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RNA-assisted sequestration of RNA-binding proteins by cytoplasmic inclusions of the C-terminal 35-kDa fragment of TDP-43

Lei-Lei Jiang, Wen-Liang Guan, Jian-Yang Wang, Shu-Xian Zhang and Hong-Yu Hu
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Original submission

First decision letter

MS ID#: JOCES/2021/259380

MS TITLE: RNA-assisted sequestration of RNA-binding proteins by the cytoplasmic inclusions of the C-terminal 35-kDa fragment of TDP-43 (TDP-35)

AUTHORS: Lei-Lei Jiang, Wen-Liang Guan, Jian-Yang Wang, Shu-Xian Zhang, and Hong-Yu Hu
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

I have now received comments on your manuscript from two expert referees. As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. I would then return it to the reviewers. Please address all issues raised by the referees as thoroughly as possible.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this work, Jiang et al. have examined the sequestration of RBP proteins by aggregates made up of the 35kDa degradation fragment of TDP-43. In particular, they have focused on cellular factor TIA1 that is important for stress granule formation and show that, at the functional level, this sequestration can disrupt maturation of specific mRNAs. In a parallel aspect of the work, the authors have investigated TDP-43 sequestration by TDP-35 aggregates by dynamic visualization within cells.

Comments for the author

In general, some results in this work are interesting. However, there are several issues that will need to be clarified:
1) First of all, in Figure 1A the authors suggest that decrease of TIA1 level is decreased in the nucleus.
However, this is not clearly visible from the Fig.1A and this raises the question whether the authors have actually quantified TIA1 nuclear signal intensity. Moreover, no statement with regards to this possible decrease was made with regards to PABPC1 that was also shown to colocalize with TDP-43 aggregates (Fig.1B). Were nuclear levels investigated also for this protein?. If not, this should be done.
2) In Figure 2, the authors should demonstrate that there is a linear relationship between the amount of oligos added to the RNAse treated mix and TIA1 sequestration by the aggregates. It is also rather disappointing that the concentration of these oligonucleotides does not seem to be specified in the text Figure legend, or Materials and Methods. The authors should therefore provide this concentration and show why they think it is ideal for the assay performed in Figure 2 (ie. that they are not oversaturating the system).
3) % of input should be provided in Figure 3. In addition, the authors should clarify what they mean by unspecified ssDNA. This should be clarified.
4) There seems to be something wrong with Figure 4C because the FISH analyses for TDP-35 4FL with Cy5-NN seems to be missing, and in the first lane of figures it is labelled Cy5-CA that should belong to Figure 4B.
5) The blots in Figure 5B and 5C are overexposed and it is hard to see the differences which are plotted in Figure 1D. In these western blots, the intensity of PRKRA does not really seem to decrease. Because the difference in protein intensity is rather small (Figure 5A) have the authors also lookd at the PRKRA mRNA levels which might yield more convincing results?.
6) Results in Figure 6 and 7 should also be complemented by co-trasfecting Flag-TDP-35 4FL mutant. Also authors should improve image intensity in Figure 7A as the cell images are mostly black.

Reviewer 2

Advance summary and potential significance to field

This study is a demonstration of how TDP-35 cytoplasmic aggregates can sequester certain RBPs, such as TIA1, via sequence-specific RNA association, and this causes a reduced function of TIA1 in the maturation of a particular RNA (PRKRA).
Moreover, TDP-35 expression also sequesters endogenous TDP-43. It builds on previous work from the same lab showing that overexpression of TDP-35 forms cytoplasmic RNA-dependent inclusions in HEK293T and HeLa cells and can sequester TDP-43. Overall, the data are clearly presented and the conclusions are mostly reasonable. The authors main claim is that the formation of TDP-35 in pathological conditions will drive TIA1 sequestration (and other RBPs, including TDP-43), causing

loss of function for these RBPs. However, the relevance of this study to ALS is difficult to reconcile. Specifically, there is a disconnect between the data/mechanism proposed here and the observation that TDP-43 inclusions in ALS patient motor neurons are devoid of RNA (Mann et al, 2019). Moreover, TIA1 aggregates have not been robustly reported in ALS patient neurons, even in a patient where a reportedly disease-causing mutation is present. Thus, a primary requirement is that the authors must comment on how the mechanism presented here correlates to what is known about ALS pathology. For example, does TDP-35 bear any post-translational modifications (ex. phosphorylation, ubiquitination) that are known to be present in cytoplasmic TDP-43 inclusions?

Comments for the author

I offer several points to help strengthen this manuscript.
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3. For the IPs, an important control would be to show a lack of interaction between TDP-35 4FL with TIA1 to support their claim that this interaction requires RNA-binding.
4. Since TIA1 is considered an important factor for stress granule assembly, does the sequestration of TIA1 interfere with stress granule formation in response to heat or arsenite? Are the kinetics the same? Also, does TDP-35 localize to stress granules?
5. Does the level of PRKRA mRNA change when TIA1 is sequestered? This would be a good measure that would strengthen the data. Also, on page 10, the authors make a claim that both TIA1 and TIAR are impacted – but no data for TIAR is provided. However, given that the impact on PRKRA protein levels is modest, does this not suggest that TIAR may be compensating? Have the authors investigated TIAR? Is there an increase in TIAR protein?
6. The authors have presented a mechanism by which TDP-43 is sequestered into the TDP-35 inclusions over time. Does the same happen with TIA1?
7. Sequestration of endogenous TDP-43 should similarly impact TDP-43 mRNA targets. Can the authors provide evidence that a well-known TDP-43 target mRNA is disrupted by TDP-35 expression? (ex. POLDIP3 or similar).
8. Many blots seem oversaturated (ex. Fig 5 PRKRA). Could the authors insert lower exposure images for these blots.
9. The introduction should consider that TDP-35 can also arise due to an alternative translational start, and not just truncation of full length protein.
10. The discussion is quite repetitive with the results. It could be streamlined, making room for the authors to better integrate the data with what is known in the field.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

In this work, Jiang et al. have examined the sequestration of RBP proteins by aggregates made up of the 35 kDa degradation fragment of TDP-43. In particular, they have focused on cellular factor TIA1 that is important for stress granule formation and show that, at the functional level, this sequestration can disrupt maturation of specific mRNAs. In a parallel aspect of the work, the authors have investigated TDP-43 sequestration by TDP-35 aggregates by dynamic visualization within cells.

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A: TIA1 is an RNA-binding protein addressed both in nucleus and cytoplasm, and its sequestration by cytoplasmic TDP-35 inclusions may lead to decrease of the nuclear localization. We have quantified the decrease of TIA1 in nuclei as provided in the inset graphs in Figure 1A and Suppl. Figure S2A in the revised version. However, PABPC1 is a cytoplasmic protein, its sequestration would not change the localization in nucleus. So, we cannot observe the nuclear localization of this protein, but we have visualized transition of PABPC1 from cytoplasmic dispersion to the TDP-35 inclusions (Figure 1B).

2. In Figure 2, the authors should demonstrate that there is a linear relationship between the amount of oligos added to the RNAse treated mix and TIA1 sequestration by the aggregates. It is also rather disappointing that the concentration of these oligonucleotides does not seem to be specified in the text, Figure legend, or Materials and Methods. The authors should therefore provide this concentration and show why they think it is ideal for the assay performed in Figure 2 (ie. that they are not oversaturating the system).
A: Since we did not know the exact amounts of overexpressed TDP-35 and endogenous TIA1, so we could just perform qualitative experiments, that is, excess amounts of ssDNA might be used in the RNase-treated mixture so that the rescue effect can reach a maximum or plateau. The final concentration of the ssDNA we applied is about 5 μM, which is specified in the Figure 2 legend of the revised version. We have also performed a dose experiment on the rescue efficiency of TIA1 by addition of ssDNA (TG+TC) as attached below, showing that the rescue effect is dependent on the ssDNA amount used and both TDP-35 and TIA1 bind specifically to ssDNA in a stoichiometric manner.

[NOTE: We have removed a figure which was provided for the referees in confidence.]

The ssDNA treatment assists on sequestration of TIA1 by the TDP-35 aggregates in a dose-dependent manner. (**A**) Supernatant/pellet fractionation for characterizing the effects of RNase plus ssDNA (TG+TC) treatments on the sequestration of endogenous TIA1. HEK 293T cells were transfected with FLAG-TDP-35, 48 hrs after transfection, the cells were harvested and lyzed with RNase plus different doses of TG+TC ssDNA (final concentration of 0, 1.0, 2.5, 5.0, 10 μM, respectively). The lysates were then subjected to supernatant/pellet fractionation and Western blotting analysis. Non-treat, without RNase treatment; TG+TC, TG+TC chimera ssDNA binding with both TDP-35 and TIA1. Sup., supernatant; Pel., pellet. *, non-specific band. (**B**) Plot of TIA1 in pellet fraction sequestered by the TDP-35 aggregates versus TG+TC ssDNA concentration. The protein amounts were estimated by the grayscale values by using *Scion Image* and normalized with that of the RNase only.

3. % of input should be provided in Figure 3. In addition, the authors should clarify what they mean by unspecified ssDNA. This should be clarified.
A: Normally we applied 5-8% of input for IP experiment as provided in the figure legends of Figure 3 and Suppl. Figure S4.

As the DNA/RNA binding sequences of TDP-43 and TIA1 are defined previously in literature (with citations in the context), we can design these specific ssDNA sequences combined in a single oligomer chain (TG+TC), which we supposed to bind both proteins specifically and stoichiometrically. Otherwise, the random ssDNA sequences we think are unspecified ssDNA, such as Cy5-NN as a control probe in Figure 4, which may also bind to both proteins weakly and non-specifically, and in a non-stoichiometric manner.

4. There seems to be something wrong with Figure 4C because the FISH analyses for TDP-35 4FL with Cy5-NN seems to be missing, and in the first lane of figures it is labelled Cy5-CA that should belong to Figure 4B.

A: In Figure 4C, we designed this experiment to compare the sequestration effects by the intrinsic RNAs in the cells overexpressing TDP-35. The CA repeat oligomer (Cy5- CA probe) was designed to detect the TG-repeat RNA that has the capacity of enhancing co-localization of TDP-35 with endogenous TIA1, whereas the control oligomer (Cy5- NN) with random sequence does not have this effect. This also indicates the specific RNA sequences (detected by specific probe) promote sequestration of endogenous TIA1 by the TDP-35 inclusions.

5. The blots in Figure 5B and 5C are overexposed and it is hard to see the differences which are plotted in Figure 1D. In these western blots, the intensity of PRKRA does not really seem to decrease. Because the difference in protein intensity is rather small (Figure 5A) have the authors also lookd at the PRKRA mRNA levels which might yield more convincing results?
A: We know that some blots shown in Figure 5 is something overexposed. We have provided an image with low exposure replacing the overexposed one and labeling with Figure 5D in the revised version. Actually, we have had triplicate experiments on the protein level alteration by TDP-35 and analyzed statistically (Figure 5E). We also performed RT-PCR experiments to check the alteration of *PRKRA* mRNA level caused by TIA1 sequestration by TDP-35, which was provided in new Figure 5A.

6. Results in Figure 6 and 7 should also be complemented by co-trasfecting Flag-TDP- 35 4FL mutant. Also, authors should improve image intensity in Figure 7A as the cell images are mostly black.
A: We previously demonstrated that TDP-35 sequesters endogenous TDP-43 into cytoplasmic inclusions [Che-MX, FEBS Lett, 2015]. This study is to observe dynamic process of the sequestration in living cells. As we know that the 4FL mutant lost this capability of sequestering endogenous TDP-43 due to its deficiency of RNA binding. We understand that in Figure 7A the individual images are too small and weak in intensity. We have provided a set of images in Figure 7A with a little higher quality, in which the image arrangement and intensity have been significantly improved.

Reviewer 2 Advance Summary and Potential Significance to Field:
This study is a demonstration of how TDP-35 cytoplasmic aggregates can sequester certain RBPs, such as TIA1, via sequence-specific RNA association, and this causes a reduced function of TIA1 in the maturation of a particular RNA (PRKRA). Moreover, TDP-35 expression also sequesters endogenous TDP-43. It builds on previous work from the same lab showing that overexpression of TDP-35 forms cytoplasmic RNA-dependent inclusions in HEK293T and HeLa cells and can sequester TDP-43. Overall, the data are clearly presented and the conclusions are mostly reasonable. The authors main claim is that the formation of TDP-35 in pathological conditions will drive TIA1 sequestration (and other RBPs, including TDP-43), causing loss of function for these RBPs. However, the relevance of this study to ALS is difficult to reconcile. Specifically, there is a disconnect between the data/mechanism proposed here and the observation that TDP-43 inclusions in ALS patient motor neurons are devoid of RNA (Mann et al, 2019).

A: TDP-35 still retains its RNA-binding ability, and then is able to form cytoplasmic inclusions and sequester other RBPs (e.g. endogenous TDP-43, TIA1, PABPC1) as assisted by RNA binding, but its RNA-binding deficient 4FL mutant loses its ability to form cytoplasmic inclusions or sequester other RBPs. The Mann et al.'s work showed that the RNA-bound TDP-43 phase separates into SGs, but the RNA-free form experiences aberrant phase transition to TDP-43 proteinopathy as in the case of TDP- 25, another C-terminal fragment without RNA-binding. Interestingly, oligonucleotide RNA antagonizes the neurotoxic phase transition of TDP-43. It is possible that in the Mann's work the excess amounts of oligonucleotides bind to the C-terminal LCD region but not to the RRM domains. This kind of RNA binding might be rather weak and non-specific but with multiple binding sites in the LCD region. We have compared this two different points in the Discussion section. Our study demonstrates that specific RNA-binding to RRM domains is beneficial to inclusion formation and sequestration of other RBPs. We have designed a ssDNA sequence linking both the sequences of TDP-35 (TDP-43) and TIA1 binding specificities to simulate the cellular TDP-35/TIA1 complex mediated by specific RNAs.

Moreover, TIA1 aggregates have not been robustly reported in ALS patient neurons, even in a patient where a reportedly disease-causing mutation is present. Thus, a primary requirement is that the authors must comment on how the mechanism presented here correlates to what is known about ALS pathology. For example, does TDP-35 bear any post-translational modifications (ex.

phosphorylation, ubiquitination) that are known to be present in cytoplasmic TDP-43 inclusions?
A: The mechanism presented here describes sequestration of endogenous TIA1 and TDP-43 and other RBPs by the cytoplasmic TDP-35 inclusions, which may impair the function of the related proteins, especially pre-RNA splicing proteins. For example, decrease of the nuclear TDP-43 level may abolish the pre-mRNA splicing function and the downstream protein expression, which is relevant to ALS pathology as demonstrated in previous research. As for TIA1, although it is not correlated to the ALS proteinopathy directly, its sequestration may cause mis-localization from nucleus to cytoplasm, loss of the function in RNA maturation and impairment of cellular homeostasis. We have not performed characterization of the TDP-35 modifications, but with a bold deduction, TDP-35 may have at least a population of ubiquitination or phosphorylation if it forms misfolded aggregates in cytoplasmic inclusions.

Reviewer 2 Comments for the Author:
I offer several points to help strengthen this manuscript.

1. The authors state emphatically that TDP-35 colocalizes with RBPs TIA1 and PABPN1 in the cytoplasm, but not when the 4FL mutation is introduced that causes TDP-35 to lose its ability to bind RNA. Addition of quantification would better support this claim. Alternatively, the authors could soften the wording of their text.
A: We have quantified the decrease of the nuclear TIA1 level in images provided in Figure 1A and Suppl. Figure S2A in the revised version.

2. Can the authors see an interaction between TDP-35 and endogenous TIA1? (All IPs shown were using over-expressed, tagged TIA1.)
A: Yes, we have performed IP experiment showing that TDP-35 interacts with endogenous TIA1 indirectly as shown in Suppl. Figure S4, as well as the exogenously overexpressed TIA1 shown in Figure 3.

3. For the IPs, an important control would be to show a lack of interaction between TDP-35 4FL with TIA1 to support their claim that this interaction requires RNA- binding.
A: The controls are important to demonstrate the interaction between TDP-35 and TIA1 in IP experiments. Figure 3A shows the interaction between TDP-35 with TIA1 assisted by ssDNA. The experiments related to the 4FL mutant are shown in Figure 3B & 3C, suggesting that 4FL does not interact with TIA1, no matter the RNase A/ssDNA treatment is done or not. Also, the 4FL control was used for examining the association of endogenous TIA1 assisted by ssDNA, as provided in Suppl. Figure S4.

4. Since TIA1 is considered an important factor for stress granule assembly, does the sequestration of TIA1 interfere with stress granule formation in response to heat or arsenite? Are the kinetics the same? Also, does TDP-35 localize to stress granules?
A: TIA1 is a marker for SGs, and it is possible that TIA1 is sequestered into the SGs formed by heat or arsenite treatment. We suppose that the cytoplasmic TDP-35 inclusions (aggregates) are more condensed than the SGs, and their dynamic behaviors, sequestration kinetics, and even cellular impacts are also different. We are not sure whether TDP-35 localizes to the SG foci and whether sequestration of TIA1 interferes with SG formation, but it deserves to be investigated in future. Cytoplasmic full-length TDP-43 may have ability to phase separates into SGs in cells assisted by RNA binding, because its N-terminal domain forms stable dimer to prevent it from aggregation. However, our previous work implied that TDP-35 is prone to aggregation due to its loss of the N-terminal dimeric domain [Jiang-LL, *Sci Rep*, 2017]. In this case, cytoplasmic TDP-43 but not TDP-35 may have a possibility to localize in the SGs. In a word, the TDP-35 inclusions are much different from the heat- or arsenite-induced SGs in condensation and mobility.

5. Does the level of PRKRA mRNA change when TIA1 is sequestered? This would be a good measure that would strengthen the data. Also, on page 10, the authors make a claim that both TIA1 and TIAR are impacted – but no data for TIAR is provided. However, given that the impact on PRKRA protein levels is modest, does this not suggest that TIAR may be compensating? Have the authors investigated TIAR? Is there an increase in TIAR protein?
A: We have performed RT-PCR experiment to examine the effect of TDP-35 over- expression on the alternative splicing level of *PRKRA* mRNA. The data showed that the TDP-35 over-expression does

reduce the splicing efficiency of *PRKRA* mRNA, as provided in Figure 5A in the revision. This implies that sequestration of TIA1 by the TDP-35 inclusions may have impact on the alternative splicing of *PRKRA* mRNA.

TIAR is a homologue of TIA1 that may compensate TIA1 for functioning in RNA maturation. The modest impact on PRKRA expression may not be explained by the compensating effect of TIAR, because we think that TIAR as well as TIA1 could be sequestered into the cytoplasmic TDP-35 inclusions, although we have not detected TIAR in this study. The sequestration of TIAR and other TIA1-like proteins and its consequent dysfunctional effect should be investigated in future. It is noted that the sequestration effect is something like RNAi knockdown on the function of the biomolecules but generally with a modest effect, because the sequestration efficiency is much lower than that of RNAi.

6. The authors have presented a mechanism by which TDP-43 is sequestered into the TDP-35 inclusions over time. Does the same happen with TIA1?

A: TDP-43 is a nucleus-addressed RBP. We chose endogenous TDP-43 as a model molecule for visualizing its sequestration by the cytoplasmic TDP-35 inclusions directly and kinetically. Perhaps it is a good system for live-cell imaging, and we have successfully obtained a movie for endogenous TDP-43 being sequestered into cytoplasmic TDP-35 inclusions from original nuclear localization. However, endogenous TIA1 is localized both in nucleus and cytosol. Although we think that it may happen to TIA1 to be sequestered into cytoplasmic inclusions, live-cell imaging of the kinetic processes is rather complicated and perhaps more difficult, because a cytoplasmic fraction of TIA1 will somehow interfere with the observation of the foci formation of sequestered TIA1.

7. Sequestration of endogenous TDP-43 should similarly impact TDP-43 mRNA targets. Can the authors provide evidence that a well-known TDP-43 target mRNA is disrupted by TDP-35 expression? (ex. POLDIP3 or similar).

A: TDP-43 is reported to regulate several target mRNAs, such as *POLDIP3* [Colombrita-C, *BBA*, 2015; Cortese-C, *Eur J Neurol*, 2018]. These target mRNAs could be applied to indicate sequestration and dysregulation of TDP-43 by cytoplasmic TDP- 35 inclusions. In our previous work, we applied a CFTR exon 9 minigene (TG13T5) to detect the dysfunction of endogenous TDP-43 by TDP-35 [Che-MX, *FASEB J*, 2011].

8. Many blots seem oversaturated (ex. Fig 5 PRKRA). Could the authors insert lower exposure images for these blots.

A: We have replaced the blots for PRKRA level with the low-exposure blots and provided in Figure 5D in the revised version.

9. The introduction should consider that TDP-35 can also arise due to an alternative translational start, and not just truncation of full length protein.

A: Yes, we acknowledge that the TDP-35 and TDP-25 fragments may originate from an alternative translation [Xiao-S, *Acta Neuropathol*, 2015], which is also referred in the Introduction section.

10. The discussion is quite repetitive with the results. It could be streamlined, making room for the authors to better integrate the data with what is known in the field.

A: This work focuses on two points, one is directly visualizing the dynamic sequestration of full-length TDP-43 by TDP-35 in living cells, the other is to characterize the RNA-assisted sequestration of TIA1. Actually, we it is not necessary to compare the TDP-35 inclusions with the TIA1-positive SGs induced by arsenite or heat treatment. The TDP-35 inclusions are caused by NTD truncation, which may be different to the arsenite treatment. These are different causes and perhaps lead to different consequences, so to our current knowledge, they might be incomparable. We have modified the discussion in some places, comparing the different RNA impacts on the formation of inclusions and SGs.

Second decision letter

MS ID#: JOCES/2021/259380

MS TITLE: RNA-assisted sequestration of RNA-binding proteins by cytoplasmic inclusions of the C-terminal 35-kDa fragment of TDP-43

AUTHORS: Lei-Lei Jiang, Wen-Liang Guan, Jian-Yang Wang, Shu-Xian Zhang, and Hong-Yu Hu
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers recognize that most of their initial criticisms have been addressed in your revised manuscript. However, reviewer #2 still raised issues that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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Authors have answered well this reviewer's comments and the manuscript has been considerably strengthened

Comments for the author

No more issues can be raised

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2. TDP-43 live imaging clearly shows sequestration of TDP-43 to TDP-35 granules. As suggested by the authors, based on their prior work (Che-MX, FASEB J 2011), we could infer that this leads to a loss of TDP-43 function in regards to a CFTR minigene.

Comments for the author

Overall, I encourage revision, but with the reviewers fully addressing these considerations:
1. Data provided to claim loss of Tia1 function is weak. The quantification of RT-PCR products in Figure 5A is unclear. To detect a change in splicing, one should compare the ratio of the major isoforms, not compare the levels of each form to the housekeeping control (GAPDH). Moreover, the gel image provided does not show clear differences. Also, there is another band (non-specific or other isoform)… Authors should either explore this with qRT-PCR probes or select another transcript that should be implicated with Tia1 loss of function.
2. While I appreciate the authors' point that TDP-35 aggregates are not stress granules, the authors have misinterpreted my earlier comment. Restated:
Tia1 has a well-defined function in stress granule formation. Thus, given their model of Tia1 loss of function, it is reasonable to evaluate whether stress granule formation is impaired when Tia1 is sequestered in TDP-35 inclusions.
Specifically, does TDP-35 expression/Tia1 sequestration correlate with alteration in the size/number/dynamics of stress granules in response to a simple heat or arsenite pulse?
3. 3The authors argue that Tia1 sequestration into TDP-35 aggregates requires a specific type of RNA. While the data provided are convincing, the relevance of these data to the greater context of ALS pathogenesis is really not clear. As highlighted previously, there is no concrete evidence that RNA localizes to the TDP-43 inclusions observed in post-mortem material. Moreover the authors have declined to provide evidence that the TDP-35 aggregates they describe here have post-translational modifications (ie. ubiquitination or phosphorylation) that would at least make some connection to TDP-43 inclusions observed in patient material. Indeed, they argue that they may not be related.... The discussion has been modified somewhat to address this point but it feels insufficient.

Second revision

Author response to reviewers' comments

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A: As in the reference [Meyer-C, et al. (2018), *Mol Cell*, 69: 622], there are three spliced isoforms of *PRKRA* mRNA labeled with I, II and III respectively in Fig. 5A (only two spliced isoforms labeled in the Meyer paper). The major isoform (I) denotes the mature *PRKRA* mRNA, which can translate into the PRKRA protein. The isoform III is relatively abundant, but in the Meyer paper, it was less abundant. The slightly different splicing patterns may originate from different cell lines or cell status. We have provided a revised Fig. 5A with graphs showing changes of the spliced (isoform I) abundance and the total mRNA caused by TDP-35 over-expression.

TIA1/TIAR not only regulates alternative splicing of *PRKRA* mRNA, but also affects its mRNA stability [Meyer-C, et al. (2018) *Mol Cell*, 69: 622]. So we could not evaluate the splicing efficiency by estimating the ratio of spliced amount over total mRNA (sum of all spliced and unspliced isoforms). We could just analyze and compare the major spliced isoform and the total mRNA, and evaluate the effect of TDP-35 over-expression upon them. Herein GAPDH was still applied for loading control.

The Meyer paper showed effect of TIA1/TIAL1 over-expression on the splicing of *PRKRA* mRNA, which may cause large changes due to its over-expression and direct function. We just adopted this system to detect the effect of TIA1/TIAR sequestration by TDP-35 over-expression indirectly. This effect is relatively moderate, because the sequestration is somewhat not much efficient; sometimes only a small population of TIA1/TIAR have been sequestered into inclusions to be functional loss.

We have considered using qRT-PCR to detect functional loss of TIA1/TIAR in this PRKRA system, however, it is difficult to design specific primers for detecting each splicing isoforms. Actually, qRT-PCR can only apply to quantitate total mRNA, but not to study mRNA splicing. We selected PRKRA as an indicator for functional loss of TIA1/TIAR, because its expression can be detected both in mRNA and protein levels. Other transcripts may be applied, but we are not sure to get evident data for TIA1 sequestration.

2. While I appreciate the authors' point that TDP-35 aggregates are not stress granules, the authors have misinterpreted my earlier comment. Restated: Tia1 has a well-defined function in stress granule formation. Thus, given their model of Tia1 loss of function, it is reasonable to evaluate whether stress granule formation is impaired when Tia1 is sequestered in TDP-35 inclusions. Specifically, does TDP-35 expression/Tia1 sequestration correlate with alteration in the size/number/dynamics of stress granules in response to a simple heat or arsenite pulse?

A: Yes, we had misunderstood the earlier comment. Since TIA1 may play a function in stress granule (SG) formation, sequestration of TIA1 into the TDP-35 inclusions (aggregates) may interfere with the formation of SGs upon heat or arsenite treatment. This is perhaps an interesting experiment for testing functional loss of TIA1, demonstrating our previous hypothesis that sequestration of TIA1 may impair its normal function in stress response. This point is added into the Discussion section. Thanks for the suggestion, our future work can apply this SG formation system to evaluate the functional loss of TIA1 upon sequestration by the TDP-35 inclusions.

3.3 The authors argue that Tia1 sequestration into TDP-35 aggregates requires a specific type of RNA. While the data provided are convincing, the relevance of these data to the greater context of ALS pathogenesis is really not clear. As highlighted previously, there is no concrete evidence that RNA localizes to the TDP-43 inclusions observed in post-mortem material. Moreover, the authors have declined to provide evidence that the TDP-35 aggregates they describe here have post-translational modifications (ie. ubiquitination or phosphorylation) that would at least make some connection to TDP-43 inclusions observed in patient material. Indeed, they argue that they may not be related.... The discussion has been modified somewhat to address this point, but it feels insufficient.

A: Both TDP-35/TDP-43 and TIA1 are RNA-binding proteins, specific types of RNA may play important roles in biomolecular condensation and sequestration of other RBPs in RBP/RNA assemblies (SGs, inclusions, etc.). It is evident that specific RNA binding assists in TDP-35 inclusion formation and sequestration of TIA1, however, the specific RNA was not detected in post-mortem material, possibly due to its instability or technical difficulties. We have provided some interpretation for this point in the Discussion section.

Post-translational modifications of TDP-43 [Buratti-E (2018) *Expert Opinion on Therapeutic Targets*, doi:10.1080/14728222.2018.1439923], such as ubiquitination [Neumann-M et al. (2006) *Science*, 314: 130; Kakihana-T et al. (2021) *iSceince*, 24: 02733] and phosphorylation [Inukai-Y et al. (2008) *FEBS Lett*, 582: 2899; Hasegawa- M et al. (2008) *Ann Neurol*, 64: 60], have been identified in cells and animals. Our recent study has also characterized the O-GlcNAcylation of TDP-43, which may promote pre-mRNA splicing activity and suppress its proteinopathies [Zhao-MJ, et al. (2021) *EMBO Rep*, 22: e51649]. Actually, phosphorylation of TDP-35 has been identified in the inclusion bodies [Nishimoto-Y et al. (2010) *J Biol Chem*, 285: 608], but in this research this point has not been involved in our discussion on RNA-assisted sequestration of RBPs. We speculate that these modifications especially ubiquitination and phosphorylation may also occur in TDP-35 during its fragmentation from TDP-43, aggregation and inclusion formation. Moreover, since TDP-43 modification occurs ubiquitously, the modified TDP-43 is supposed to be sequestered into the cytoplasmic TDP-35 inclusions in patient material, which may be implicated in the disease pathology. Whether TDP-35 modification has impact on its sequestration of TIA1 and other RBPs and how it is related to disease pathology remain to be investigated.

Third decision letter

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MS TITLE: RNA-assisted sequestration of RNA-binding proteins by cytoplasmic inclusions of the C-terminal 35-kDa fragment of TDP-43

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ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.