification for the quantification of PS incorporation into NS. The manual classification of PSs as segregated or incorporated could be misleading. Therefore, I would like to suggest the authors to train a model using e.g. Ilastik in order to distinguish PS and NS, then use CellProfiler. This approach would help the presentation of results in a less biased manner. I understand this could be a bit tricky, but the manual classification should be followed only if automated options failed.

Final Decision Letter:

Dear Dr Hirose,

I am pleased to inform you that your manuscript, "Shell protein composition specified by lncRNA NEAT1 domains dictates the formation of paraspeckles as distinct membraneless organelles", has now been accepted for publication in Nature Cell Biology- Congratulations!

Thank you for sending us the final manuscript files to be processed for print and online production, and for returning the manuscript checklists and other forms. Your manuscript will now be passed to our production team who will be in contact with you if there are any questions with the production quality of supplied figures and text.

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With kind regards,

Sabrya

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