JCB

Stress granules regulate stress-induced paraspeckle assembly

Haiyan An, Jing Tong Tan, and Tatyana Shelkovnikova

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May 17, 2019

Re: JCB manuscript #201904098

Dr. Tatyana A Shelkovnikova
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Medicines Discovery Institute
Main Building
Park Place
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Dear Dr. Shelkovnikova,

Thank you for submitting your manuscript entitled "Stress granules regulate stress-induced paraspeckle assembly". Your manuscript has been assessed by expert reviewers, whose comments are appended below.

You will see that although the reviewers express some clear enthusiasm for the work and your proposed conclusions, they each raise a number of significant concerns which preclude publication of the current version of the manuscript in JCB. In particular, you'll note that all three reviewers acknowledge that further validation is needed to confirm that these proposed proteins are indeed common to both SGs and PSs. They each also point to a number of missing controls which must be included in a revised manuscript as well as several added experiments that are needed to support the main conclusions of the study. We hope that you will be able to address these and each of the other reviewer comments in full.

Please note that a substantial amount of additional experimental data likely would be needed to satisfactorily address the concerns of the reviewers. It may therefore be necessary to extend your manuscript to a full Research Article. Our typical timeframe for revisions is three to four months; if submitted within this timeframe, novelty will not be reassessed. We would be open to resubmission at a later date; however, please note that priority and novelty would be reassessed.

If you choose to revise and resubmit your manuscript, please also attend to the following editorial points. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:
Text limits: Character count for a Report is < 20,000; a full Research Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

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If you choose to resubmit, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Joan Steitz, PhD
Monitoring Editor
JCB

Tim Spencer, PhD
Deputy Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

An et al describe a compositional and functional link between stress granules (SG) and paraspeckles (PS). This is a novel and interesting observation that may have implications for the pathogenesis of neurodegenerative disease.

My main concern is that the purification protocol will produce a NONO- or SFPQ-interactome that may not be synonymous with PSs. It will be important to verify that each of the proteins identified are components of NEAT1-containing PSs (e.g., immunofluorescence microscopy combined with FISH). Without this confirmation, these proteins may be part of an interactome assembled during cell lysis, but not components of PSs.

It is not surprising that KD of G3BP1 does not affect SGs or PSs as G3BP2 can do the job if it is available. The authors should examine whether cells lacking both G3BP1 and G3BP2 are able to assemble PSs.

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Manuscript: Stress granules regulate stress-induced paraspeckle assembly
The authors of this manuscript identified new paraspeckle proteins by establishing a paraspeckle isolation technique and performing mass spectrometry of isolated paraspeckle proteins. The data shows a correlation with the known stress granule proteome. The main findings suggest a new role for stress granule in regulating paraspeckle assembly by sequestering negative paraspeckle regulators.
The strength if the work is a potential novel mechanism by which stress granules regulate the formation of paraspeckles. The weakness of the work is that the evidence for some conclusions are limited. Thus, the following comments need to be addressed before further consideration at JCB. This review is from Roy Parker and I would be willing to clarify these comments for the reviews directly if needed.

Major Comments:
1) A key point of the work is that they have identified novel paraspeckle proteins by purification/mass spec of paraspeckles. However, to validate that these proteins are actually in paraspeckles, at least couple of the newly identified paraspeckle proteins should be verified to be in paraspeckles by immunofluorescence. I suggest this a) since many known paraspeckles proteins are not identified in their mass spec analysis, and b) it remains possible they are purifying a related RNP that contains many of the same proteins as stress granules.
2) The correlation of stress granule formation and paraspeckle formation is the most important and interestingly contribution of this work. Given that, robust data that this is a causative relationship and not simply a correlation is needed. I suggest the authors examine the formation of stress granules and paraspeckles in the g3bp1/2ΔΔ cells lines (Kedersha et al., 2016, JCB), since this cell line fails to make stress granules, and therefore by the authors model should fail to induce paraspeckles with stress. Moreover, since the g3bp1/2ΔΔ cell lines still form stress granules in response to sorbitol, it is strongly predicted that sorbitol treatment should still induce stress granules and paraspeckles in response to sorbitol treatment. This combination of results would provide a robust demonstration that stress granule formation per se is the inducer of paraspeckle formation.
Minor Comments:
3) Fig. 4H: the authors state that 10 min AS (NaAsO2) stress fail to induce paraspeckle numbers. However, only NEAT+area is quantified in this panel. Is the paraspeckle count also not significant? I would recommend to show the count or both, as done before. Additionally, the NEAT1 FISH image shows a couple of FISH spots. Thus, a control figure with no AS stress will help the reader see that no changes are happening.
4) „data not shown" was used several times. Given this possibility of supplemental data, please try to avoid that and add data as much as possible. E.g. FUS IF in Fig. 2C or PS phases in other cell lines.
5) Table S1 is subdivided in multiple sheets. It will help the reader, if it's clearly stated, which sheet contains which data.

6) Fig.1E: to have it uniform, add reference for known PSPs into the figure or legend (as done for NEAT1 interactors or in Fig. 2A)

7) Fig.2C: Add GFP label to G3BP1 to distinguish overexpression; are the two images in top panel for CPSF6 the same image?

8) Fig. 5A and B; it well help the reader if the images in A) are arranged in the same order as the normalization in B). FUS is missing in the normalization in B).

9) Was the LC-MS/MS done in both cell lines? Unclear in the material and method section, while in the main text, it's stated that HEK293 cells were used.

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An et al. describe a possible regulatory crosstalk between nuclear paraspeckles and cytoplasmic stress granule (SG). They first identified multiple proteins which are commonly contained in stress granules (SGs) and paraspeckle-like foci obtained by NONO- or SFPQ-GFP trap followed by MS analysis. Pharmacological treatments of the cells to block SG formation or those to prolong the SG maintenance suggested that the cytoplasmic SG state affects nuclear paraspeckle assembly. Then, the authors argue that SGs regulate paraspeckle assembly through sequestration of the negative regulators of paraspeckle assembly. Overall, the proposed mechanism regarding the crosstalk between stress inducible nuclear and cytoplasmic cellular bodies are interesting and provocative, however, the data presented in all figures of the manuscript do not sufficiently support several critical arguments given by the authors.

Major points
1. Figure 1: The authors treated the proteins obtained by the GFP-trap of NONO or SFPQ as the paraspeckle proteins (PSPs), however, they need more carefully evaluate the precipitates that they obtained by GFP trap. NEAT1 is the most reliable core molecule of paraspeckles, so the authors should check if the PSs pelleted (shown in Figure 1B) contain NEAT1 by RNA-FISH, and also check the enrichment of NEAT1 in the final beads fraction by RT-qPCR.

2. Figure 1: The authors should also confirm if the identified proteins are localized in paraspeckles the cells as expected. It should be noted that the levels of paraspeckle-localized NONO or SFPQ may be limited less than 10% of total NONO or SFPQ in the cells. Therefore, it is likely that majority of the co-precipitated proteins by GFP trap are just the interactors of NONO or SFPQ in the nucleoplasm.

3. Figure 2: (Related to 2) The authors should carefully confirm the paraspeckle localization of the SG proteins determined by LC-MS/MS, since it is still possible that the sticky SG proteins artificially associate with NONO/SFPQ complexes during the extract preparation.

4. Figure 3 and 4: In most cases reported so far, the paraspeckle size and numbers are determined by the transcription levels of NEAT1. It should be clarified if the NEAT1 transcription is modulated by SG states or the PSP sequestration into SGs more directly affect paraspeckle size and numbers without affecting NEAT1 transcription.

5. Figure 4: To modulate the SG states, the authors employed several chemical compounds that globally affect translation that give pleiotropic effects to various cellular pathways, not limited in SG formation. Only the pharmacological analyses cannot give the conclusion that the PS assembly was affected by modulation of SG. SG formation should be blocked by more specific ways. The authors examined the effect of G3BP1 RNAi and detected little effect on both SGs and paraspeckles in

Figure 4I. The authors should refer to the literature showing that the double KD of G3BP1 and 2 can block SG formation (e.g. Matsuki et al. Genes to Cells 18: 135-146, 2013).

6. Figure 5: The author's hypothesis that the negative regulators of PS assembly are sequestrated by SGs looks very interesting. However, the authors should more carefully investigate the role of each candidate of the negative regulator. The effect of RNAi of each negative regulator should be confirmed with the second siRNA sets (or CRISPR). Particularly, regarding UBPAL2 the previous paper cited by the authors (Naganuma et al., EMBO J 2012) has never reported that UBPAL2 was a negative regulator of paraspeckle assembly, it just showed that the UBAPL2 KD reduced NEAT1_1 level without affecting NEAT1_2 and PS levels.

7. Figure 5D: The majority of PSPs in nucleoplasm would buffer the effect on the level of paraspeckle localization when SG sequestrates them. The overexpression experiment needs quantitative data. Transfection of the control plasmids (e.g. combination of other RBP plasmids) should be set to exclude the possibility that the transfection of excess plasmids (and/or the overexpressed RBPs) nonspecifically block paraspeckle formation for example through blocking transcription. The expression of NLS-attached UBAPL2 would be worth to investigate the possible function of this protein as the negative regulator. The nucleo-/cytoplasmic ratio of UBAPL2 should be monitored before/after the stress and also with/without chemicals.

Minor points
Figure 2D CPSF6 panels: The field of cell image looks like up-side-down in right and left panels (?).
Figure 3A graph: The data on poly(I:C) should be included in the graph.

Reviewer #1 (Comments to the Authors (Required)):

An et al describe a compositional and functional link between stress granules (SG) and paraspeckles (PS). This is a novel and interesting observation that may have implications for the pathogenesis of neurodegenerative disease.

My main concern is that the purification protocol will produce a NONO- or SFPQ-interactome that may not be synonymous with PSs. It will be important to verify that each of the proteins identified are components of NEAT1-containing PSs (e.g., immunofluorescence microscopy combined with FISH). Without this confirmation, these proteins may be part of an interactome assembled during cell lysis, but not components of PSs.

We appreciate this concern. However, it is highly unlikely that PS-like structures broke up and were assembled *de novo* during lysis, since our analysis showed that these structures are very stable, even at 37^{0}C, and only harsh treatments such as urea or SDS could dissolve them. To ensure that PS-like structures do not dissolve and then re-appear during lysis, we monitored their behaviour during lysis. In the nuclear lysis buffer, nuclei break up and eventually release PS-like structures; after their release from the nucleus, PS-like structures distribute evenly in the field of view but stay intact (Fig R1). This data is for Reviewer's attention but we included a modified version of this figure in the manuscript (Fig. 1 B). Moreover, we examined PS-like structures stability at room temperature, and their numbers were not changed 4 h after lysis and decreased only 16 h after lysis (these data were included in the manuscript text). The possibility that some proteins join these structures in the lysate cannot be ruled out completely, but we believe that this is a limitation of any protocol for biochemical isolation of membraneless assemblies.

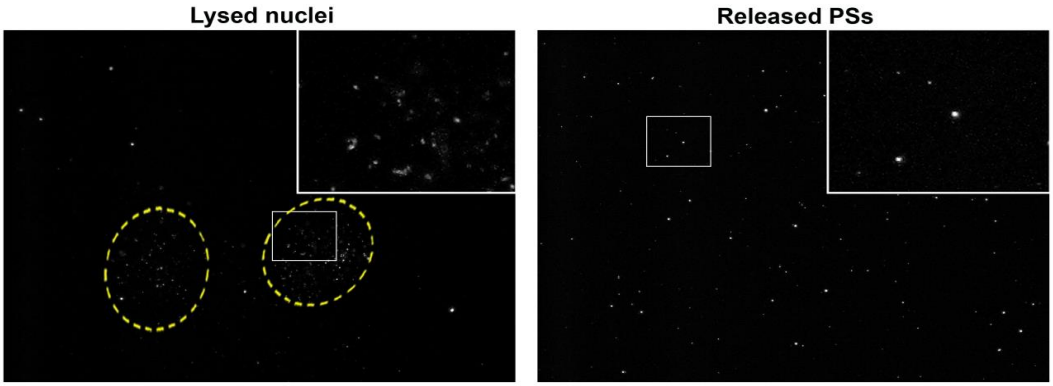

Figure R1. PS-like structures during lysis of HEK293 cells. Left, partially lysed cell nuclei at the beginning of lysis. Right, PS-like structures after 30 min of lysis.

As requested, we performed validation of several proteins identified in our analysis, using a combination of NEAT1 RNA-FISH and immunocytochemistry, and these data have been included as Fig. 1 J. However, we would like to draw the Reviewer's attention to the statement in the previous version of our manuscript, which emphasises our aim to identify minor and transiently recruited components which might not be readily detected in PSs using immunofluorescence:

"…While these 43 validated PSPs are presumably most strongly associated with PSs, other proteins may be present in PSs at a level below microscopic detection limit and/or associate with PSs transiently."

This statement was slightly modified and moved to another part of the Results due to new material added, and in its current form it reads:

"However, it should be noted that PSPs identified in the fluorescent protein screen (Naganuma et al., 2012) are likely most strongly associated with PSs, whereas some of the new proteins from the current study may be present in PSs at a level below microscopic detection limit and/or associate with PSs transiently."

It is not surprising that KD of G3BP1 does not affect SGs or PSs as G3BP2 can do the job if it is available. The authors should examine whether cells lacking both G3BP1 and G3BP2 are able to assemble PSs.

We are grateful to the Reviewer for this suggestion. We have now performed analysis of PS assembly after double knockdown of G3BP1 and G3BP2 in neuroblastoma cells. Depletion of both proteins simultaneously was sufficient to significantly perturb SG formation in response to NaAsO2, with cells displaying only residual SGs. In cells with both G3BP proteins downregulated, we indeed observed significantly diminished PS assembly after 3 h of recovery from NaAsO2 stress and after 4 h of MG132 treatment. These data are in line with observations made using chemical inhibitors of SG formation (CHX and emetine) and strengthen our conclusion that the assembly of microscopically visible SGs is required for efficient PS formation during stress. These results have been included as Fig. 4 D,E and Fig. S2 C.

Reviewer #2 (Comments to the Authors (Required)):

Manuscript: Stress granules regulate stress-induced paraspeckle assembly

The authors of this manuscript identified new paraspeckle proteins by establishing a paraspeckle isolation technique and performing mass spectrometry of isolated paraspeckle proteins. The data shows a correlation with the known stress granule proteome. The main findings suggest a new role for stress granule in regulating paraspeckle assembly by sequestering negative paraspeckle regulators.
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We appreciate this concern (also raised by other Reviewers). Several proteins identified in this analysis have been now validated using a combination of NEAT1 RNA-FISH and immunostaining in human fibroblasts, which possess large nucleus with multiple PSs best suited for co-localisation analysis. These data have been included in the manuscript as Fig. 1 J.

2) The correlation of stress granule formation and paraspeckle formation is the most important and interestingly contribution of this work. Given that, robust data that this is a causative relationship and not simply a correlation is needed. I suggest the authors examine the formation of stress granules and paraspeckles in the g3bp1/2ΔΔ cells lines (Kedersha et al., 2016, JCB), since this cell line fails to make stress granules, and therefore by the authors model should fail to induce paraspeckles with stress. Moreover, since the g3bp1/2ΔΔ cell lines still form stress granules in response to sorbitol, it is strongly predicted that sorbitol treatment should still induce stress granules and paraspeckles in response to sorbitol treatment. This combination of results would provide a robust demonstration that stress granule formation per se is the inducer of paraspeckle formation.

We are grateful for this suggestion, and the use of simultaneous depletion of G3BP1/2 proteins was suggested by other Reviewers as well. We found that siRNA-mediated knockdown of G3BP1 and G3BP2 efficiently depletes both proteins (72 h post-transfection) and is sufficient to disrupt SG formation in response to NaAsO2. Using this approach, we have shown that depletion of both G3BP proteins significantly decreases NaAsO2- and MG132-induced PS hyper-assembly. These results are now included as Fig. 4 D,E. Regarding sorbitol, as shown in Fig. 3 A, induction of PSs by sorbitol is limited, which is probably due to induction of small, non-canonical SGs by this agent (please note that in Fig. 3 A, after addition of RocA data, the difference between control and sorbitol-treated cells, after correction for multiple comparisons, is no longer significant). Given this small effect on PSs, it is hardly possible to test how G3BP depletion would affect PSs in sorbitol-treated cells.

Minor Comments:
3) Fig. 4H: the authors state that 10 min AS (NaAsO2) stress fail to induce paraspeckle numbers. However, only NEAT+area is quantified in this panel. Is the paraspeckle count also not significant? I would recommend to show the count or both, as done before. Additionally, the NEAT1 FISH image shows a couple of FISH spots. Thus, a control figure with no AS stress will help the reader see that no changes are happening.

Yes, the PS count was significantly lower than in cells treated for 20 or 60 min, albeit slightly higher than in control cells. This was not included before due to space limitations but now this data has been added.

In control (naïve) neuroblastoma cells PSs are also present, although their numbers are very low or they can be undetectable in some cells (as shown in Fig. 3 A,C; Fig. 4 A,F). We have now added a panel for non-treated cells for comparison. Also, the entire panel Fig. 4 H has been moved to supplementary (currently Fig. S2 E).

4) „data not shown" was used several times. Given this possibility of supplemental data, please try to avoid that and add data as much as possible. E.g. FUS IF in Fig. 2C or PS phases in other cell lines.

We have now added all the data which were not included in the previous version: NEAT1 FISH in HEK293 cells (Fig. 1 A); FUS IF (Fig. 2 C and Fig. S1); data for fibroblasts (Fig. 3 G); data for emetine (Fig. S2 B).

5) Table S1 is subdivided in multiple sheets. It will help the reader, if it's clearly stated, which sheet contains which data.

We have now added references to the sheet title in each case.

6) Fig.1E: to have it uniform, add reference for known PSPs into the figure or legend (as done for NEAT1 interactors or in Fig. 2A)

We have added references to figure legend (current Fig. 1 H) (Since three references will have to be given, there is not enough space on the figure itself).

7) Fig.2C: Add GFP label to G3BP1 to distinguish overexpression; are the two images in top panel for CPSF6 the same image?

GFP label added. The images for CPSF6 are the same image but one of the panels was rotated to prevent overlap between the inset and the cell in the main panel, whereas the other one was not rotated in the same way, by mistake. We are grateful for spotting this, it has been corrected.

8) Fig. 5A and B; it well help the reader if the images in A) are arranged in the same order as the normalization in B). FUS is missing in the normalization in B).

The images have been re-arranged. FUS quantitation was added.

9) Was the LC-MS/MS done in both cell lines? Unclear in the material and method section, while in the main text, it's stated that HEK293 cells were used.

LC-MS/MS was done in HEK293 cells (this is now clearly stated in the Methods section), since we were struggling to obtain a sufficient amount of PS-like structures from SH-SY5Y cells due to lower transfection efficiency/expression levels as compared to HEK293 cells. However, we purified PS-like structures from HEK293 and SH-SY5Y cells in small scale experiments, and they were similar in size/morphology and stability between SH-SY5Y and HEK293 cells. Subsequent PS analysis in response to stress (RNA-FISH, immunocytochemistry) was done in SH-SY5Y cells and fibroblasts because they are flatter cells with bigger nuclei, more suitable analysis of subnuclear structures.

Reviewer #3 (Comments to the Authors (Required)):

An et al. describe a possible regulatory crosstalk between nuclear paraspeckles and cytoplasmic stress granule (SG). They first identified multiple proteins which are commonly contained in stress granules (SGs) and paraspeckle-like foci obtained by NONO- or SFPQ-GFP trap followed by MS analysis. Pharmacological treatments of the cells to block SG formation or those to prolong the SG maintenance suggested that the cytoplasmic SG state affects nuclear paraspeckle assembly. Then, the authors argue that SGs regulate paraspeckle assembly through sequestration of the negative regulators of paraspeckle assembly. Overall, the proposed mechanism regarding the crosstalk between stress inducible nuclear and cytoplasmic cellular bodies are interesting and provocative, however, the data presented in all figures of the manuscript do not sufficiently support several critical arguments given by the authors.

Major points

1. Figure 1: The authors treated the proteins obtained by the GFP-trap of NONO or SFPQ as the paraspeckle proteins (PSPs), however, they need more carefully evaluate the precipitates that they obtained by GFP trap. NEAT1 is the most reliable core molecule of paraspeckles, so the authors should check if the PSs pelleted (shown in Figure 1B) contain NEAT1 by RNA-FISH, and also check the enrichment of NEAT1 in the final beads fraction by RT-qPCR.

PS pellet (after 17,000g centrifugation of nuclear lysates) would contain unlabelled PSs as well, and hence NEAT1. Therefore, we have verified the presence NEAT1 (total and NEAT1_2 isoform) in the final PS preparations, by RT-PCR. As a negative control, we used SG core preparations. These data have now been added as Fig. 1 E.

2. Figure 1: The authors should also confirm if the identified proteins are localized in paraspeckles the cells as expected. It should be noted that the levels of paraspeckle-localized NONO or SFPQ may be limited less than 10% of total NONO or SFPQ in the cells. Therefore, it is likely that majority of the co-precipitated proteins by GFP trap are just the interactors of NONO or SFPQ in the nucleoplasm.

We understand this concern however, our purification protocol does allow isolation of a pure fraction of PS-like particles. Perhaps it was not explained sufficiently in the experimental procedures, but the nucleoplasmic fraction containing soluble complexes of NONO and SFPQ was excluded prior to affinity purification. To achieve this, we first isolated the nuclei, lysed them, enriched PS-like structures by centrifugation (separating from soluble fraction) and washed these PS pellets, to remove soluble proteins. Only after this, the fraction containing PS-like structures was combined with the beads. We have changed the cartoon

(current Fig. 1 C) to reflect this and added a respective sentence to the Results section.

We have now performed validation of several proteins identified in our analysis, as per Reviewers' request, and these data have been now added to Fig. 1 J. Please also see reply to comment 1 by Reviewer #1, including Fig. R1.

3. Figure 2: (Related to 2) The authors should carefully confirm the paraspeckle localization of the SG proteins determined by LC-MS/MS, since it is still possible that the sticky SG proteins artificially associate with NONO/SFPQ complexes during the extract preparation.

Proteins selected for validation experiments are also SG components (new Fig. 1 J). However, we agree that the possibility that some proteins may associate with these structures in the lysates cannot be ruled out completely, but we believe this is the limitation of any protocol designed for biochemical isolation of membraneless assemblies.

4. Figure 3 and 4: In most cases reported so far, the paraspeckle size and numbers are determined by the transcription levels of NEAT1. It should be clarified if the NEAT1 transcription is modulated by SG states or the PSP sequestration into SGs more directly affect paraspeckle size and numbers without affecting NEAT1 transcription.

SG formation affects not only PS size and numbers but also NEAT1 transcription, as shown in Figs 3 E and 4 C.

5. Figure 4: To modulate the SG states, the authors employed several chemical compounds that globally affect translation that give pleiotropic effects to various cellular pathways, not limited in SG formation. Only the pharmacological analyses cannot give the conclusion that the PS assembly was affected by modulation of SG. SG formation should be blocked by more specific ways. The authors examined the effect of G3BP1 RNAi and detected little effect on both SGs and paraspeckles in Figure 4I. The authors should refer to the literature showing that the double KD of G3BP1 and 2 can block SG formation (e.g. Matsuki et al. Genes to Cells 18: 135-146, 2013).

Double G3BP KD experiments have been done, and results are consistent with those obtained using chemical inhibitors (included as new Fig. 4 D,E and Fig S2 C). For details, please see comments #2 by Reviewers #1 and #2.

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investigate the role of each candidate of the negative regulator. The effect of RNAi of each negative regulator should be confirmed with the second siRNA sets (or CRISPR). Particularly, regarding UBPAL2 the previous paper cited by the authors (Naganuma et al., EMBO J 2012) has never reported that UBPAL2 was a negative regulator of paraspeckle assembly, it just showed that the UBAPL2 KD reduced NEAT1_1 level without affecting NEAT1_2 and PS levels.

As suggested, we have verified the effect of siRNA-mediated depletion using a second siRNA set (included as Fig. S3 A). We apologise for inaccurate interpretation of UBAP2L data from Naganuma et al. and calling UBAP2L a known negative PS regulator. The reason for that is that at least one set of UBAP2L siRNA did increase NEAT1_2 levels in the original study (Naganuma et al., 2013: Figure 3F). However, we transferred UBAP2L to the category of newly identified negative regulators.

7. Figure 5D: The majority of PSPs in nucleoplasm would buffer the effect on the level of paraspeckle localization when SG sequestrates them. The overexpression experiment needs quantitative data. Transfection of the control plasmids (e.g. combination of other RBP plasmids) should be set to exclude the possibility that the transfection of excess plasmids (and/or the overexpressed RBPs) nonspecifically block paraspeckle formation for example through blocking transcription. The expression of NLS-attached UBAPL2 would be worth to investigate the possible function of this protein as the negative regulator. The nucleo-/cytoplasmic ratio of UBAPL2 should be monitored before/after the stress and also with/without chemicals.

Quantification for these experiments has been added as suggested by this Reviewer (Fig. 5 E). As control plasmids, we used two RNA-binding proteins whose knockdown does not significantly affect PSs - TAF15 (nuclear, category 3 protein– Naganuma et al. 2012) and ATXN2 (siRNA knockdown results included as Fig. S3 B). Either protein or the combination of the two did not affect stress-induced PSs, these data have been added as Fig. S3 C. As suggested, we generated a plasmid construct to express UBAP2L tagged with SV40 NLS at its C-terminus (Fig. R2A). This approach is known to efficiently drive their nuclear import of proteins in SH-SY5Y cells (Kodama, Kondo et al. 2005; Kontopoulos, Parvin et al. 2006; Song, Kim et al. 2016). However we found that attachment of NLS to UBAP2L was not sufficient to cause nuclear retention of the protein in the nucleus in SH-SY5Y cells. This is likely due to a very large size of UBAP2L protein (1087aa, 115kDa). Instead, SV40 NLS increases UBAP2L aggregation capacity, for unknown reasons (Fig. R2B). However, we did examine subcellular distribution of endogenous UBAP2L under conditions of stress and in the presence of SG modulators using

immunocytochemistry, and none of the treatments affected almost exclusively cytoplasmic localisation of UBAP2L (included as Fig. S3 D). This suggests that the inhibitory effect of UBAP2L on PSs is executed via its cytoplasmic functions rather than direct association with PSs, which has been pointed out in the manuscript text. It will be important to address UBAP2L function as a negative modulator of PS assembly in future, but such detailed studies of this specific protein are beyond the scope of this study.

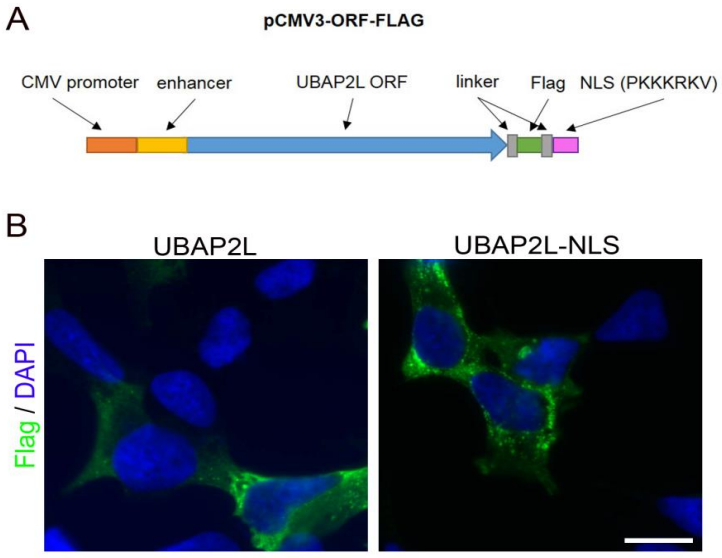

Figure R2. SV40 NLS attached to UBAP2L does not target the protein to the nucleus.

Minor points
Figure 2D CPSF6 panels: The field of cell image looks like up-side-down in right and left panels (?).

Yes, the images for CPSF6 are the same image, but one CPSF6 panel was rotated to prevent overlap between the inset and the cell in the main panel, whereas the merged panel was not rotated in the same way, by mistake. We are grateful for spotting this, it has been corrected now.

Figure 3A graph: The data on poly(I:C) should be included in the graph.

The data has been included. We additionally demonstrated that treatment with an eIF4A inhibitor rocaglamide A (Roc A) which induces SGs (Kedersha et al., 2016) also triggers PS hyper-assembly. These data were also added to Fig. 3 A.

August 23, 2019

RE: JCB Manuscript #201904098R

Dr. Tatyana A Shelkovnikova
Cardiff University
Medicines Discovery Institute
Main Building
Park Place
Cardiff CF10 3AT
United Kingdom

Dear Dr. Shelkovnikova:

Thank you for submitting your revised manuscript entitled "Stress granules regulate stress-induced paraspeckle assembly". The paper has now been assessed again by the original three reviewers. As you will see, reviewers #1 and 2 are largely satisfied with the revisions but reviewer#3 has a few lingering concerns about your interpretations of the data. It is likely that addressing these issues will not require any new experiments but simply involve new analysis/presentation of the data (rev3's pt#1), including the total NEAT1 levels in figures 4 and 5 (pt#3), and modifications to the text (points #1 and 2). Please also be sure to address the two minor issues raised by reviewer #2.

Assuming your ability to adequately address these lingering issues, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis, including cropped blots like those in figure 2E.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure

legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts.

5) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

6) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

9) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

10) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and video files: See our detailed guidelines for preparing your production-ready images, http://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

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Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Joan Steitz, PhD
Monitoring Editor
JCB

Tim Spencer, PhD
Interregnum Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The authors have addressed my concerns.

Reviewer #2 (Comments to the Authors (Required)):

The resubmission by An et al is a considerable improvement over the original, with all of my issues adequately addressed.
Two very minor items noted:
1) Fig. 1E: Lysate 1 and 2 are on the first view a bit confusing. It seems there are two lysis steps. Maybe better to call them lysate- PS and lysate- SG
2) Fig. 1J: MATR3 is labeled in blue, while others are labeled in black

Reviewer #3 (Comments to the Authors (Required)):

The manuscript was improved by addition of new data. However, I still recognize some ambiguities in two points below.

1. Figure 1: RT-PCR and FISH confirmed that NEAT1 is enriched in the final PS preparation. However, it is still unclear the purity of the PS preparation, it cannot measure how much contamination of the nucleoplasmic NONO/SFPQ interactors (or artificially interacted ones) is in the final preparation. Indeed at least three proteins tested in Fig. 1J are unlikely paraspeckle-localized, which are possible the contaminated NONO/SFPQ interactors rather than transiently associated paraspeckle proteins. The authors can show line-scan data for colocalization and should mention the above possibility, and carefully include them into "the list of PSPs".

2. Figure 5: The author's argument on connection between PS and SG was strengthened by G3BP1 double KD experiment. However, unfortunately, the argument of the next part regarding the role of "PS negative regulators" has not been supported by the presented data. The authors need to show the requirement of SG localization of these proteins for induction of PS formation. At least the authors should mention it in Discussion.

3. The authors need to show the quantitative data of NEAT1 level not only in Figure 3 but also in Figure 4 and 5.

28th August 2019

Dr Joan Steitz

Dr Tim Spencer

Journal of Cell Biology

Dear Editors,

Thank you for offering to publish our manuscript #201904098 pending final revisions. We have now addressed Reviewers' comments on the revised version, as outlined below. Changes made are highlighted in the manuscript text. We have also formatted the manuscript as per the JCB guidelines.

We hope that in its current form, you will find our manuscript suitable for publication in the Journal of Cell Biology.

Yours sincerely,

Tatyana Shelkovnikova, on behalf of authors

Reviewer #2

1) Fig. 1E: Lysate 1 and 2 are on the first view a bit confusing. It seems there are two lysis steps. Maybe better to call them lysate- PS and lysate- SG

We have renamed the lysates as suggested.

2) Fig. 1J: MATR3 is labeled in blue, while others are labeled in black

The original reason for labelling MATR3 in a different colour was that it was included as a "positive control". We have now indicated this in the figure legend instead, and added green labelling for proteins in this panel.

Reviewer #3

1. Figure 1: RT-PCR and FISH confirmed that NEAT1 is enriched in the final PS preparation. However, it is still unclear the purity of the PS preparation, it cannot measure how much contamination of the nucleoplasmic NONO/SFPQ interactors (or artificially interacted ones) is in the final preparation. Indeed at least three proteins tested in Fig. 1J are unlikely paraspeckle-localized, which are possible the contaminated NONO/SFPQ interactors rather than transiently associated paraspeckle proteins. The authors can show line-scan data for colocalization and should mention the above possibility, and carefully include them into "the list of PSPs".

Line profiles, which clearly show co-localisation, have now been added to Fig. 1J. As outlined in our previous Response to Reviewer's comments, nucleoplasmic SFPQ/NONO interactors should have been largely removed during PS washes. It should be noted however that some proteins are likely present in PSs by virtue of their interaction with a primary PS component (e.g. nucleoplasmic NONO/SFPQ interactors); and if such proteins have been captured in our analysis, they would be covered by the definition "transiently associated PS proteins". As per this Reviewer's request, this has been mentioned in the main text.

2. Figure 5: The author's argument on connection between PS and SG was strengthened by G3BP1 double KD experiment. However, unfortunately, the argument of the next part regarding the role of "PS negative regulators" has not been supported by the presented data. The authors need to show the requirement of SG localization of these proteins for induction of PS formation. At least the authors should mention it in Discussion.

The principal finding of our manuscript is the previously unappreciated crosstalk between SGs and PSs which has been thoroughly documented in our study. We found that sequestration of negative regulators of PS assembly into SGs may serve as a plausible molecular mechanism underlying this phenomenon. Pilot data in support of this mechanism, obtained using overexpressed proteins, have been included in the manuscript. We agree that more extensive and sophisticated experiments, such as quantitative analysis of protein shuttling between the two granules, should be carried out in future studies (but are beyond the scope of the current study), and we are looking into design of such experiments. Because we do not provide such extended data in the current manuscript, we formulated the text in all sections accordingly, e.g. the subheading reads "Stress granules *may* regulate paraspeckle assembly via sequestration of specific proteins." As suggested, we have included a sentence stating the need for further in-depth analysis of negative regulators in the Discussion section.

3. The authors need to show the quantitative data of NEAT1 level not only in Figure 3 but also in Figure 4 and 5.

Data for NEAT1 levels are as such already provided in the graphs showing NEAT1+ area measurements for all experiments in Figs 4 and 5. From our extensive experience with PS analysis, measurement of the NEAT1-positive area from an RNA-FISH experiment provides the most accurate and reliable readout for changes in NEAT1 levels, as compared, for example, to the indirect method, NEAT1 qRT-PCR.