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Paraspeckles are constructed as block copolymer micelles

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5th Jan 2021

Re: EMBOJ-2020-107270
Paraspeckles are constructed as block copolymer micelles through microphase separation

Dear Prof. Hirose,

Thank you again for submitting your manuscript for consideration by The EMBO Journal. Please excuse the delay in communicating this decision to you, which is due to delayed referee responses on account of the pandemic and the recent holiday season. We have now however received two referee reports on your study, which are included below for your information. Given the referees' comments, we would like to invite you to prepare and submit a revised manuscript.

As you will see, the reviewers are overall positive and appreciate the additional insights and refined model for paraspeckle structure and formation. Nonetheless both referees raise several points that should be addressed and discussed in a revised version. Referee #3 in particular points out several aspects that should be discussed in further detail and in the context of previous studies. In addition, you have chosen to separate reporting the theoretical model and the experimental validation into two studies. While we do not find that this is an issue that would preclude publication per se, you should however provide additional information on the model and the assumptions derived from it, in the study submitted here, as is also indicated by referee #3. In addition, please carefully consider and reply to all referee comments in a detailed point-by-point response when submitting the revised manuscript.

Please note that it is our policy to allow only a single round of major revision. We realize that lab work worldwide is currently affected by the COVID-19/SARS-CoV-2 pandemic and that an experimental revision may be delayed. If you foresee any potential issues that may significantly delay a revision, please contact us to discuss this. Please also feel free to contact me should you have any other questions.

Thank you again for the opportunity to consider your work for publication. I look forward to receiving your revised manuscript.

Kind regards,

Stefanie

Stefanie Boehm
Editor
The EMBO Journal

Referee #1:

The manuscript by Yamazaki et al. reports an in-depth analysis of domain contributions of the lncRNA NEAT1 to paraspeckle fine structure. It then explores the ability of a recent coarse-grained biophysical model of the group (available as preprint) to understand this data. They then do a number of follow-up experiments to test predictions of the model and show that their observations in cells are in excellent agreement with these data. I found myself designing experiments along the way that would test certain aspects of the model, and I was not disappointed; the authors did them all. This manuscript was a pleasure to read and provides very clear insights into the mechanisms underlying paraspeckle fine structure, changes thereof as a function of lncRNA expression levels, and previous and current observations of the behaviors of certain mutants. Importantly, the manuscript raises awareness to the fact that natural biomolecular condensates are not simple disordered liquids, a simplifying assumption that has been prevalent at the beginnings of the phase separation field. It is time to discover how the complexity of polymeric biomolecules gives rise to many different internal structures. This manuscript is a beautiful example for this.

The manuscript is very clearly written, and the conclusions are warranted by the data. I only have two minor comments below:

The authors say that the D5'/D3' mutant cells form "large spherical condensates with disordered internal structures". The microscopy images don't look like there is actually mixing between the two colors; they still occupy two different spaces, but they are now not organized into a core and a shell. This should be addressed, and the implications explained in the manuscript.

The authors mention that the current model may be interesting in the context of the recently reported numerous possible functions of paraspeckles, but they do not address any connections. I find it very important for this manuscript to discuss the functional implications of the tri-block copolymer model for paraspeckle function. What is a possible reason for the micellar structure? Why does the outer corona of paraspeckles need to be soluble? Is there a reason why a limitation of the size of paraspeckles would be functionally useful?

Referee #2 (Report for Author)

In this work, the authors take on the problems of (a) deriving a mechanistic basis for the distinctive spatial organization of NEAT1_2 in paraspeckles; (b) sorting out the determinants of paraspeckle shape; and (c) providing a soft matter physics framework that explains the transition from spherical to cylindrical structures. The work is very interesting and highly relevant. There are issues that arose during review that would benefit from being addressed in a suitably revised version.

1. On pg. 4, the authors write "These characteristic shapes and internal organization of paraspeckles are distinct from those of condensates formed by LLPS, which are usually spherical and have non-ordered internal structures." This assertion flies in the face of what we know about condensates and core-shell architectures that are realized via phase separation. Please see: https://doi.org/10.1016/j.molcel.2019.06.044 - for aspherical condensates,
https://www.pnas.org/content/early/2019/03/28/1821038116 - for the observation of core-shell architectures with simple protein-RNA mixtures,
http://iopscience.iop.org/article/10.1088/1367-2630/aab8d9 - for simple systems and explanations of core-shell behavior, http://jcs.biologists.org/content/130/24/4180 - for clear demonstration of multi-layered organization of nuclear speckles, and
http://www.cell.com/cell/fulltext/S0092-8674(16)30492-5 - for the demonstration that nucleolar substructures can be explained as the result of coexisting phases of viscous and viscoelastic liquids.

2. Conclusions in Figure 1 rest on the results of RNA FISH experiments. Were the choices for the deletion constructs made based on the FISH probes that are available? And how does one rule out the possibility that random localization and / or not observing localization to the shell derives not from a localization issue, but from a deficit in recognition? This is an earnest question and it raises the corollary of whether one can confirm the inferences from deletions using mutagenesis.

3. The data in Figure 3 are interpreted as being supportive of a random distribution of the double deletion mutants. Two questions arise from the images presented. First, the merged image shows non-overlapping organization of the 3' and 5' ends. This is to be expected based on topological considerations. However, it would help to know what the expected organization would be for suitable null model. Second, the histograms parse the occupancy in core vs. shell. How is the core and shell delineated in paraspeckles that do not show a core-shell organization?

4. The triblock model is very interesting, and details of this model are offered in a preprint. The model rests on a few assumptions, the most noteworthy being that the middle region of NEAT1_2, when bound to PSPs will behave like an effectively hydrophobic system. A more precise statement would be that protein associated middle regions will associate preferentially with one another over solvent, whereas the 3' and 5' ends will preferentially interact with the solvent than with themselves or PSPs. Can this be tested directly? A strategy for getting at the relative hydrophobicities of PSP bound middle regions and the potentials of mean force for interactions between PSP bound middle regions would be helpful. Perhaps the work of Feric et al., cited above might provide some inspiration.

5. It would help to clarify what the authors mean by excluded volume interactions in the current context. Do they mean steric overlap or do they mean the excluded volume also referred to as the effective solvation volume, which one would calculate as the integral of the Meyer-f function?Please see the work of Harmon et al., cited above. If they mean the latter, then it does come down

to differential solvation effects, which are presumably lost in the deletion constructs. Similar observations have recently been reported by the Banerjee group - see: https://doi.org/10.1073/pnas.1922365117.

6. Since the "hydrophobicity" of the middle region is governed by protein-RNA interactions, it would be useful to know whether the protein levels increase as the expression levels of NEAT1_2 increases due to proteosome inhibition. Without these data (which I may have missed) one cannot be sure of the congruence between theory and experiment. Also, there is a conflation between the application of a thermodynamic framework and the actual experiments where the transcriptional rates are increased. Is it implicitly assumed that the rate of transcription, as far as PS assembly is concerned, is at steady state, so the only thing that matters is the inhibitor dose specific steady state level of NEAT1_2 and the proteins that associate with the middle region?

5. While this might, to some extent, be outside the scope of the current MS, the question is how does the Flory-Huggins style theory compare with other attempts to describe sphere to cylinder transitions in micellar systems? The work of May and Ben-Shaul (see http://dx.doi.org/10.1021/jp003021o) comes to mind. This theory makes specific predictions regarding the sizes of the end caps indicating that the diameter of the caps should be larger than the internal diameter of the cylinder. Are similar predictions made by the current theory?

6. The discussion introduces the interaction between RBPs and the 5' and 3' ends. This again raises the question of why / how the apparent hydrophobicity of the 5' and 3' ends, bound to proteins, would be significantly different from the hydrophobicity of the middle region, which should also have proteins bound to them. There is a symmetry breaking operation that was not discussed or measured.

Referee #3:

There is much excitement by the prevalence of liquid-like assemblies as an organizational mechanism within the cell. The formation of these mebraneless structures is often attributed to LLPS due to the similarities to phase transition when (partially) reconstituted in vitro using a select group of proteins. In this manuscript, the authors study nuclear structure paraspeckles as an example of these liquid forming system. They apply an interesting combination of experiment and theory to deduce the mechanism of formation. Interestingly, they find that paraspeckels, are not, in fact, the result of LLPS (as suggested by Peng and Weber 2019), but, instead, are polymer micelles. The finding is interesting, but I feel that the authors are missing an opportunity that would enhance the significance of their findings.

The authors use the term "microphase separation" to describe the formation of paraspeckles. This term strikes me as an oxymoron because the discontinuous behavior defining phase transitions can only occur in an infinite system, which is the opposite of the finding in this manuscript that finite-sized assemblies are preferred. Rather than dismiss this fine work on a semantic point, I suggest the authors take this chance to discuss the biological implications of this distinction. In particular, in

many cases cells require a mechanism to control the size of phase separated structures that would otherwise grow without bound. Several mechanisms for size limitation have previously been reported in the literature. Examples include the elastic energy of the cytoskeleton (experiments by Dufresne and coworkers 2018, 2020 and theory by Wei et al PRL 2020), kinetic limitations on coarsening, stoichiometric constraints, and multiple nucleation sites (for example, ribosomal DNA gene arrays that initiate the formation of nucleoli). This manuscript adds micellization to this list, which perhaps provides a tighter control over sizes, but that control is limited to molecular dimensions. Going into finer detail, micellization provides separate mechanisms for size control. In this case, the authors find that the excluded volume/polymer entropy in the shell provides a repulsive force that favors a curved surface. A separate mechanism, which the authors seem to have considered but rejected, is the stretching entropy of polymers in the core (for example: Phan and Schmit, Biophys. J. 2020). Given the rush in the field to label everything as LLPS, I strongly encourage the authors to provide a more nuanced discussion of the similarities and differences between LLPS and micellization, with particular attention on how the finite size of the micelle provides a more gradual transition than a phase transition and a discussion of the advantages and limitations of these various mechanisms for size control.

I have some reservations about the authors' decision to publish the experiment and theoretical results in separate manuscripts, as these methods/findings are intimately intertwined. I appreciate that the audiences for the two sets of results are different, but in this case it is not possible to judge their findings without an assessment of the theory. I have taken a cursory look at the theory preprint and am satisfied with the physical model, but I have not given it the attention to review it in detail. At a minimum, I feel that the authors need to do a better job at explaining the physical contributions that enter the model. I feel a figure would be helpful here. Perhaps something similar to Fig. 1 of Yamamoto 2020, but labeled to emphasize energetic contributions rather than physical dimensions.

The free energy expression appearing in this manuscript needs further explanation. The first two terms are self-evident, but the third and fourth term are less obvious. The third term has the appearance of a difference in chemical potentials, but the kinetic factors in the logarithm differ from the concentrations that I would expect. Also, a brief justification for the Gibbs factor in the final term would be appropriate.

The authors should comment on the roles of specific NEAT1 RNA-binding proteins within the scaffold model and within their Flory-Huggins model.

The experiments were done exclusively in haploid HAP1 cells which grow significantly slower than diploid and aneuploid cells. These cells posit reduction in absolute gene expression levels and cell size. Their nuclear compartmentalization is certainly affected by these facts. The authors should provide clear justification for their model reflecting proteomic and RNA abundance in diploid cells.

Responses to the Reviewers' comments

The authors appreciate 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 revisions. Our point-by-point responses to the comments are provided below in red text.

Referee #1:

The manuscript by Yamazaki et al. reports an in-depth analysis of domain contributions of the lncRNA NEAT1 to paraspeckle fine structure. It then explores the ability of a recent coarse-grained biophysical model of the group (available as preprint) to understand this data. They then do a number of follow-up experiments to test predictions of the model and show that their observations in cells are in excellent agreement with these data. I found myself designing experiments along the way that would test certain aspects of the model, and I was not disappointed; the authors did them all. This manuscript was a pleasure to read and provides very clear insights into the mechanisms underlying paraspeckle fine structure, changes thereof as a function of lncRNA expression levels, and previous and current observations of the behaviors of certain mutants. Importantly, the manuscript raises awareness to the fact that natural biomolecular condensates are not simple disordered liquids, a simplifying assumption that has been prevalent at the beginnings of the phase separation field. It is time to discover how the complexity of polymeric biomolecules gives rise to many different internal structures. This manuscript is a beautiful example for this.

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Thank you for pointing out this important issue. We think that there are two interpretations for this observation. First, our previous report showed that the NEAT1_2

RNPs form bundles (West et al., JCB (2016) 214, 817), where each 5' terminal, middle and 3' terminal domains of NEAT1_2 self-assemble. Thus, our observation can be interpreted that even when the 5' and 3' terminal regions are truncated in the Δ5'/Δ3' mutant, NEAT1_2 still bundles and moves around within the paraspeckles. As a result, the NEAT1_2 terminal regions were detected as clusters (Fig 3B). Second, when A and C blocks enter the hydrophobic core of the paraspeckles they tend to gather within the core because of their hydrophilicity. We have now added these points in the **Results** of the manuscript (page 8, lines 15–21):

In contrast to the highly ordered core-shell NEAT1_2 organization of the paraspeckles in WT cells, SRM observations clearly showed that the core-shell organization of the NEAT1_2 was totally lost in the Δ5'/Δ3' mutant cells (Fig 3B and C). In addition, the 5' and 3' terminal regions of NEAT1_2 occupied different spaces within the paraspeckle, which might reflect the bundles of NEAT1_2 RNPs (West et al., 2016) and/or the hydrophilic nature of the NEAT1_2 5' and 3' regions to gather within the hydrophobic core.

We also changed “large spherical condensates with disordered internal structures” to “large spherical condensates without core-shell architectures” (page 13, lines 14–15).

The authors mention that the current model may be interesting in the context of the recently reported numerous possible functions of paraspeckles, but they do not address any connections. I find it very important for this manuscript to discuss the functional implications of the tri-block copolymer model for paraspeckle function. What is a possible reason for the micellar structure? Why does the outer corona of paraspeckles need to be soluble? Is there a reason why a limitation of the size of paraspeckles would be functionally useful?

Thank you for pointing out this important issue. We now mention several functional implications of the triblock copolymer micelle model in the **Discussion** of the manuscript as provided below (page 19, line 13 – page 20, line 7).

For example, it has been reported that paraspeckles that form in close proximity to the NEAT1 gene locus are released from the locus and distribute widely to the nucleoplasm (Mao et al., 2011), which is likely to be important for genome-wide targeting of the paraspeckles to chromatins (Bonetti et al., 2020; Cai et al., 2020; Li et al., 2017). Herein,

we demonstrated that micellization limits the size of the paraspeckle and increases the number of the paraspeckles. Moreover, spherical Δ5'/Δ3' mutant paraspeckles form a few large condensates per nucleus, which are presumably localized near NEAT1_2 transcription sites. Thus, these features of the size and the number of the paraspeckles likely contribute to the genome-wide targeting of the paraspeckles to chromatins and facilitate interactions with other nucleoplasmic complexes. In addition, the hydrophilic surface of the micellar structure is important for dispersion or motility of paraspeckles in the nucleoplasm by solubilizing the paraspeckles in the nucleoplasm. A further possible importance of micellization of the paraspeckle is that fusion of the paraspeckles is repressed. Because NEAT1 is a highly expressed lncRNAs and the paraspeckles globally interact with chromatins, formation of too large condensates and frequent coalescences of paraspeckles might influence various nuclear events such as chromatin organization. These features of micelles might minimize such adverse effects. A recent report of the CasDrop system has shown that fusion of condensates formed by LLPS reorganizes nuclear chromatin architectures (Shin et al., 2018). Therefore, there may be such an influence if coalescence of the paraspeckles frequently occurs. Another possible importance is that the micellar structure of the paraspeckle may be suitable for the sequestration of specific RNA molecules to or kinetic enhancement of pre-miRNA processing onto the surface of the paraspeckles (Hirose et al., 2014; Imamura et al., 2014; Jiang et al., 2017). Many paraspeckles produced by micellization are also expected to increase the surface area of the paraspeckles, which would be beneficial for efficient sequestration and/or chromatin interaction. Hence, it would be interesting to investigate these possibilities in the future.

Referee #2

In this work, the authors take on the problems of (a) deriving a mechanistic basis for the distinctive spatial organization of NEAT1_2 in paraspeckles; (b) sorting out the determinants of paraspeckle shape; and (c) providing a soft matter physics framework that explains the transition from spherical to cylindrical structures. The work is very interesting and highly relevant. There are issues that arose during review that would benefit from being addressed in a suitably revised version.

1. On pg. 4, the authors write "These characteristic shapes and internal organization of paraspeckles are distinct from those of condensates formed by LLPS, which are usually

spherical and have non-ordered internal structures." This assertion flies in the face of what we know about condensates and core-shell architectures that are realized via phase separation. Please see: https://doi.org/10.1016/j.molcel.2019.06.044 - for aspherical condensates, https://www.pnas.org/content/early/2019/03/28/1821038116 - for the observation of core-shell architectures with simple protein-RNA mixtures, http://iopscience.iop.org/article/10.1088/1367-2630/aab8d9 - for simple systems and explanations of core-shell behavior, http://jcs.biologists.org/content/130/24/4180 - for clear demonstration of multi-layered organization of nuclear speckles, and http://www.cell.com/cell/fulltext/S0092-8674(16)30492-5 - for the demonstration that nucleolar substructures can be explained as the result of coexisting phases of viscous and viscoelastic liquids.

Thank you for your suggestion. We have changed the sentence as follows and in accordance with the suggestion (page 4, lines 27–32):

These characteristic shapes and the internal organization of paraspeckles are distinct from those of the typical condensates formed by LLPS that are spherical and have non-ordered internal structures; although, phase-separated condensates with core-shell or multi-layered architectures have also been reported (e.g., Boeynaems et al., 2019; Fei et al., 2017; Feric et al., 2016; Harmon et al., 2018; Powers et al., 2019).

2. Conclusions in Figure 1 rest on the results of RNA FISH experiments. Were the choices for the deletion constructs made based on the FISH probes that are available? And how does one rule out the possibility that random localization and / or not observing localization to the shell derives not from a localization issue, but from a deficit in recognition? This is an earnest question and it raises the corollary of whether one can confirm the inferences from deletions using mutagenesis.

Additionally, we prepared several FISH probes according to the deletion mutants. We do not believe in bias due to deficit in recognition because data obtained with probes detecting truncated ends were consistent between two independent methods, RNA-FISH (using optical microscopy) and EM-ISH (using electron microscopy), with different probe sets. These methods are also different in terms of sample preparation: penetration of probes on whole objects (RNA-FISH) and in a surface-reaction on sectioned objects (EM-ISH). Furthermore, in EM-ISH, we also used the method to

remove proteins from the ultrathin-sections by protease treatment to improve accessibility of the probes to the NEAT1_2 lncRNAs. Thus, our interpretations from the experiments are reasonable.

3. The data in Figure 3 are interpreted as being supportive of a random distribution of the double deletion mutants. Two questions arise from the images presented. First, the merged image shows non-overlapping organization of the 3' and 5' ends. This is to be expected based on topological considerations. However, it would help to know what the expected organization would be for suitable null model.

We have answered a similar question raised by reviewer #1 (see responses to the comments raised by reviewer #1). We think that there are two possibilities for this observation. First, each of the NEAT1_2 ends forms bundles and the bundled 5' and 3' ends of NEAT1_2 are visualized by FISH as clusters within the paraspeckles. Second, the A and C blocks (5' and 3' NEAT1_2 ends) tend to gather together because of their hydrophilicities when they enter the paraspeckle core. We have added the following sentences on these points in the **Results** of the manuscript (page 8, lines 15–21):

In contrast to the highly ordered core-shell NEAT1_2 organization of the paraspeckles in WT cells, SRM observations clearly showed that the core-shell organization of the NEAT1_2 was completely lost in the Δ5'/Δ3' mutant cells (Fig 3B and C). In addition, the 5' and 3' terminal regions of NEAT1_2 occupied different spaces within the paraspeckle, which might reflect the bundles of NEAT1_2 RNPs (West et al., 2016) and/or the hydrophilic nature of the NEAT1_2 5' and 3' regions to gather within the hydrophobic core.

Second, the histograms parse the occupancy in core vs. shell. How is the core and shell delineated in paraspeckles that do not show a core-shell organization?

Thank you for pointing this out. We defined the core/shell or the outer/middle/inner layers of paraspeckles in electron microscopic images by dividing the surface area into two or three domains, each of which has the same area, respectively, as used in our previous study (Souquere et al., MBoC (2010) 21, 4020). This method was used to analyze all mutants used in this study. We have added the following sentences on this explanation in the **Quantification and statistical analysis of the Materials and Methods** (page 26, line 31– page 32, line 2):

The procedure to count the population of gold-particles in core/shell domains or outer/middle/inner layers has been described previously (Souquere et al., 2010). Briefly, the surface area of the paraspeckle was divided into two (for core/shell domains) or three (for the outer/middle/inner layers) domains that have the same area and the number of gold-particles within each of these areas was counted.

4. The triblock model is very interesting, and details of this model are offered in a preprint. The model rests on a few assumptions, the most noteworthy being that the middle region of NEAT1_2, when bound to PSPs will behave like an effectively hydrophobic system. A more precise statement would be that protein associated middle regions will associate preferentially with one another over solvent, whereas the 3' and 5' ends will preferentially interact with the solvent than with themselves or PSPs. Can this be tested directly? A strategy for getting at the relative hydrophobicities of PSP bound middle regions and the potentials of mean force for interactions between PSP bound middle regions would be helpful. Perhaps the work of Feric et al., cited above might provide some inspiration.

Thank you for your thoughtful suggestion. Our previous study showed that the NEAT1_2 middle domain, corresponding to the B block, is the preferential binding region for NONO and SFPQ proteins, which are essential for paraspeckle assembly (Naganuma et al., (2012) EMBO J 31, 4020; Sasaki et al., PNAS (2009) 106, 2525; Yamazaki et al., Mol Cell (2018) 70, 1038). These proteins form hetero- or homo-oligomers with other proteins of the DBHS family (NONO, SFPQ and PSPC1) with high affinity (Huang et al., JBC (2018) 293, 6593; Knott et al., NAR (2016) 44, 3989; Lee et al., NAR (2015) 43, 3826; Passon et al., PNAS (2012) 109, 4846). In addition, the crystal structure of the NONO/PSPC1 heterodimer, a typical DBHS protein dimer, revealed that it forms a unique oligomerized crystal in solution, which would be the structural basis for the paraspeckle core. Importantly, dimerization of DBHS proteins is mediated mainly by hydrophobic interactions (Passon et al., PNAS (2012) 109, 4846). Thus, we added this point in the **Results** of our manuscript (page 10, lines 11–12). In contrast, the mechanism of hydrophilic shell formation remains unresolved. Currently, we are searching for PSP(s) that specifically associate with the 5' and 3' terminal regions of NEAT1_2 to form the shell. Once the shell-forming PSPs are identified, analyses of biophysical properties of these proteins should provide more mechanistic insights into the formation of the core-shell structure.

5. It would help to clarify what the authors mean by excluded volume interactions in the current context. Do they mean steric overlap or do they mean the excluded volume also referred to as the effective solvation volume, which one would calculate as the integral of the Meyer-f function? Please see the work of Harmon et al., cited above. If they mean the latter, then it does come down to differential solvation effects, which are presumably lost in the deletion constructs. Similar observations have recently been reported by the Banerjee group - see: https://doi.org/10.1073/pnas.1922365117.

We define the excluded volume by the volume integral of the Meyer-f function as many treatments of polymer solutions and it represents differential solvation effects. To clarify this point, we have added the following sentence in the **Triblock copolymer micelle model, Materials and Methods** (page 23, lines 22–25)**:**

The magnitudes of the latter interactions are represented by the excluded volumes of A units v_{A} and C units v_C, which take into account the entropy of mixing of polymer units and solvent molecules and the interaction energy difference between the different molecular species and the same molecular species (Doi 1996).

We also thank reviewer #2 for providing the relevant reference.

6. Since the "hydrophobicity" of the middle region is governed by protein-RNA interactions, it would be useful to know whether the protein levels increase as the expression levels of NEAT1_2 increases due to proteosome inhibition. Without these data (which I may have missed) one cannot be sure of the congruence between theory and experiment.

Our previous report showed that MG132 treatment enhances NEAT1_2 transcription by transcriptional activation through the NEAT1 promoter region but does not increase the expression levels of multiple paraspeckle core proteins (Hirose et al., MBoC (2014) 25, 169). To clarify this point, we added a sentence (light blue) in the **Results** as provided below (page 13, lines 20–25).

We measured the Sx values of paraspeckles by SRM when changing the NEAT1_2 expression levels using various concentrations of a transcriptional activator of NEAT1_2, the proteasome inhibitor MG132, which activates NEAT1 transcription without changing

the expression levels of essential PSPs, whereas MG132 (5 μM) was used in previous experiments as shown in Figures 1–3 (Hirose et al., 2014).

Also, there is a conflation between the application of a thermodynamic framework and the actual experiments where the transcriptional rates are increased. Is it implicitly assumed that the rate of transcription, as far as PS assembly is concerned, is at steady state, so the only thing that matters is the inhibitor dose specific steady state level of NEAT1_2 and the proteins that associate with the middle region?

As noted by reviewer #2, our theory assumes that the transcription rate is at steady state and the only thing that matters is the inhibitor specific steady state rate (not level) of NEAT1_2 transcription and the proteins that associate with the middle region. We have added the following paragraphs to **Triblock copolymer micelle model, Materials and Methods** (page 24, line 25 – page 25, line 26)**:**

The growth of the paraspeckle was analyzed by using the Master equation

$$\frac{d}{dt}q_1(t) = -J_1(t) \#(1)$$

$$\frac{d}{dt}q_n(t) = -J_n(t) + J_{n-1}(t) \#(2)$$

with $n = 2,3,\cdots$, and

$$J_n(t) = k_0\phi_p q_n(t) - k_0 e^{-\frac{F_n+F_1-F_{n+1}}{k_BT}}(n+1)q_{n+1}(t) + k_{\mathrm{tx}}q_n(t), \#(3)$$

where $q_n(t)$ is the probability that the paraspeckle is composed of n NEAT1_2 transcripts, and $J_n(t)$ is the flux of the probability. The first term on the right side of eq. (3) is the rate that a NEAT1_2 transcript associates with the paraspeckle from solution. The second term is the rate that a NEAT1_2 in the paraspeckle dissociates and the third term is the rate that a nascent NEAT1_2 transcript associates with the paraspeckle. The factor $n+1$ in the second term accounts for the fact that any of the $n+1$ NEAT1_2 transcripts dissociate with equal probability. k_0 is the rate constant that accounts for the association of NEAT1_2 with the paraspeckle. ϕ_p is the volume fraction of NEAT1_2 in solution. F_n is the free energy $F_n(\alpha)$, which has been already minimized with respect to α. k_{B} is the Boltzmann constant, T is the absolute temperature and k_{tx} is the rate constant that accounts for the production of nascent NEAT1_2 transcripts. The first and second terms of eq. (3) are determined so that they satisfy the

detailed balance when transcription is suppressed, $k_{\mathrm{tx}} \to 0$. In the following, we set $\phi_p \to 0$ because most NEAT1_2 transcripts are incorporated into paraspeckles.

In the steady state, $J_n(t) \to 0$, the solution of eqs. (1) and (2) has the form

$$q_n = \frac{1}{Z_{\mathrm{st}}} e^{-\frac{\mathcal{F}_n}{k_B T}}, \#(4)$$

where $\mathcal{F}_n$ is the effective free energy of the paraspeckle composed of n NEAT1_2 transcripts

$$\mathcal{F}_n = F_n - nF_1 - (n-1)k_{\mathrm{B}}T \log\frac{k_{\mathrm{tx}}}{k_0} + k_{\mathrm{B}}T \log(n!) \#(5)$$

and Z_{st} is the effective partition function

$$Z_{\mathrm{st}} = \sum_n e^{-\frac{\mathcal{F}_n}{k_B T}} . \#(6)$$

The most probable number n of NEAT1_2 transcripts in the steady state is derived by minimizing the effective free energy $\mathcal{F}_n$. Our model predicts the number n of transcripts in a paraspeckle, the fraction α of A blocks in the shell, the radius of the paraspeckle in the steady state as a function of the transcription rate k_{tx}, and the number of segments in the A blocks.

7. While this might, to some extent, be outside the scope of the current MS, the question is how does the Flory-Huggins style theory compare with other attempts to describe sphere to cylinder transitions in micellar systems? The work of May and Ben-Shaul (see http://dx.doi.org/10.1021/jp003021o) comes to mind. This theory makes specific predictions regarding the sizes of the end caps indicating that the diameter of the caps should be larger than the internal diameter of the cylinder. Are similar predictions made by the current theory?

Thank you for raising this interesting comment. This comment may be important for cylindrical paraspeckles that have a relatively small aspect ratio for cases where the free energy caused by excluded volume interactions between A units and those between C units is very large. However, our experiments, including our unpublished tomographic studies and a high number of longitudinal sections of the paraspeckles by EM, suggest that the cap of cylindrical paraspeckles is well approximated by a hemisphere. It might be relevant to the fact that our theory and experiments treat polymer micelles, while May and Benshaul treat micelles as small molecules. We are currently preparing a model to predict the sphere-to-cylinder morphological transitions by using this approximation. We would like to add this discussion to the preparing

manuscript of the latter theory.

8. The discussion introduces the interaction between RBPs and the 5' and 3' ends. This again raises the question of why / how the apparent hydrophobicity of the 5' and 3' ends, bound to proteins, would be significantly different from the hydrophobicity of the middle region, which should also have proteins bound to them. There is a symmetry breaking operation that was not discussed or measured.

The NEAT1_2 sequence is not uniform but has specific RNA domains containing sequences and/or secondary structures that serve as binding sites of partner RNA-binding proteins. The NEAT1_2 middle domain has RNA sequences and/or secondary structures that interact with NONO, SFPQ and FUS proteins essential for paraspeckle assembly (Yamazaki et al., Mol Cell (2018) 70, 1038). In contrast, the 5' and 3' terminal domains of NEAT1_2 likely interact with the RBPs to give hydrophilicity to these domains. Identification of the partner proteins represents the next step and the experiments for this purpose are ongoing in our laboratory. To clearly present this point, we have added text (light blue) in the **Discussion** of the manuscript (page 16, lines 10–13):

Thus, it is likely that proteins interacting with RNA sequences and/or secondary structures in the 5' and 3' shell-forming domains determine the hydrophilic nature of these domains and thus the localization of these domains in the shell (Fig 7B).

Referee #3:

There is much excitement by the prevalence of liquid-like assemblies as an organizational mechanism within the cell. The formation of these membraneless structures is often attributed to LLPS due to the similarities to phase transition when (partially) reconstituted in vitro using a select group of proteins. In this manuscript, the authors study nuclear structure paraspeckles as an example of these liquid forming system. They apply an interesting combination of experiment and theory to deduce the mechanism of formation. Interestingly, they find that paraspeckles, are not, in fact, the result of LLPS (as suggested by Peng and Weber 2019), but, instead, are polymer micelles. The finding is interesting, but I feel that the authors are missing an opportunity that would enhance the significance of their findings.

The authors use the term "microphase separation" to describe the formation of paraspeckles. This term strikes me as an oxymoron because the discontinuous behavior defining phase transitions can only occur in an infinite system, which is the opposite of the finding in this manuscript that finite-sized assemblies are preferred. Rather than dismiss this fine work on a semantic point, I suggest the authors take this chance to discuss the biological implications of this distinction. In particular, in many cases cells require a mechanism to control the size of phase separated structures that would otherwise grow without bound. Several mechanisms for size limitation have previously been reported in the literature. Examples include the elastic energy of the cytoskeleton (experiments by Dufresne and coworkers 2018, 2020 and theory by Wei et al PRL 2020), kinetic limitations on coarsening, stoichiometric constraints, and multiple nucleation sites (for example, ribosomal DNA gene arrays that initiate the formation of nucleoli). This manuscript adds micellization to this list, which perhaps provides a tighter control over sizes, but that control is limited to molecular dimensions.

Thank you for providing these very important points. We have discussed the differences and similarities between microphase separation and micellization. In a restricted sense, microphase separation often refers to the formation of a pattern of microphases across the entire system and occurs discontinuously across the spinodal line, whereas, in micellization, micelles assemble locally and the number of micelles increases continuously as the concentration of amphiphiles increase (Mai and Eisenberg, 2012; Safran, 2003). In a broad sense, micellization can be regarded as a type of microphase separation because the assemblies have an optimal size and shape in both processes. We have decided to use both terms partly because using the term “micellization” alone may cause confusion or a misunderstanding to readers working in biological research fields. This is because in a biological context micellization is used mainly for micellization of phospholipids. Additionally, we think that a comparison between macroscopic phase separation and microphase separation would be conceptually helpful for readers. We added micellization in the revised manuscript including abstract, Figures 4B and 7B, and keywords of the manuscript, and microphase separation was substituted or removed in some cases including the title. Moreover, we claimed “Thus, paraspeckles are most likely formed through micellization, a type of microphase separation” in the **Discussion** of the manuscript (page 15, lines 9–10). As suggested, we have also described the biological implications from features of micellization as presented below (page 15, lines 23–33).

Cells usually require a mechanism to control the size of phase-separated structures that would otherwise grow without bound by coarsening and coalescence. Several mechanisms for size limitation, such as the elastic energy of the cytoskeleton, kinetic limitations on coarsening, stoichiometric constraints, multiple nucleation sites and emulsification, have been reported (Berry et al., 2018; Berry et al., 2015; Brangwynne et al., 2009; Dar and Pappu, 2020; Ranganathan and Shakhnovich, 2020; Rosowski et al., 2020; Sabari et al., 2020; Style et al., 2018; Wei et al., 2020). Our study shows that paraspeckles employ a different mechanism – micellization to control their size. Micellization likely provides a tighter control over sizes, which are defined by the block sizes, when compared with that of other reported mechanisms, and this control is limited to molecular dimensions.

Going into finer detail, micellization provides separate mechanisms for size control. In this case, the authors find that the excluded volume/polymer entropy in the shell provides a repulsive force that favors a curved surface. A separate mechanism, which the authors seem to have considered but rejected, is the stretching entropy of polymers in the core (for example: Phan and Schmit, Biophys. J. 2020).

Stretching free energy suppresses the growth of paraspeckles and drives sphere-to-cylinder morphological transitions. This free energy scales as $N_B^{-1/3}$ with increasing number N_B of units in B blocks and is usually neglected in many treatments of polymer micelles (see Halperin and Alexander, 1989 and Semenov et al., 1995). We therefore excluded this contribution for the first approximation. However, as suggested by reviewer #3, the number of units of B blocks in NEAT1_2 may be sufficiently small such that the stretching free energy contributes significantly. We therefore summarized the results of the model calculations when taking into account the contributions of the stretching free energy in Figure 5EV. We also added a paragraph shown below in **Triblock copolymer micelle model, Materials and Methods** (page 24, lines 5–16)**.**

Polymer blocks in the core stretch as the radius of the paraspeckle increases and this increases the stretching free energy (Halperin and Alexander, 1989; Semenov et al., 1995; Zhulina et al., 2005). The stretching free energy also contributes to the structure of paraspeckles (see also Fig EV5). The stretching free energy of B blocks decreases the radius of paraspeckles (Fig EV5C) because this energy term decreases the number of transcripts in paraspeckles (Fig EV5D). The stretching free energy therefore plays a

similar role to the free energy due to the excluded volume interactions between A blocks and those between C blocks in the shell. The stretching free energy is small for cases in which B blocks are relatively long (Halperin and Alexander, 1989; Semenov et al., 1995). For simplicity, we thus excluded the stretching free energy in the calculation presented in the manuscript; although, this energy term is significant for cases where the B blocks are relatively short.

We have also added a paragraph in the **Discussion** as below (page 18, lines 3–9).

As introduced above, the elastic free energy in the B blocks may contribute to the structure and size of the paraspeckle. Thus, we constructed a different version of the triblock copolymer micelle model of the spherical paraspeckle by also taking into account the elastic free energy in the B blocks (Fig EV5A). The data generated from this model gave essentially the same results as the data from the original triblock copolymer model (Fig EV5B-E) (see the Materials and Methods for details). Thus, our model represents and predicts features of the paraspeckle effectively.

Given the rush in the field to label everything as LLPS, I strongly encourage the authors to provide a more nuanced discussion of the similarities and differences between LLPS and micellization, with particular attention on how the finite size of the micelle provides a more gradual transition than a phase transition and a discussion of the advantages and limitations of these various mechanisms for size control.

Thank you for your suggestion. We have added these points in the **Discussion** of the manuscript as below (page 15, line 23 – page 16, line 6).

Cells usually require a mechanism to control the size of phase-separated structures that would otherwise grow without bound by coarsening and coalescence. Several mechanisms for size limitation, such as the elastic energy of the cytoskeleton, kinetic limitations on coarsening, stoichiometric constraints, multiple nucleation sites and emulsification, have been reported (Berry et al., 2018; Berry et al., 2015; Brangwynne et al., 2009; Dar and Pappu, 2020; Ranganathan and Shakhnovich, 2020; Rosowski et al., 2020; Sabari et al., 2020; Style et al., 2018; Wei et al., 2020). Our study shows that paraspeckles employ a different mechanism – micellization to control their size. Micellization likely provides a tighter control over sizes, which are defined by the block sizes, when compared with that of other reported mechanisms, and this control is

limited to molecular dimensions. In addition to size control, micellization differs from LLPS; micellization is a continuous process where the number of assemblies increase continuously, whereas an LLPS is a discontinuous process, in which phase separation occurs across a certain threshold (which is called the binodal line), although the nucleation dynamics of the assembly on the short time scale is similar for both processes. These distinct features of micellization may contribute to the functions of the paraspeckle (discussed below).

I have some reservations about the authors' decision to publish the experiment and theoretical results in separate manuscripts, as these methods/findings are intimately intertwined. I appreciate that the audiences for the two sets of results are different, but in this case it is not possible to judge their findings without an assessment of the theory. I have taken a cursory look at the theory preprint and am satisfied with the physical model, but I have not given it the attention to review it in detail. At a minimum, I feel that the authors need to do a better job at explaining the physical contributions that enter the model. I feel a figure would be helpful here. Perhaps something similar to Fig. 1 of Yamamoto 2020, but labeled to emphasize energetic contributions rather than physical dimensions.

Thank you for your suggestion. We again discussed the possibility of combining the two manuscripts. We, however, decided not to do this because we would have to sacrifice detailed theoretical background information because of manuscript length limitations. Instead, as suggested by reviewer #3 and the editor, we have explained the theoretical points and assumptions in detail to make this manuscript easier for readers to understand. We have added energetic contributions in Figure 4B, as suggested. Additionally, we have added a schematic (Fig EV5A) of the energetic contributions for another version of the triblock copolymer micelle model of the paraspeckle that takes into consideration the elastic free energy in the B blocks. Furthermore, we have added explanations and discussion about the micellization/microphase separation in the revised manuscript. We therefore believe that these changes should help readers to understand the theoretical background seamlessly without referring to our theoretical paper.

The free energy expression appearing in this manuscript needs further explanation. The first two terms are self-evident, but the third and fourth term are less obvious. The third term has the appearance of a difference in chemical potentials, but the kinetic factors in

the logarithm differ from the concentrations that I would expect. Also, a brief justification for the Gibbs factor in the final term would be appropriate.

As suggested, our effective free energy reduces to the free energy of micelles in a solution when transcription is suppressed, $k_{\mathrm{tx}} \to 0$, and the third term of eq. (3) (i.e., logarithmic term) in the revised manuscript is the concentration. With our model, nascent transcripts associate with a growing paraspeckle at a constant rate k_{tx}. The rates of association of the transcripts in solution and nascent transcripts to the paraspeckle have similar forms (see the first and third terms of eq. (3) below), and the transcription dynamics is thus taken into account by an additional effective concentration k_{tx}/k_0 to the effective free energy. In contrast to micelles in solution, most of the NEAT1_2 transcripts are included in paraspeckles and thus the rate that free NEAT1_2 transcripts in the nucleoplasm associate with the growing paraspeckle can be ignored, $\phi_p \to 0$. Therefore, we have the form of the third term of eq. (3) in the revised manuscript. The probability of transcripts dissociating from the paraspeckle increases with the number n of transcripts in the paraspeckle. This results in the fourth term of eq. (3) in the revised manuscript. We added two paragraphs in **Triblock copolymer micelle model, Materials and Methods** (page 24, line 25 – page 25, line 26) to clarify the above points.

The growth of the paraspeckle was analyzed by using the Master equation

$$\frac{d}{dt}q_1(t) = -J_1(t) \#(1)$$

$$\frac{d}{dt}q_n(t) = -J_n(t) + J_{n-1}(t) \#(2)$$

with $n = 2,3,\cdots$, and

$$J_n(t) = k_0\phi_p q_n(t) - k_0 e^{-\frac{F_n+F_1-F_{n+1}}{k_BT}}(n+1)q_{n+1}(t) + k_{\mathrm{tx}}q_n(t), \#(3)$$

where $q_n(t)$ is the probability that the paraspeckle is composed of n NEAT1_2 transcripts, and $J_n(t)$ is the flux of the probability. The first term on the right side of eq. (3) is the rate that a NEAT1_2 transcript associates with the paraspeckle from solution. The second term is the rate that a NEAT1_2 in the paraspeckle dissociates, and the third term is the rate that a nascent NEAT1_2 transcript associates with the paraspeckle. The factor $n+1$ in the second term accounts for the fact that any of the $n+1$ NEAT1_2 transcripts dissociate with equal probability. k_0 is the rate constant that accounts for the association of NEAT1_2 to the paraspeckle. ϕ_p is the volume fraction

of NEAT1_2 in solution. F_n is the free energy $F_n(\alpha)$, which has been already minimized with respect to α. k_{B} is the Boltzmann constant, T is the absolute temperature, and k_{tx} is the rate constant that accounts for the production of nascent NEAT1_2 transcripts. The first and second terms of eq. (3) are determined so that they satisfy the detailed balance when transcription is suppressed, $k_{\mathrm{tx}} \to 0$. In the following, we set $\phi_p \to 0$ because most NEAT1_2 transcripts are incorporated in paraspeckles.

In the steady state, $J_n(t) \to 0$, the solution of eqs. (1) and (2) has the form

$$q_n = \frac{1}{Z_{\mathrm{st}}} e^{-\frac{\mathcal{F}_n}{k_B T}}, \#(4)$$

where $\mathcal{F}_n$ is the effective free energy of the paraspeckle composed of n NEAT1_2 transcripts

$$\mathcal{F}_n = F_n - nF_1 - (n-1)k_{\mathrm{B}}T \log\frac{k_{\mathrm{tx}}}{k_0} + k_{\mathrm{B}}T \log(n!) \#(5)$$

and Z_{st} is the effective partition function

$$Z_{\mathrm{st}} = \sum_n e^{-\frac{\mathcal{F}_n}{k_B T}}. \#(6)$$

The most probable number n of NEAT1_2 transcripts in the steady state is derived by minimizing the effective free energy $\mathcal{F}_n$. Our model predicts the number n of transcripts in a paraspeckle, the fraction α of A blocks in the shell, the radius of the paraspeckle in the steady state as a function of the transcription rate k_{tx}, and the number of segments in the A blocks.

The authors should comment on the roles of specific NEAT1 RNA-binding proteins within the scaffold model and within their Flory-Huggins model.

The feature of A, B and C blocks result from (or correspond to) the feature of RBP(s) bound to each of these blocks. In our previous study, we showed that the complex of RNA and RBPs can be treated as one polymer for cases where the binding energy between each RBP and RNA is sufficiently large. To emphasize these points, we have added the following sentences in **Triblock copolymer micelle model, Materials and Methods** (page 23, lines 2–15).

In our theory, NEAT1_2 is treated as an ABC triblock copolymer. The B blocks are localized in the core and the C blocks are localized in the shell. A fraction, α, of the A blocks are in the shell and the other fraction, $1-\alpha$, are in the core. A blocks and C blocks form distinct domains in the shell. The feature of each block results from the

nature of RBPs bound to the block. Here, the B blocks tend to associate with each other because RBPs that are prone to dimerize/oligomerize to assemble the core, such as NONO, bind to these blocks (Yamazaki et al. 2018), whereas A and C blocks have higher affinity toward the nucleoplasm because RBPs that exhibit hydrophilic properties bind to these blocks to form the shell. We treat simple cases by considering that the number of RBPs in the nucleoplasm is large, the binding energy between RBPs and NEAT1_2 transcripts is large, and the binding of RBPs to NEAT1_2 is fast. In such cases, the complexes can be treated as copolymers, where RBPs are implicitly taken into account in the interaction parameters of polymer units (Yamamoto et al. 2020a).

The experiments were done exclusively in haploid HAP1 cells which grow significantly slower than diploid and aneuploid cells. These cells posit reduction in absolute gene expression levels and cell size. Their nuclear compartmentalization is certainly affected by these facts. The authors should provide clear justification for their model reflecting proteomic and RNA abundance in diploid cells.

Since 2014, we have been using near-haploid HAP1 cells and never recognized that these cells grow significantly slower than other diploid and aneuploid cell lines. The HAP1 cell is derived from the near-haploid KBM7 human myeloid leukemia cancer cell line (Essletzbicher et al., Genome Res (2014) 24, 2059; Carette et al., Nature (2011) 477, 340). Thus, HAP1 cells are different from other haploid cells such as haploid ES cells because they are derived from cancer cells. HAP1 cells grow fast and are usually split to 1:10 to 1:15 every 2 to 3 days as Horizon Discovery (a company distributing HAP1 cells) recommends, which is a similar growth rate to widely used cancer cell lines including HeLa cells and is consistent with other reports (e.g., Yaguchi et al., JCB (2018) 217, 2463).

In addition, our EM observations, as an unbiased way to inspect cellular structures, did not show any significant differences in intracellular structures including paraspeckles (e.g., organization of NEAT1_2, organization and distribution of multiple PSPs, size and shape of paraspeckles, MG132 responsiveness) and many other subnuclear structures (e.g., nuclear speckles, nucleoli and chromatins) in HAP1 and other cell lines such as HeLa cells.

The HAP1 cell line is also used widely in a variety of experiments including haploid genetic screening for a wide range of biological processes, which are unlikely affected by ploidy (e.g., Blomen et al., Science (2015) 350, 1092; Carette et al., Nature (2011) 477, 340; Jae et al., Science (2014) 344, 1506; Kravtsova-Ivantsiv et al., Cell

(2015) 161, 333; Marceau et al., Nature (2016) 535, 159; Nieuwenhuis et al., Science (2017) 358, 1453).

Furthermore, near-haploid HAP1 cells undergo diploidization spontaneously during cell culture (Yaguchi et al., JCB (2018) 217, 2463; Olbrich et al., PNAS (2017) 114, 9367). As it takes at least 1 month to establish HAP1 NEAT1 mutant clones, our clones undergo diploidization. We did not observe any correlation between the ploidy and the phenotypes analyzed in this study. Thus, we do not think that ploidy and its associated cellular fitness significantly influenced the phenotypes we analyzed in the work presented and in our theoretical model.

1st Revision - Editorial Decision 10th Mar 2021

Thank you for submitting your revised manuscript, we have now received the reports from the three initial referees (see comments below). I am pleased to say that they overall find that their comments have been satisfactorily addressed and now support publication. Therefore I would now like to ask you to address a number of editorial issues that are listed in detail below. Please make any changes to the manuscript text in the attached document only using the "track changes" option. Once these remaining issues are resolved, we will be happy to formally accept the manuscript for publication.

Referee #1:

The authors have addressed my comments satisfactorily. This is a very interesting paper. Seeing the concept of micellation/micro-phase separation applied to a relevant biomolecular condensate will be of benefit for the phase separation community.

Referee #2:

The authors have addressed all of the concerns I raised. The issue of protein binding and RNA sequence / secondary structure together giving rise differential hydrophobicity and block copolymeric architecture is fascinating. This work opens new doors to our thinking and should be published with no further revisions.

Referee #3:

The authors have done an excellent job of addressing my comments. I have a minor quibble with the authors' claim that micellization is a subset of microphase transition (due to the finite size of a micelle). However, I strongly agree with the sentiment expressed by the authors in their response: "We have decided to use both terms partly because using the term "micellization" alone may cause confusion or a misunderstanding to readers working in biological research fields. This is because in a biological context micellization is used mainly for the micellization of phospholipids. Additionally, we think that a comparison between macroscopic phase separation and microphase separation would be conceptually helpful for readers."
On page 19, in discussing the role of NEAT1 in the formation of paraspeckles the authors should also discuss the capability of Neat1 to form paraspeckle de novo (Nature Cell Biol. 13, 167-73) DOI: 10.1038/ncb2157.
I agree that these phenomena are close cousins, and making this connection justifies the mild imprecision. I support publication in the present form.

Graduate School of Frontier Biosciences
Osaka University

Dr. Stefanie Boehm
The EMBO Journal
Meyerhofstrasse 1
D-69117 Heidelberg
Germany

March 15, 2021

Dear Dr. Boehm

Thank you very much for the positive comments and inviting us the final revision of our manuscript (EMBOJ-2020-107270R). We addressed all the editorial concerns (#1~7 in the decision letter) and made changes to the manuscript text using the track changes option. We also prepared a synopsis image and text, four bullet points and blurb text.

The reviewer #3 gave an additional minor comment, our response to the comment is as follows.

On page 19, in discussing the role of NEAT1 in the formation of paraspeckles the authors should also discuss the capability of Neat1 to form paraspeckle de novo (Nature Cell Biol. 13, 167-73) DOI: 10.1038/ncb2157.

In the paper (Nature Cell Biol. 13, 167-73), the authors use NEAT1_1, a short isoform of NEAT1, and show that tethering MS2-tagged NEAT1_1 to a specific genomic site induce nuclear foci containing three paraspeckle proteins, NONO, SFPQ (PSF), and PSP1, which are all DBHS family proteins. However, their image data do not tell whether these foci contain core-shell architectures like paraspeckles due to the resolution of the images. In addition, our previous work clearly shows that NEAT1_1 alone is not sufficient to induce paraspeckles (Figure 1A in Naganuma et al., EMBO J (2012) 31, 4020). Thus, we think that the data shown in the Nature Cell Biology paper are not convincing to show de novo formation of paraspeckles. Further, we already discuss de novo formation of paraspeckles by NEAT1_2 at NEAT1 gene locus by citing the paper (Mao et al., Nature Cell Biol (2011) 13, 95). Therefore, we did not include the reference and the related discussion in our manuscript.

We hope that our revised manuscript will now be acceptable for publication in the *EMBO Journal*.

Thank you for your consideration.

Graduate School of
Frontier Biosciences
Osaka University

Yours sincerely,

Tetsuro HIROSE, Ph.D.

2nd Revision - Editorial Decision 18th Mar 2021

Thank you again for submitting the final revised version of your manuscript. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Tomohiro Yamazaki, Tetsuro Hirose
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-10720

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical method was used to determine sample size. Sample sizes were chosen based on literatures and pur previous studies.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Samples were not randamized.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No blinding was applied.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes. we performed statistic analyses using Prism7 software. The statistic methods used in this study are shown in the Materials and Methods.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	When statistical tests were performed, we used non-parametric tests to avoid assumptions.
Is there an estimate of variation within each group of data?	Same as above.

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Same as above.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Information of the antibodies used in this study are shown in Materials and Methods and Appendix Table S2.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Information of the cell lines used in this study are shown in Materials and Methods and Appendix Table S2.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Programs used in theoretical modeling are deposited in Figshare (the detail is shown in the Data and code availability in the Materials and Methods.).
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

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