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Loss of TDP-43 oligomerization or RNA binding elicits distinct aggregation patterns

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Hi Magda,

Thank you for submitting your MS to the EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see the referees find the analysis interesting and are supportive of publication here. However, they also raise a number of different issues that should be resolved. It would be helpful to discuss the raised points further and I am available to do so via email or video. Let me know when is a good time for you.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

I thank you for the opportunity to consider your work for publication. I look forward to discussing the revisions further.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

I have attached a guide with helpful tips on how to prepare the revised version

I realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (22nd Sep 2022).

As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work, to discuss how to proceed.

If you require more time to complete the revisions let me know as as I can grant an extension.

Referee #1:

The manuscript by Perez-Berlanga et al. reports the exciting finding that TDP-43 oligomerization and RNA-binding modulates TDP-43 aggregation. TDP-43 is a major deposited protein in neurodegenerative disorders, in particular ALS and FTD, and the pathological events that lead to its cytosolic deposition are still not well understand. Therefore, the findings by Perez-Berlanga et al. should be a great interest to the neurodegeneration /protein aggregation community.

There are, however, a few significant issues that need to be addressed before publication. Before listing the major and minor points, I would like to point out one major issue: The majority of experiments were performed under conditions where TDP-43 WT and the oligomerization / RNA-binding-deficient mutants show vastly different protein levels (due to the more rapid degradation of the mutants). So the described loss of-function (e.g. in splicing regulation) or loss of partitioning into membrane-less organelles (e.g. stress granules or Cajal bodies) could simply be due to the reduced levels. Therefore, at least a few key experiments should be performed under conditions where this problem is circumvented and similar levels are reached, and this major caveat should be clearly pointed out to the reader. Moreover, the manuscript could benefit from some "data trimming", as not all data shown seems necessary for the main story, which makes the manuscript not that easy to follow and a bit lengthy. I believe the manuscript would benefit from focusing the story on the exciting main finding, namely that TDP-43 oligomerization

and RNA-binding govern TDP-43 stability (Fig. 1) and that preventing the degradation of such oligomerization/RNA-binding deficient mutants by proteasome inhibition leads to the formation of cytosolic or nuclear TDP-43 aggregates (Fig. 7-8).

Specific points:

(1) Wording in title and abstract and beginning of discussion: I don't see that the manuscript anywhere shows that TDP-43 oligomerization and RNA-binding "are co-dependent" (title) or "interconnected" (discussion on p. 9) or that TDP-43 oligomerization "is connected to, and conformationally modulated by, RNA-binding" (abstract). The short title "Oligomerization modulates TDP-43 aggregation patterns" reflects the main finding much better. The authors should consider an alternative title that better reflects their main finding, e.g. "Loss of oligomerization and RNA-binding elicits distinct TDP-43 pathologies" and these main findings should also be brought out better in the abstract, which does not read very clear so far.

(2) Figure 1 and Figure S1: It would be nice to show the endogenous TDP-43 levels in both the WT and mutant-expressing cell lines by Western blots - authors comment that WT GFP-TDP-43 displayed a 4-fold increase compared to endogenous TDP-43 (Fig. S1D), so it would be informative to make the same comparison of steady state levels for all 4 proteins (WT, 6M, RRMm, 6M/RRMm). They later on do show endogenous TDP-43 levels when talking about autoregulation in Fig. 6, but I was missing this information when going over Fig. 1 and S1.
With respect to Fig. 1F: Is there a reason why the RRMmut was not included in the CD spectroscopy analysis? Was it not possible to purify the RRMmutant proteins? When looking at the NMR data in Fig. S1J (overlay of HSQC spectra from RRMs (WT vs. mutant), it looks as if there is not a complete overlap of signals (many red dots shifted compared to the blue dots) - authors should comment on this and explain why from these spectra one can conclude that the RRM mutations do not interfere with folding of TDP-43, as stated on page 3.

(3) Figure 3C: It is rather unusual to leave on the MBP tag when performing phase separation assays with TDP-43-MBP (see e.g. two other papers in the EMBO J. that used the same construct, PMID: 29438978 and PMID: 35112738), this is probably also the reason why relatively few condensates are seen for WT TDP-43 at 10 µM. The in vitro phase separation assays should be performed with removal of the MBP tag (TEV cleavage) to assess TDP-43 phase separation in the absence of the MBP tag. If the RRMmut proteins can be purified as recombinant proteins and are available in the lab, it would be informative to also include these mutants in the analysis and compare phase separation behavior of all three mutants studied throughout this paper.

(4) As pointed out above, THE major issue is several experiments (e.g. in Fig. 3, 5, 6) is that the observed effects cannot be clearly attributed to the introduced mutations, but could simply come from the much lower protein levels of the mutant proteins. In particular it is known that phase separation and partitioning into membrane-less organelles is highly dependent on the protein concentration, hence the reduction in nuclear droplets (Fig. 3E/F), Cajal body or paraspeckle localization (Fig. 5E/F), or stress granules localization (Fig. 6G/H) could be due to the much lower protein levels of the mutants. The same issue exists when analyzing splicing regulation of RNA targets (Fig. 6) - the lack of splicing functionality could simply be caused by the reduced levels - I believe this is an issue to be addressed (see suggestions below), as the knockdown control experiment shown in Fig. S6D is not ideal, as done under different experimental conditions (knockdown by siRNAs might only become effective later on, when splicing regulation has already occurred).
To address this major issue, the authors should come up with a strategy that allows them to compare the different proteins at the same levels. I could envision the following options: a) looking at early timepoints (e.g. 4h) after dox induction, when the levels are still somewhat similar? b) titrating the dox concentration massively down for the wild-type, to achieve similar steady state protein levels; c) transiently transfecting the constructs (possibly using a lower DNA concentrations for the WT) to achieve similar expression levels; d) in the microscopy-based assays, would it be possible to find cells that have similar fluorescence intensities (based on quantification of the mean fluorescence intensity) and focus analyses on these cells only?
It would be important to replicate at least some key experiments with such a "same protein level" strategy, to confirm that the observed effects are not just due to reduced levels.

(5) There seem to be some discrepancies in Fig. 4 (PLA data): For endogenous TDP-43, the PLA signal is not enriched in nuclear bodies (Fig. 4E), whereas when the PLA assay is performed with a GFP antibody on cells expressing GFP-TDP-43 WT, the PLA signal is mainly seen in nuclear droplets (Fig. 4F). Can the authors explain this discrepancy, or provide additional controls that the PLA signals obtained with the TDP-43- or GFP antibody are specific?

(6) A discrepancy exists between the oligomerization data shown in Fig. S2C and Fig. 3G: In Fig. S2C, the RRMm protein does not show any higher order oligomers (e.g. dimers) after DSG cross-linking, whereas in Fig. 3G at least some dimers/oligomers are visible. A similar discrepancy exists for the 6M mutant: Why does the 6M mutant in Fig. S2C show similarly many dimers/oligomer as WT, but not in Fig. 3G (or published data by the same team, shown in Afroz et al. 2017)? Is there a labelling error in Fig. S2C, or how can these variable results be explained?

(7) Another discrepancy can be noted between Fig. 6G (T10-TDP-43 with 6M seems exclusively nuclear) and Fig. 4H, where the same construct seems quite strongly mislocalized to the cytoplasm. Can this be explained by experimental conditions? What is the reason why SG recruitment was analyzed with the GFP triFC assay and not simply by analyzing SG recruitment of the different GFP-tagged mutants or NLS-mutant proteins shown in Fig. S6G? The latter could be a good alternative, and

(8) The data shown in Fig. 7/8 are very impressive, and it would be nice to demonstrate that the observed relocalization of mutant TDP-43 goes along with a stabilization of its levels after MG132 treatment, e.g. by doing a Western blot or CHX-chase experiment -/+MG132 - does the proteasome inhibitor restore the levels of the mutant proteins to WT levels and prevent rapid degradation of the mutants?

Finally, some non-essential suggestions that could enhance the manuscript or possibly could be done in a follow-up study (just curiosity-based questions):
• Do the mutant TDP-43 inclusions (Fig. 7, 8) become C-terminally phosphorylated or poly-ubiquitinated, as in ALS/FTD patients?
• It is interesting that the cytoplasmic inclusions are surrounded by a vimentin cage (aggresome marker) - is there any evidence for such a vimentin cage or other aggresome markers in human post-mortem material of ALS/FTD patients)?

Minor points:
(9) Figure labeling could be a bit more self-explanatory, e.g. what are the red and yellow circles in Fig. S1A? In Fig. 7 A,C it would be nice to indicate in the figure that MG132 was present for 24h in all conditions, as it helps the reader to understand the result better at first glance. In Fig. S6F it would be nice to see at one glance that here a set of mutNLS proteins was used (like indicated in Fig. S6G/H).

(10) Legends: Some important experimental details are missing from some legends, e.g. what timepoint is shown in Fig. 8B and Fig. 8D/E?

(11) Use of certain phrases and designation of inhibitors:
- Page 4, last paragraph: 1,6-hexanediol should not be introduced as "an LLPS-suppressive alcohol", as 1,6-HD does not generally suppress all LLPS, but only weak hydrophobic interactions (e.g. LLPS driven by polar or charged interactions will NOT be affected by 1,6-HD). This compound should also be used with great caution, as it has been shown to disrupt nuclear transport (PMID: 17418788) and more recently kinase and phosphatase activity (PMID: 33814344), so effects seen with this drug could also be due to these general impairments instead of a direct LLPS suppression...
- Figure 2J/K: Ivermectin is mentioned on page 4 as a "nuclear import inhibitor", it would be informative to say what precisely it inhibits, and why it was only used on TDP-43 WT. Alternatively, this experiment could be taken out of the manuscript, as it does not seem essential to support their main story and distracts rather than contributing much information.
- Page 8, end of second paragraph: "several classes of proteasome inhibitors, but not an autophagy one". It would be more informative to say that bafilomycin A was used (an acidification inhibitor, NOT a specific autophagy inhibitor).

(12) Some fluorescence microscopy figures could benefit from display in black-and-white instead of color, e.g. the green signals in Fig. 3E or 4C (HEK cells), or the nuclear body markers shown in purple in Fig. 5 look very weak and are hard to see, especially in a printed version. The signals will be better visible if shown in black-and white.

(13) Scale bar missing from Fig. 6G (legend says 10 µm, but no bar shown in the figure).

(14) Legends in Fig. S7C and D does not fit to figure, so I believe C and D were swapped. Same for the legends to Figure 2F and H.

(15) Page 9, end of first paragraph: I believe the reference to Fig. S7B (human neuron data) is not correct, as HEK293 cell data are shown in Fig. S7B. This correct figure number should be given here.

Referee #2:

In their manuscript "TDP-43 oligomerization and RNA binding are codependent but their loss elicits distinct pathologies", Pérez-Berlanga et al. decipher the relation between TDP-43 RNA binding and oligomerization and its function in splicing and stress granule association. Using TDP-43 mutants that are deficient in RNA binding and/or oligomerization, the authors find that oligomerization and RNA binding effect the nuclear:cytoplsmic distribution of TDP-43, the formation of nuclear speckles, and the types and localization of 'inclusions' formed. The findings presented bring new insights in the complex biology of TDP_43 and help to understand the differential functions that TDP-43 has, thereby also contributing to the understanding of TDP-43 pathology in FTD/ALS.

Major points:
- The Manuscript is well written and the data well presented, and the authors succeed to address the complexity of TDP-43 biology by looking at the implications of TDP-43 in cell biology and stress response. Because the results are complex, it is sometimes a bit hard to follow the rationale / story line of the manuscript. To make it easier to for the reader to understand, I would suggest to start the manuscript with the impact of oligomerization and RNA binding on the important biological functions of TDP-43, namely splicing and stress granule association (currently Fig.6) and. , because this establishes the relevance of the

addressed questions and the model of TDP-43 mutants. Then move to the mechanistic part of the manuscript, in which the authors show the nuclear body association, LLPS, and oligomerization, which help to build a model for the functional role of TDP_43 RNA binding and oligomerization. Then, the authors could move on to effects in the pathological context and show changes in the nucleocytoplasmic distribution and inclusion distribution and kind. This is a suggestion, which in my eyes would help to give structure to the manuscript for a better understanding of the complex data.

- Lane 53+: The authors suggest that passive diffusion out of the nucleus is increased when RNA binding or oligomerization is inhibited in TDP-43 mutants. This interesting idea seems supported by nuclear etention of these TDP-43 variants upon chemical crosslinking. However, to further validate this idea without the potential confound of unspecific protein crosslinking, passive diffusion and nuclear transport can be selective blocked e.g. by WGA before preparing the nuclear enriched fraction.

- ActD treatment may put stress on cells and induce stress-related translocation of TDP-43 into the cytoplasm, similar to H2O2 treatment. Also, ActD may reduce the amount of available nuclear transport factors and thereby inhibit nuclear. Import of TDP-43. Please show that the import of the different TDP-43 constructs are similar at base line, that the import of other cargo is generally intact after ActD treatment, and that the levels of importin-alpha/beta are not changed upon ActD treatment. Please also show the depletion of RNA from the nucleus upon ActD treatment.

- Lane 92/93, Figure S3A/B: the sizes of TDP-34 nuclear 'droplets' seems very small, sometimes only a few pixel. A size/volume analysis becomes unreliable at this resolution and contrast. Please show in supplemental data how the analysis was done

- LLPS in vitro data in Figure 3 are performed with TDP-43 carrying a MBP tag, which inhibits TDP-43 LLPS. These experiments should be repeated after cleavage of the tag, and, if possible, also for the other constructs. How does RNA binding influence TDP-43 LLPS when oligomerization or RNA binding is inhibited?

- In the title, the authors talk about 'distinct pathologies' of TDP-43 but they do not show any evidence of human pathology related TDP-43. In their cell lines, they observe different inclusions located in nucleus or cytosol. Cytosolic inclusions are appearing upon proteasome inhibition in aggresomes in 6M mutants that are anyways primarily located in the cytoplasm. This is kind of expected from previous studies showing that accumulation of (aggregating) proteins in the cytosol upon proteasome inhibition get sorted into the aggresome. Whether this is also the case in TDP-43 pathology in the human brain remains speculative. Therefore, I ask to remove the word 'pathologies' from the title and replace it by 'cellular inclusions'.

Minor points:
- Many figure panels showing fluorescent images have a low contrast and are dark, and are therefore hard to see. Examples: 2B, 4E, 5 all panels, 8B. I. Suggest the authors to either switch to grey scale. Images for 1-color images, or enhance the contrast where possible, e.g. when showing distribution or nuclear/cyt ratio.
- To proof the robustness of the observations on TDP-43 foci in the nucleus - e.g. for colocalization with Cajal body markers, number and size analysis - it would be helpful if the authors could provide galleries of more example nuclei images in the supplemental data.
- Multiple figure panels: please show all data points or non-truncated violin plots in graphs
- Lane 44: the authors talk about 'nuclei isolation' and the 'nuclear fraction' although they work with 'nuclear enrichment' and 'nuclear enriched fractions' if I understand right from the methods. This may seem like a trivial comment but is important when interpreting the results since cytosolic TDP-43 may in part 'contaminate' the. Nuclear enriched fraction.
- Figure 4, and matching text part: the authors state that TDP-43 forms 'conformational distinct oligomers', however, they do not apply structural methods to test TDP-43 conformation or oligomer structure. The word 'conformation' refers to protein structure or folding states. The authors do observe specific stoichiometries of oligomers though, which I guess they are referring to. Please correct the wording accordingly.
- Instead of referring to 'monomeric GFP-TDP-43' and 'RNA-binding deficient GFP-TDP-43' in the text, it would help the understanding if the authors stick to the abbreviations/nomenclature introduced in the beginning of the manuscript: WT, 6M, RRMm and 6M&RRMm
- Lane 268/269: the oligomerization domain of TDP-43 may catalyze incorporation of TDP-43 into Canal bodies and paraspeckles, independent of actual oligomerization. The authors should either test this or rephrase the text, since it it not clear whether TDP-43 oligomerization is really necessary for joining these nuclear bodies.
- Lane 272-279: I suggest to speak about TDP-43 foci instead of droplets since the liquid nature of the TDP-43 species is not proven or tested. Furthermore, the unidentified TDP-43 foci containing RRMm are likely to contain RNA - like all known nuclear bodies - which may not bind TDP-43 specifically but still be important for the formation of the formations. Polyanionic RNA can coordinate and recruit proteins into condensates even without specific binding activity.
- Figures S4E, S5B would benefit from a logarithmic y-axis
- Figure S2C: why does the RRMm but not the 6M mutant has oligomeric TDP-43? shouldn't that be the opposite way? Is the western blot labeling mixed up?

Pérez-Berlanga et al: Point-by-point response to the referees' comments

Referee #1:

The manuscript by Perez-Berlanga et al. reports the exciting finding that TDP-43 oligomerization and RNA-binding modulates TDP-43 aggregation. TDP-43 is a major deposited protein in neurodegenerative disorders, in particular ALS and FTD, and the pathological events that lead to its cytosolic deposition are still not well understood. Therefore, the findings by Perez-Berlanga et al. should be a great interest to the neurodegeneration /protein aggregation community.

We gratefully acknowledge the referee's positive assessment of our work.

A. There are, however, a few significant issues that need to be addressed before publication. Before listing the major and minor points, I would like to point out one major issue: The majority of experiments were performed under conditions where TDP-43 WT and the oligomerization / RNA-binding-deficient mutants show vastly different protein levels (due to the more rapid degradation of the mutants). So the described loss-of-function (e.g. in splicing regulation) or loss of partitioning into membrane-less organelles (e.g. stress granules or Cajal bodies) could simply be due to the reduced levels. Therefore, at least a few key experiments should be performed under conditions where this problem is circumvented and similar levels are reached, and this major caveat should be clearly pointed out to the reader.

We thank the referee for pointing out this critical issue that we have considered throughout our studies. Please see **point #6** below for a detailed response. In brief, to address this issue we took the following approaches:

1. We have performed experiments with equal protein levels of the variants, which occurs after 4hrs of expression. This was done for assessing the TDP-43 oligomers biochemically (**Figures 3H-I, Figure S3G**), for quantifying TDP-43 nuclear droplets (new **Figures S3D-E**) and for variant incorporation into nuclear bodies (new **Figure S5E-F**) and stress granules (new **Figure S6I-J**).

2. We have manually selected cells with equal protein levels of the variants on imaging. This was done for quantifying the levels of TDP-43 dimers by PLA in **Figure 4F** and GFP complementation in **Figures 4H, and S4F**.

3. We have correlated the observed phenotype with the protein levels of each variant. This was done for quantifying the droplets in **Figures 3F and** new **S3E**, the TDP-43 dimers by PLA in **Figure 4G** and the GFP complementation in **Figure S4G**.

4. We have used siRNA knockdown of WT GFP-TDP-43 simultaneously with doxycycline induction to show that similarly low levels of the WT protein are sufficient for autoregulation (**Figure S6D**), while the

monomeric or RNA-binding-deficient variants are not. Reversely, we have previously shown that strong overexpression of our monomeric (6M) variant does not support splicing regulation (PMID: 28663553).

B. Moreover, the manuscript could benefit from some "data trimming", as not all data shown seems necessary for the main story, which makes the manuscript not that easy to follow and a bit lengthy. I believe the manuscript would benefit from focusing the story on the exciting main finding, namely that TDP-43 oligomerization and RNA-binding govern TDP-43 stability (Fig. 1) and that preventing the degradation of such oligomerization/RNA-binding deficient mutants by proteasome inhibition leads to the formation of cytosolic or nuclear TDP-43 aggregates (Fig. 7-8).

We thank the referee for this comment, which we have considered. We agree that the role of oligomerization and RNA binding in TDP-43 stability and in the formation of different types of aggregates is exciting and must be highlighted in our manuscript. We have now done this by strongly revising the title and abstract of the manuscript (see also **point #1** below). However, we think that the data shown in **Figures 2-6** are equally important for understanding the role of these different modalities of TDP-43 on its physiological functions and eventually on the formation of distinct aggregates. We are afraid that omitting the data showing the role(s) of oligomerization in subcellular localization (**Figure 2**), LLPS (**Figures 3-5**) and splicing regulation (**Figure 6**) would significantly diminish the value of our study, as these are important pieces of information leading to our conclusion of the distinct pathways of aggregation, among others. We hope that the referee can agree that including the data is the preferred strategy.

Specific points:

1. Wording in title and abstract and beginning of discussion: I don't see that the manuscript anywhere shows that TDP-43 oligomerization and RNA-binding "are co-dependent" (title) or "interconnected" (discussion on p. 9) or that TDP-43 oligomerization "is connected to, and conformationally modulated by, RNA-binding" (abstract). The short title "Oligomerization modulates TDP-43 aggregation patterns" reflects the main finding much better. The authors should consider an alternative title that better reflects their main finding, e.g. "Loss of oligomerization and RNA-binding elicits distinct TDP-43 pathologies" and these main findings should also be brought out better in the abstract, which does not read very clear so far.

Our response: Following the reviewer's suggestion we revised the title of the manuscript to the shorter version: "*Loss of TDP-43 oligomerization or RNA binding elicits distinct aggregation patterns*". In addition, as suggested, we thoroughly revised the abstract. The conclusion that TDP-43 oligomerization and RNA binding are "co-dependent" or "interconnected" derive from the data in **Figures 3H-I** and **Figures 4A-B** showing reduced TDP-43 oligomerization upon loss of RNA binding. Data showing conformational modulation of TDP-43 oligomers by their RNA binding status are displayed in **Figures 4F-G** and **S4C**. We have rephrased the text to emphasize the conclusions from these data in the corresponding Results section

(page 6, lines 377-381): *"This contrast between RRMm and the WT TDP-43 supports the notion of a distinct conformation of TDP-43 dimers in the absence of RNA (RRMm). Overall, detection and quantification of dimeric TDP-43 species by a combination of different imaging and biochemical methodologies supports the view that RNA binding is required for the proper orientation of TDP-43 dimers."*

2. Figure 1 and Figure S1: It would be nice to show the endogenous TDP-43 levels in both the WT and mutant-expressing cell lines by Western blots - authors comment that WT GFP-TDP-43 displayed a 4-fold increase compared to endogenous TDP-43 (Fig. S1D), so it would be informative to make the same comparison of steady state levels for all 4 proteins (WT, 6M, RRMm, 6M/RRMm). They later on do show endogenous TDP-43 levels when talking about autoregulation in Fig. 6, but I was missing this information when going over Fig. 1 and S1.

Our response: We thank the referee for the suggestion. We have now included information on the levels of the GFP-TDP-43 variants in respect to endogenous TDP-43 levels in the text (page 3, lines 143-146): "*However, despite equal RNA levels (**Figure S1B**), protein levels of the GFP-TDP-43 mutants were noticeably lower than their WT counterpart (**Figures 1B-C and S1E-G**), and displayed 1.5-, 1- and 0.5-fold levels compared to endogenous TDP-43 for GFP-TDP-43 6M, RRMm and 6M&RRMm, respectively (**Figure S1E-G**)*."

Additionally, the corresponding blot showing the levels of endogenous TDP-43 for each isogenic line both in the absence and the presence of doxycycline has now been included in new **Figure 1B**, and referenced accordingly in the text when describing the autoregulation properties of TDP-43 (page 8, line 641).

3. With respect to Fig. 1F: Is there a reason why the RRMmut was not included in the CD spectroscopy analysis? Was it not possible to purify the RRMmutant proteins? When looking at the NMR data in Fig. S1J (overlay of HSQC spectra from RRMs (WT vs. mutant), it looks as if there is not a complete overlap of signals (many red dots shifted compared to the blue dots) - authors should comment on this and explain why from these spectra one can conclude that the RRM mutations do not interfere with folding of TDP-43, as stated on page 3.

Our response: Here the referee's guess is correct. Recombinant full-length TDP-43-MBP RRMm was unfortunately not available due to solubility issues during its purification and therefore could not be included in CD spectroscopy experiments. Regarding the HSQC spectra of the TDP-43 RRMs, the chemical shift differences that are observed between the spectra recorded with the WT and the mutated protein (RRMm) are due to the mutations of the five phenylalanine residues to alanine in TDP-43. Each of these phenylalanines induces a change in the environment affecting all the residues located in proximity to these mutated amino acids. As the mutated residues are all aromatic, their substitution induces important chemical shift perturbations of the backbone amides of all these residues, which explains why the two spectra do not completely overlap. The fact that dispersed peaks are still present in the spectrum recorded with the mutated RRMs (RRMm) shows that the protein is folded and the dispersion is such that it indicates

the formation of α-helix and β-strand structures. In case of unfolding, all NMR signals would be observed around 8 ppm and would not be dispersed as it is observed with the mutated and the WT protein. We have now clarified this in the new legend of **Figure S1J**: "*(J) Overlay of 2D ^{1}H-^{15}N HSQC spectra from purified His-tagged, ^{15}N-isotopically labeled TDP-43 RRMs WT (blue) and RRMm RRMs (red). The presence of dispersed peaks in the spectra indicates that both WT and RRMm RRMs are folded, and are compatible with the formation of α-helix and β-strand structures. In case of unfolding, all ^{1}H NMR signals would pool around 8 ppm*."

4. Figure 3C: It is rather unusual to leave on the MBP tag when performing phase separation assays with TDP-43-MBP (see e.g. two other papers in the EMBO J. that used the same construct, PMID: 29438978 and PMID: 35112738), this is probably also the reason why relatively few condensates are seen for WT TDP-43 at 10 μM. The in vitro phase separation assays should be performed with removal of the MBP tag (TEV cleavage) to assess TDP-43 phase separation in the absence of the MBP tag.

Our response: The referee raises an important point, namely the influence of the MBP tag in the phase separation behavior of TDP-43. We originally chose this approach because droplets formed by recombinant full-length TDP-43 upon cleaving its solubility tag are rather unstable and rapidly mature to aggregates, so the window to observe phase separation prior to aggregation is rather short. Therefore, previously reported *in vitro* TDP-43 droplets do not usually show a purely round shape, reflecting not only a phase separated state but a mix between LLPS and droplet maturation. We note that other groups have also previously reported TDP-43 LLPS with the uncleaved solubility tag, including MBP (PMID: 30826182) and SUMO (PMID: 30100264).

However, to exclude that the solubility tag interferes with the LLPS ability of TDP-43 *in vitro*, we have followed the referee's suggestion and repeated the experiments, after removing the MBP tag upon treatment with TEV protease. These new experiments fully confirmed our original observations and our conclusion that NTD interaction and oligomerization is necessary for the phase separation of full length TDP-43 *in vitro*. We have now replaced the previous **Figure 3C-D** with the new data and revised the text corresponding to this experiment accordingly (page 5, lines 258-260): "*Indeed, at a reported physiological concentration of 10 μM (25, 56), purified full-length TDP-43 phase separated into droplets, which also dissolved in the presence of 1,6-HD (**Figure 3C-D and S3C**)*."

5. If the RRMmut proteins can be purified as recombinant proteins and are available in the lab, it would be informative to also include these mutants in the analysis and compare phase separation behavior of all three mutants studied throughout this paper.

Our response: As indicated in **point #3** above, we were not successful in purifying the full-length TDP-43-MBP with mutations in the RRMs (RRMm), due to solubility issues during its purification.

6. As pointed out above, THE major issue in several experiments (e.g. in Fig. 3, 5, 6) is that the observed effects cannot be clearly attributed to the introduced mutations, but could simply come from the much lower protein levels of the mutant proteins. In particular it is known that phase separation and partitioning into membrane-less organelles is highly dependent on the protein concentration, hence the reduction in nuclear droplets (Fig. 3E/F), Cajal body or paraspeckle localization (Fig. 5E/F), or stress granules localization (Fig. 6G/H) could be due to the much lower protein levels of the mutants. The same issue exists when analyzing splicing regulation of RNA targets (Fig. 6) - the lack of splicing functionality could simply be caused by the reduced levels - I believe this is an issue to be addressed (see suggestions below), as the knockdown control experiment shown in Fig. S6D is not ideal, as done under different experimental conditions (knockdown by siRNAs might only become effective later on, when splicing regulation has already occurred).

To address this major issue, the authors should come up with a strategy that allows them to compare the different proteins at the same levels. I could envision the following options: a) looking at early time points (e.g. 4h) after dox induction, when the levels are still somewhat similar? b) titrating the dox concentration massively down for the wild-type, to achieve similar steady state protein levels; c) transiently transfecting the constructs (possibly using a lower DNA concentrations for the WT) to achieve similar expression levels; d) in the microscopy-based assays, would it be possible to find cells that have similar fluorescence intensities (based on quantification of the mean fluorescence intensity) and focus analyses on these cells only?

It would be important to replicate at least some key experiments with such a "same protein level" strategy, to confirm that the observed effects are not just due to reduced levels.

Our response: The reviewer raises a very important point that we have thoroughly considered and addressed. We thank the reviewer for the long list of specific recommendations for experimental approaches that could help us address this point. As we detail below, we have followed each of these recommendations and have added new data to clarify this important point.

We agree with the reviewer that looking at the time point of 4 hours after protein expression induction by DOX (see **point #6a** above) or selecting cells with similar fluorescence intensities in imaging experiments (see **point #6d** above) is the fairest comparison between conditions, as this ensures comparable protein levels for the four TDP-43 variants used in our study (WT, 6M, RRMm and 6M&RRMm). Indeed, we have followed both of these approaches for key experiments, as the referee recommends. In the original manuscript we have used the 4 hours time point when studying the TDP-43 oligomerization status by DSG cross-linking, as is shown in **Figures 3H-I, S3G and 6I-K**. Furthermore, in the original manuscript, we have selected cells with comparable fluorescence intensities for analysis when measuring PLA signal intensity (**Figures 4F-G**) and GFP complementation (**Figures 4H-I and S4F-G**), both reporting on the dimerization capacity of the TDP-43 variants.

Moreover, we would like to emphasize that for the analysis of droplet counts mentioned by the reviewer, we correlated the droplet count to the nuclear TDP-43 variant levels in the same cell (see **point #6d** above) at 4 hours (new **Figure S3E**), or 48 hours (**Figure 3F**) of protein expression. From these plots, it is clear that at the same protein levels (nuclear TDP-43 levels on the x-axis) the droplet counts are comparable for GFP-TDP-43 WT and RRMm, whereas droplets are virtually absent in the monomeric TDP-43 6M condition. This proves that the absence of TDP-43 6M droplets is independent of the nuclear protein concentration of each variant, a result supported by the *in vitro* experiment that was performed at equal protein concentrations for WT and 6M TDP-43 protein (**Figure 3C-D**). Similar correlations of measured parameters to nuclear TDP-43 levels are shown for the PLA (**Figure 4G**) and GFP complementation (**Figure S4G**) signal, allowing the direct comparison of the four TDP-43 variants at the same protein levels.

To further address the reviewer's concern, we have combined multiple of the suggested approaches and added new experiments to the revised manuscript. We have repeated various experiments at the 4 hour time point, namely 1) the quantification of nuclear droplets formed by each of the variants (new **Figure S3D-E**), 2) the analysis of TDP-43 variant incorporation into Cajal bodies (new **Figures S5E-F**), 3) the incorporation of TDP-43 variants into stress granules using the isogenic TDP-43 mutNLS lines as a model (new **Figures S6I-J**, see also this referee's **point #9** below).

We have tried the titration of DOX suggested by the reviewer (see **point #6b** above). Unfortunately, changing the DOX concentration for GFP-TDP-43 WT (up to 1000-fold difference) has not been sufficient to achieve steady state protein levels similar to those of the mutants (data not shown). Additionally, different DOX concentrations in the medium of the four stable lines would introduce a variable between conditions that is not dependent on the expressed protein itself, since DOX has been shown to alter the metabolism and proliferation of HEK293 cells (PMID: 23741339), which could influence readouts.

Transient transfection of low amounts of plasmid by lipofection (see **point #6c** above) yields the same difference in protein expression as observed for the variants in the stable isogenic lines. Therefore, this approach does not solve the differential protein levels of the variants. Furthermore, the alternative use of different concentrations of DNA for transfection could result in different toxicity from the transfection itself (or other effects) between conditions, which may influence the results. Additionally, differences in the amount of transfected plasmid would make the cellular transcription and translation machineries work at different paces further complicating equal comparison between the conditions. Overall, we aimed at avoiding transient transfection when possible, due to the high protein overexpression that it results in, as protein concentration really influences TDP-43 oligomerization (PMID: 31118120).

Lastly, regarding the reviewer's comment on the unsuitability of knocking down (KD) GFP- TDP-43 WT with a siRNA targeted to GFP (siGFP) to achieve similar protein levels as the mutants to fairly compare their splicing activities, we would like to point out that the experimental conditions are indeed comparable. In our knockdown experiments, the induction by doxycycline was done simultaneously with the application of the

siRNAs to minimize the possibility of a transient high expression of WT protein leading to splicing regulation at seemingly reduced protein levels. In addition, in our previous study (PMID: 28663553), we analyzed several splicing targets of TDP-43 after transient transfection of WT or 6M variants and showed the lack of splicing regulation by 6M, despite the equally high levels of monomeric TDP-43.

Taken together, the old and newly added set of experiments using different approaches to obtain similar levels, or to account for different protein levels of the TDP-43 variants convincingly show that our conclusions (e.g. on LLPS properties and functionality) are not the mere result of reduced protein levels of TDP-43 6M, RRMm and 6M&RRMm, but arise from intrinsic properties of these TDP-43 variants.

7. There seem to be some discrepancies in Fig. 4 (PLA data): For endogenous TDP-43, the PLA signal is not enriched in nuclear bodies (Fig. 4E), whereas when the PLA assay is performed with a GFP antibody on cells expressing GFP-TDP-43 WT, the PLA signal is mainly seen in nuclear droplets (Fig. 4F). Can the authors explain this discrepancy, or provide additional controls that the PLA signals obtained with the TDP-43- or GFP antibody are specific?

Our response: We believe there is a confusion in this point stemming from the colors originally used in **Figures 4E and 4F** in the original manuscript. In **Figure 4E**, the endogenous TDP-43 staining was previously shown in green and the PLA in magenta. However, in **Figure 4F** the GFP-TDP-43 signal was shown in magenta and the PLA in green. While the labels were correctly placed, we understand that the choice of colors was misleading. We have now exchanged the colors in our new **Figure 4E** to match those in **Figure 4F**. For both cases (endogenous TDP-43 and GFP-TDP-43 WT), the PLA signal identifying TDP-43 dimers only partially colocalizes with nuclear TDP-43 droplets, indicating that TDP-43 oligomerization does not only occur in the nuclear droplets. Moreover, following the reviewer's suggestion, we have included an additional control showing that the fluorescence signal observed in the PLA assay using the monoclonal GFP antibody is specific, as no PLA signal is detected when the expression of GFP-TDP-43 is not induced (–DOX) (see the new **Figure S4B**).

8. A discrepancy exists between the oligomerization data shown in Fig. S2C and Fig. 3G: In Fig. S2C, the RRMm protein does not show any higher order oligomers (e.g. dimers) after DSG cross-linking, whereas in Fig. 3G at least some dimers/oligomers are visible. A similar discrepancy exists for the 6M mutant: Why does the 6M mutant in Fig. S2C shows similarly many dimers/oligomers as WT, but not in Fig. 3G (or published data by the same team, shown in Afroz et al. 2017)? Is there a labeling error in Fig. S2C, or how can these variable results be explained?

Our response: We thank the referee for noticing this discrepancy. There was indeed a label mix-up in the previous **Figure S2C** (**S2D** in the revised manuscript) that has now been corrected. The results shown in **Figure S2D** are in line with those shown in **Figure 3H** and **Figure S3F-G**.

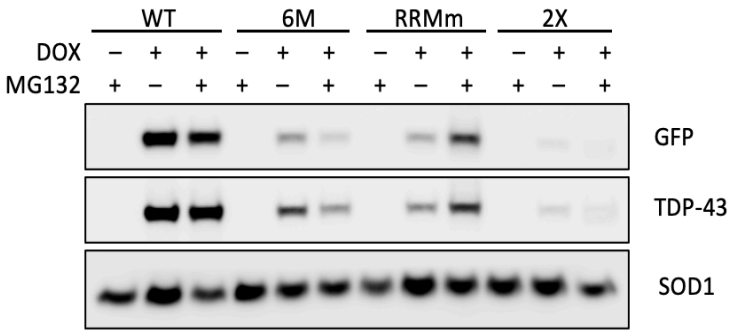
9. Another discrepancy can be noted between Fig. 6G (T10-TDP-43 with 6M seems exclusively nuclear) and Fig. 4H, where the same construct seems quite strongly mislocalized to the cytoplasm. Can this be explained by experimental conditions? What is the reason why SG recruitment was analyzed with the GFP triFC assay and not simply by analyzing SG recruitment of the different GFP-tagged mutants or NLS-mutant proteins shown in Fig. S6G? The latter could be a good alternative, and

Our response: We thank the referee for raising this point. We think that the difference may lie in the different cellular models used in **Figure 6G** (HeLa) and **Figure 4H** (NSC-34). Indeed, it was recently published that the nucleocytoplasmic levels of endogenous TDP-43 vary across different cell types (PMID: 35858577, Figure 1D), possibly due to differences in the TDP-43 import rate.

Regarding the triFC assay, we resorted to this technique to understand not only whether oligomerization is required for TDP-43 incorporation into stress granules, but also if TDP-43 remains in an oligomeric state inside them. We have now modified the text to reflect this point better (page 8, lines 685-687): "*The presence of reconstituted GFP fluorescence in the SGs further indicates that TDP-43 oligomerization is not only required for its incorporation into but also that TDP-43 exists as a dimer in SGs.*" At the reviewer's suggestion, we applied arsenite stress also on the isogenic GFP-TDP-43 mutNLS HEK293 lines (see the new **Figure S6I-J**). This new experiment confirmed the results obtained in the triFC assay (**Figures 6G-H**) in an independent model, further strengthening the conclusion that the incorporation of TDP-43 into stress granules requires its cytoplasmic oligomerization and RNA binding (see page 8, lines 704-706): *"Additionally, exposure of these isogenic lines to oxidative stress further confirmed the requirement of cytoplasmic oligomerization (6M and 6M&RRMm mutNLS) and RNA binding (RRMm mutNLS) for TDP-43 incorporation into stress granules (**Figure S6I-J**)."*

10. The data shown in Fig. 7/8 are very impressive, and it would be nice to demonstrate that the observed relocalization of mutant TDP-43 goes along with a stabilization of its levels after MG132 treatment, e.g. by doing a Western blot or CHX-chase experiment -/+MG132 - does the proteasome inhibitor restore the levels of the mutant proteins to WT levels and prevent rapid degradation of the mutants?

Our response: We thank the reviewer for this suggestion. Indeed, we have performed the Western blot analysis of the treatment of the HEK293 stable lines inducibly expressing GFP-TDP-43 with the proteasome inhibitor MG132 (see **Rebuttal Figure 1** below).



***Rebuttal Figure 1**: Western blot analysis of the isogenic cell lines described in Figure 1A after inducing GFP-TDP-43 expression for 48 h and treating them with 2.5 μM MG132 for the last 24 h.*

As expected, we observed the accumulation of GFP-TDP-43 RRMm upon proteasome inhibition. However,

unexpectedly, this is not the case for the monomeric variants (6M and 6M&RRMm), both of which present a more cytoplasmic cellular localization (**Figures 2A-C**), and the WT protein. We are currently investigating what may be the reason behind this surprising difference in behavior. However, due to the big load of data included in this manuscript, as both referees have pointed out, we could not expand further and will continue with this new set of experiments as a followup project.

Finally, some non-essential suggestions that could enhance the manuscript or possibly could be done in a follow-up study (just curiosity-based questions):

11. Do the mutant TDP-43 inclusions (Fig. 7, 8) become C-terminally phosphorylated or poly-ubiquitinated, as in ALS/FTD patients?

Our response: We thank the referee for this relevant question. To probe the resemblance of the induced (mutant) TDP-43 inclusions in our human neuronal and HEK293 models to pathological aggregates in the ALS/FTD patient CNS, we immunolabeled fixed cells with antibodies against ubiquitin and TDP-43 C-terminally phosphorylated at S403/404 or S409/410. This extensive characterization has now been added to **Figure 7** (new panels **7E-G**) and the new **Supplementary Figure 8** in the revised manuscript. In summary, our new data show robust ubiquitination of both cytoplasmic (neurons and HEK293) and nuclear inclusions (neurons and a subset of HEK293). Interestingly, whereas there was also overt positivity for the phospho-S403/404 TDP-43 epitope for both cytoplasmic and a subset of nuclear inclusions in both neurons and HEK293, positivity for the phospho-S409/410 epitope was only observed in a fraction of more dense inclusions in neurons, as well as in GFP-TDP-43 mutNLS HEK293 cells. This is in agreement with our previously published observations that phosphorylation of the low complexity region of TDP-43 occurs in an N-to-C-terminal pattern (PMID: 34806807) and highlights the maturation of TDP-43 aggregates in the cellular models presented in this work. Based on this new set of experiments, we conclude that the distinctly localized TDP-43 inclusions formed in our cellular models harbor the pathological features that characterize TDP-43 aggregates in the CNS of ALS/FTD patients (see pages 9-10, lines 767-794): *Subsequently, we probed the different TDP-43 inclusions formed upon proteasomal inhibition in the isogenic cell lines and human neurons with markers that identify nuclear and cytoplasmic TDP-43 aggregates in the CNS of ALS/FTLD patients, namely ubiquitin (2, 3) and TDP-43 C-terminally phosphorylated at serines (S) 403/404 or 409/410 (66). Both in the isogenic HEK293 lines (**Figure S8A**) and in human neurons (**Figure S8B**), MG132-induced cytoplasmic inclusions were starkly immunopositive for ubiquitin. Neuronal nuclear inclusions (NIIs) containing TDP-43 (WT and RRMm) were also ubiquitin positive (**Figure S8B**), similar to a subset of the nuclear aggregates arising upon MG132 treatment in HEK293 (**Figure S8A**). Likewise, cytoplasmic TDP-43 inclusions in both the cell lines (**Figure 7E**) and human neurons (**Figure 7F**) were overtly phosphorylated at the S403/404 TDP-43 epitope. Immunopositivity for phospho-S403/404 was also observed for some NIIs (**Figure 7F**) and a subset of the smaller nuclear inclusion in the isogenic lines*

*(**Figure 7E**). Phosphorylation at the more C-terminal S409/410 TDP-43 epitope was observed in a fraction of both cytoplasmic and nuclear inclusions in neurons (**Figure 7G**) and cytoplasmic inclusions formed by GFP-TDP-43 mutNLS in the isogenic HEK293 cell line (**Figure S8C**). More specifically, the larger and denser inclusions were found immunopositive for phospho-S409/410. In contrast, none of the inclusions found in the GFP-TDP-43 HEK293 lines (WT, 6M, RRMm, 6M&RRMm) were labeled by the phospho-S409/410 antibody, despite their immunopositivity for phospho-S403/404 (**Figures 7E and S8C**). This is in line with the previously reported sequential N- to C-terminal phosphorylation pattern in the LCR of TDP-43 (7) and indicates a process of TDP-43 aggregate maturation in our human neurons and isogenic HEK293 cell lines. Taken together, the distinctly localized TDP-43 inclusions formed in our cellular models harbor the pathological features that characterize TDP-43 aggregates in the CNS of ALS/FTLD patients."*

12. It is interesting that the cytoplasmic inclusions are surrounded by a vimentin cage (aggresome marker) - is there any evidence for such a vimentin cage or other aggresome markers in human post-mortem material of ALS/FTD patients)?

Our response: We thank the reviewer for this very interesting suggestion. Indeed, the cytoplasmic TDP-43 inclusions that we found in human neurons (**Figures 7C, 7F-G and S8B**) resemble the vimentin-caged TDP-43-positive aggresomes induced in HEK293 cells (**Figures 8D-E**) in their 1) morphology (round), 2) subcellular localization (juxtanuclear) and 3) effect on the nucleus (characteristic inward budding of the adjacent nuclear membrane). In addition, like aggresomes, the cytoplasmic TDP-43 aggregates in human neurons stain positive for ubiquitin. Although the importance of aggresome formation to neurons and outside of cell culture setups is not yet fully understood, a recent study provided the first evidence of *in vivo* aggresome formation characterized by vimentin-positive cage formation in a dividing neural stem cell (NSC) in the mouse dentate gyrus (PMID: 32109376). However, in contrast to high vimentin expression in neural stem cells and (mature) glia, we found that neurons in our human neural cultures express only low levels of vimentin (see Hruska-Plochan *et al*., 2021, BioRxiv [https://doi.org/10.1101/2021.12.08.471089]: Figure. 2a and Extended Data Figure 5b and g), fully in line with data from the adult human brain (see the Human Protein Atlas for e.g. cortex and hippocampus). This indicates that in neurons aggresome caging is likely mediated by intermediate filament proteins other than vimentin. Therefore, dissecting the involvement of neuron-specific intermediate filament proteins (the neurofilament triplet proteins, α-internexin and peripherin) in aggresome caging in human neurons in culture and inclusion formation in the TDP-43 proteinopathy central nervous system is of high interest for future research, yet beyond the scope of the current story. Notably, neuropathological studies do support an interplay between neuronal intermediate filament proteins and TDP-43 aggregates in ALS/FTD patients (PMIDs: 15170578, 18287500, 28466273). Furthermore, like aggresomes, TDP-43 aggregates in patients are immunopositive for ubiquitin (PMID: 17084815, 17023659) and p62 (PMIDs: 19496940, 31633109, 18584184, 21118398) and more recently immunoreactivity for the aggresome marker HDAC6 was reported for a subset of TDP-43 inclusion types

(PMID: 33097688), further sparking interest in a more thorough investigation of aggresome marker abundance in TDP-43 proteinopathy patient tissue in future studies.

Minor points:

13. Figure labeling could be a bit more self-explanatory, e.g. what are the red and yellow circles in Fig. S1A? In Fig. 7 A,C it would be nice to indicate in the figure that MG132 was present for 24h in all conditions, as it helps the reader to understand the result better at first glance. In Fig. S6F it would be nice to see at one glance that here a set of mutNLS proteins was used (like indicated in Fig. S6G/H).

Our response: We thank the referee for these helpful suggestions. We have now modified the labels of **Figures S1A and S6F** (and corresponding **Figure 6I-K**) accordingly. Images in **Figures 7A and C** have been rotated and the label reflecting the treatment has been placed on top to facilitate readability. The corresponding **Figure S7C** has also been modified for consistency.

14. Legends: Some important experimental details are missing from some legends, e.g. what time point is shown in Fig. 8B and Fig. 8D/E?

Our response: Figure 8B is a live imaging experiment and the time points corresponding to each frame are stamped onto the pictures. To clarify this point, we have stated this in the updated legends of **Figures 8B, S9D and S9E.**

15. Use of certain phrases and designation of inhibitors:

- Page 4, last paragraph: 1,6-hexanediol should not be introduced as "an LLPS-suppressive alcohol", as 1,6-HD does not generally suppress all LLPS, but only weak hydrophobic interactions (e.g. LLPS driven by polar or charged interactions will NOT be affected by 1,6-HD). This compound should also be used with great caution, as it has been shown to disrupt nuclear transport (PMID: 17418788) and more recently kinase and phosphatase activity (PMID: 33814344), so effects seen with this drug could also be due to these general impairments instead of a direct LLPS suppression...

Our response: We agree with the reviewer that a more precise explanation of the mechanism of action of 1,6-HD is valuable to the reader. We have now included in the text the mechanism of 1,6-HD-mediated LLPS disruption, as well as the additional cellular effects of 1,6-HD. Furthermore, we added additional references to publications using 1,6-HD in conjunction with other methods that have previously proven that nuclear TDP-43 punctae are liquid droplets. We would like to highlight that in addition to the sensitivity of the nuclear TDP-43 punctae to 1,6-HD treatment, also the dependence of WT and RRMm GFP-TDP-43 punctae on their protein concentration (**Figures 3F and S3E**) (PMID: 30682370) and the observed punctae

fusion and fast recovery after photobleaching in live cell imaging setups (**Figure 8B**) underline the liquid nature of the nuclear TDP-43 droplets.

16. Figure 2J/K: Ivermectin is mentioned on page 4 as a "nuclear import inhibitor", it would be informative to say what precisely it inhibits, and why it was only used on TDP-43 WT. Alternatively, this experiment could be taken out of the manuscript, as it does not seem essential to support their main story and distracts rather than contributing much information.

Our response: Ivermectin is an importin α/β-mediated nuclear import inhibitor, and this has now been specified in the text with its corresponding reference (page 4, lines 216-220): "*Indeed, similar results were observed in human neurons, where the combined treatment of ActD and ivermectin, an importin α/β-mediated nuclear import inhibitor (49), increased the cytoplasmic shift of endogenous TDP-43 as compared to treatment with ActD alone (**Figure 2J-K**).*"

17. Page 8, end of second paragraph: "several classes of proteasome inhibitors, but not an autophagy one". It would be more informative to say that bafilomycin A was used (an acidification inhibitor, NOT a specific autophagy inhibitor).

Our response: We agree that bafilomycin A1 is not a specific autophagy inhibitor, but its inhibition of lysosomal acidification and blocking of the fusion between autophagosomes and lysosomes (PMID: 9639028) results in the disruption of autophagy. We have thus rewritten the text as follows (page 9, line 680-683): "*The observed TDP-43 aggregation patterns were specific to the inhibition of the UPS degradation pathway, as several classes of proteasome inhibitors, but not disruption of autophagy with bafilomycin A1, yielded similar outcomes in the isogenic cell lines (**Figure S7B-C**).*"

18. Some fluorescence microscopy figures could benefit from display in black-and-white instead of color, e.g. the green signals in Fig. 3E or 4C (HEK cells), or the nuclear body markers shown in purple in Fig. 5 look very weak and are hard to see, especially in a printed version. The signals will be better visible if shown in black-and white.

In line with the reviewer's suggestion, we have done this in **Figures 3A, 3C, 3E, 4C, 8B,** new **S2C**, new **S3D**, **S9B and S9D**.

19. Scale bar missing from Fig. 6G (legend says 10 µm, but no bar shown in the figure).

We thank the referee for noticing the missing scale bar. This issue has been corrected accordingly.

20. Legends in Fig. S7C and D do not fit the figure, so I believe C and D were swapped. Same for the legends to Figure 2F and H.

We thank the referee for spotting the mismatch between the figures and their corresponding legends. Indeed, the captions for **Figures 2F and H** and **Figures S7B-D** were mixed up, but have now been corrected.

21. Page 9, end of first paragraph: I believe the reference to Fig. S7B (human neuron data) is not correct, as HEK293 cell data are shown in Fig. S7B. This correct figure number should be given here.

The reference to **Figure S7B** was actually meant to direct the reader to **Figure S7C**, and has been changed accordingly in the text. We thank the referee for noticing this incongruence.

Referee #2:

In their manuscript "TDP-43 oligomerization and RNA binding are codependent but their loss elicits distinct pathologies", Pérez-Berlanga et al. decipher the relation between TDP-43 RNA binding and oligomerization and its function in splicing and stress granule association. Using TDP-43 mutants that are deficient in RNA binding and/or oligomerization, the authors find that oligomerization and RNA binding effect the nuclear:cytoplasmic distribution of TDP-43, the formation of nuclear speckles, and the types and localization of 'inclusions' formed. The findings presented bring new insights in the complex biology of TDP-43 and help to understand the differential functions that TDP-43 has, thereby also contributing to the understanding of TDP-43 pathology in FTD/ALS.

We thank the referee for the accurate summary and positive evaluation of our study.

Major points:

1. The Manuscript is well written and the data well presented, and the authors succeed to address the complexity of TDP-43 biology by looking at the implications of TDP-43 in cell biology and stress response. Because the results are complex, it is sometimes a bit hard to follow the rationale / story line of the manuscript. To make it easier to for the reader to understand, I would suggest to start the manuscript with the impact of oligomerization and RNA binding on the important biological functions of TDP-43, namely splicing and stress granule association (currently Fig.6) and. , because this establishes the relevance of the addressed questions and the model of TDP-43 mutants. Then move to the mechanistic part of the manuscript, in which the authors show the nuclear body association, LLPS, and oligomerization, which help to build a model for the functional role of TDP-43 RNA binding and oligomerization. Then, the authors could move on to effects in the pathological context and show changes in the nucleocytoplasmic distribution and inclusion distribution and kind. This is a suggestion, which in my eyes would help to give structure to the manuscript for a better understanding of the complex data.

Our response: We thank the referee for these comments and suggestions. We recognize that the study addresses several aspects of TDP-43 pathobiology and is complex. We considered different ways of streamlining the information, also in light of the comments of Referee #1 on this point (see above, referee #1, point #B on top). We think that it is important to start by explaining the role of oligomerization and RNA binding in the half-life of the protein (as Referee #1 also noted), before we move on to explaining the impact on splicing and stress granule association. However, we have added several new explanatory sentences and data that we hope improve the readability of the manuscript. Importantly, we have revised the title and abstract of the manuscript to point our readers to the key findings of our study.

2. Lane 53+: The authors suggest that passive diffusion out of the nucleus is increased when RNA binding or oligomerization is inhibited in TDP-43 mutants. This interesting idea seems supported by nuclear retention of these TDP-43 variants upon chemical crosslinking. However, to further validate this idea without

the potential confound of unspecific protein crosslinking, passive diffusion and nuclear transport can be selectively blocked e.g. by WGA before preparing the nuclear enriched fraction.

Our response: We thank the referee for raising this point. We agree that protein-protein interactions independent from self-oligomerization could, in principle, contribute to nuclear retention of TDP-43. However, DSG cross-linking of monomeric GFP-TDP-43 (6M), as shown in **Figures S2D-E**, confirms that their contribution is minimal. Indeed, cross-linking of monomeric GFP-TDP-43 (6M) to other proteins also retains GFP-TDP-43 6M in the nucleus, yet this nuclear retention is significantly reduced when compared to its oligomerizing counterparts (4- and 3-fold less retention, respectively for WT and RRMm; **Figure S2E**), supporting the notion that self-oligomerization is the main driver of nuclear TDP-43 retention.

Nuclear import is abolished when the cells are lysed in the first step of nucleocytoplasmic fractionation. The use of ivermectin, an inhibitor of importin α/β-mediated nuclear import, on neurons showed that, upon RNA synthesis inhibition, endogenous TDP-43 partially relocates to the cytoplasm as observed by immunocytochemistry (**Figures 2J-K**), supporting the results observed by nucleocytoplasmic fractionation in western blot (**Figures 2F-G and S2D-E**). Unfortunately, passive diffusion cannot be selectively blocked, as wheat germ agglutinin (WGA) only blocks active import, and not passive diffusion (PMID: 2446896).

3. ActD treatment may put stress on cells and induce stress-related translocation of TDP-43 into the cytoplasm, similar to H2O2 treatment. Also, ActD may reduce the amount of available nuclear transport factors and thereby inhibit nuclear import of TDP-43. Please show that the import of the different TDP-43 constructs are similar at baseline, that the import of other cargo is generally intact after ActD treatment, and that the levels of importin-alpha/beta are not changed upon ActD treatment. Please also show the depletion of RNA from the nucleus upon ActD treatment.

Our response: To address the reviewer's comments, in the revised manuscript, we now show that ActD does not alter the levels (**Figures S2F-G**) or the distribution (**Figures S2H-I**) of importins α1 (KPNA2) and β1 (KPNB1), which together form the importin complex recognizing the NLS of TDP-43 (PMIDs: 35767952, 20020773), nor the localization of an independent non-RNA-binding cargo of KPNA2/KPNB1, c-myc (PMIDs: 30115078, 30323892) (**Figures S2J-K**). This has been specified accordingly in the main text (page 4, lines 207-211): "*Importantly, this ActD-induced translocation of TDP-43 to the cytoplasm was not due to altered levels (**Figure S2F-G**) or distribution (**Figure S2H-I**) of the importins involved in nuclear TDP-43 import. Moreover, ActD treatment did not affect the localization of c-myc, which is also transported to the nucleus by the same importin complex but does not bind RNA (**Figure S2J-K**)*." Additionally, the experiment depicted in **Figures 2J-K** further shows that ActD does not reduce the availability of nuclear transport factors, as the combined treatment of ActD together with the import inhibitor IVM results in a more pronounced cytoplasmic accumulation of TDP-43 compared to ActD treatment alone.

Additionally, a recent publication (PMID: 35767952) has described the mechanism of recognition of the TDP-43 nuclear localization signal by importin complex α1/β and shows that such interaction disrupts TDP-

43 dimerization. Thus, binding of TDP-43 to the importin complex is enhanced in the monomeric state, such as the one that our 6M and 6M&RRMm mutants present, making it unlikely that the loss of nuclear localization observed with these variants is due to problems in its nuclear import.

To probe the total nuclear RNA content upon ActD treatment, we used an RNA-specific dye (**Figure S2C**). Although we did not detect a significant alteration in total nuclear RNA levels, we did observe the disappearance of RNA punctae within nucleoli, the sites of ribosomal RNA biosynthesis (PMIDs: 29589958, 35858577). This proves that nascent RNA levels are decreased upon ActD treatment in our model. We have reflected this observation in the revised text as follows (page 3, lines 203-205): "*Conversely, when we pretreated the cells with actinomycin D (ActD) to block transcription and decrease the levels of newly synthesized pre-mRNAs –the main RNA targets bound by TDP-43 in the nucleus (20, 21)– (**Figure S2C**) (48) [...]*." In line with the specific reduction of newly synthesized rather than total nuclear RNA content, a recent publication showed the efficacy of ActD to decrease nascent RNA levels in HeLa and NIH 3T3 cells and primary neurons using click chemistry (PMIDs: 18840688, 35858577). Since TDP-43 preferentially binds UG-rich intronic sites on pre-mRNA, the drop in nascent rather than total RNA levels is the cause of the nuclear exit of TDP-43 upon ActD treatment.

4. Lane 92/93, Figure S3A/B: the sizes of TDP-34 nuclear 'droplets' seems very small, sometimes only a few pixels. A size/volume analysis becomes unreliable at this resolution and contrast. Please show in supplemental data how the analysis was done

Our response: Droplet analysis was performed on high resolution images, and only bodies larger than 0.003 µm3 (corresponding to 2 square pixels) were considered as droplets. The detailed protocol for droplet analysis can be found in the *Supplementary materials and methods* file (page 7, *Image analysis*) and the newly added **Figure S3A** now visually summarizes the analysis process.

5. LLPS in vitro data in Figure 3 are performed with TDP-43 carrying a MBP tag, which inhibits TDP-43 LLPS. These experiments should be repeated after cleavage of the tag, and, if possible, also for the other constructs. How does RNA binding influence TDP-43 LLPS when oligomerization or RNA binding is inhibited?

Our response: We thank the reviewer for bringing up this important point, which was also raised by the other reviewer (see above, **referee #1, point #4**).

As explained above, droplets formed by recombinant full-length TDP-43 upon cleaving its solubility tag are rather unstable due to the intrinsic aggregation propensity of TDP-43, and the window to observe pure TDP-43 LLPS *in vitro* is rather short. Previously reported TDP-43 droplets don't show the typical round shape that are usually associated with biomolecular condensates, indicating a mixture of LLPS droplets and LLPS-dependent maturation. For this reason, we originally chose to perform this experiment with the uncleaved solubility tag and a crowding agent, as done by other groups using MBP (PMID: 30826182), or SUMO

(PMID: 30100264) tags. However, to exclude that the solubility tag interferes with the LLPS of TDP-43 *in vitro*, we have now repeated the experiment in the presence of the TEV protease to remove the MBP tag. These experiments confirmed our original observations. We now replaced **Figures 3C-D** with the new data and updated the text accordingly to (page 5, lines 258-260): "*Indeed, at a reported physiological concentration of 10 μM (25, 56), purified full-length TDP-43 phase separated into droplets, which also dissolved in the presence of 1,6-HD (**Figure 3C-D and S3C**).*"

Regarding the question of the effect of RNA on the LLPS of TDP-43 *in vitro* raised by the referee, we have performed a set of experiments with different RNA lengths and concentrations. Our initial experiments show that the *in vitro* data support our conclusions from the cellular experiments of this manuscript. GU-rich sequences indeed abolish phase separation of the WT full-length TDP-43 in physiological concentrations, while AC-rich RNA have no influence. In the case of the oligomerization-deficient mutant (6M), which is LLPS-deficient, the presence of different types of RNA has no impact. Since these data are not strictly necessary for our main conclusions and given the large amount of data that are already included in the manuscript, we decided against adding this information on this manuscript. Instead, this information will be included in a separate manuscript, focusing on different RNA binding modalities of TDP-43, which is currently in preparation.

6. In the title, the authors talk about 'distinct pathologies' of TDP-43 but they do not show any evidence of human pathology related TDP-43. In their cell lines, they observe different inclusions located in nucleus or cytosol. Cytosolic inclusions are appearing upon proteasome inhibition in aggresomes in 6M mutants that are anyways primarily located in the cytoplasm. This is kind of expected from previous studies showing that accumulation of (aggregating) proteins in the cytosol upon proteasome inhibition get sorted into the aggresome. Whether this is also the case in TDP-43 pathology in the human brain remains speculative. Therefore, I ask to remove the word 'pathologies' from the title and replace it by 'cellular inclusions'.

Our response: We have taken the referee's point into account and have replaced 'pathologies' with 'aggregation patterns' in the manuscript title (page 1, line 2).

Minor points:

7. Many figure panels showing fluorescent images have a low contrast and are dark, and are therefore hard to see. Examples: 2B, 4E, 5 all panels, 8B. I. Suggest the authors to either switch to grayscale. Images for 1-color images, or enhance the contrast where possible, e.g. when showing distribution or nuclear/cyt ratio.

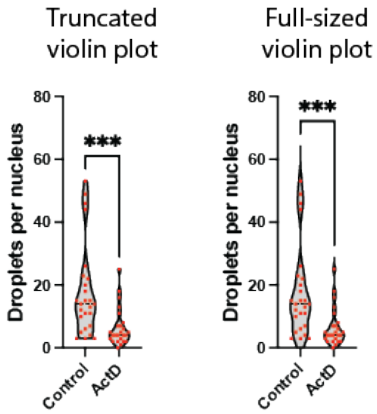
Our response: We agree with the referee and have now converted **Figures 3A, 3C, 3E, 4C, 8B, S9B and S9D** to grayscale. Moreover, we replaced the images in **Figure 2B** with new ones that better represent the differences in nucleocytoplasmic distribution of the different TDP-43 variants.

8. To prove the robustness of the observations on TDP-43 foci in the nucleus - e.g. for colocalization with Cajal body markers, number and size analysis - it would be helpful if the authors could provide galleries of more example nuclei images in the supplemental data.

Our response: We agree with the referee that more representative images would strengthen our conclusions regarding the subnuclear localization of TDP-43 shown in **Figure 5**. To this end, we have now included image galleries for the coilin and *NEAT1* stainings in new **Figures S5A-B** that support the quantifications shown in **Figures 5B-C**.

9. Multiple figure panels: please show all data points or non-truncated violin plots in graphs

Our response: In agreement with EMBO guidelines for data presentation (*"We recommend that the actual individual data from each experiment should be plotted if n<5, alongside an error bar"*), we have included all individual experimental data points in all bar graphs. We resorted to violin plots when the experimental N was ≥8, for aesthetic purposes. In both bar and violin plots, the size of the N and the statistical analysis performed has been precisely detailed in the figure legend. Truncated violin graphs do not hide any data points (see **Rebuttal Figure 2** for **Figure 4D**) and were chosen over full-sized ones for visualization purposes.



***Rebuttal Figure 2**: Violin plot corresponding to Figure 4D in its truncated (left) and full-sized (right) versions.*

10. Lane 144: the authors talk about 'nuclei isolation' and the 'nuclear fraction' although they work with 'nuclear enrichment' and 'nuclear enriched fractions' if I understand right from the methods. This may seem like a trivial comment but is important when interpreting the results since cytosolic TDP-43 may in part 'contaminate' the nuclear enriched fraction.

Our response: We thank the referee for raising this point. To clarify this, we have now modified the text accordingly:

- Page 4, lines 186-187: "*…upon mild lysis and nuclei enrichment by centrifugation…*"
- Page 17, lines 1188-1189: "*The final pellet fraction enriched in nuclei was resuspended…*"

However, we would like to stress that the contamination of the nuclear fraction with cytoplasmic components is minimal with this protocol, since it includes a washing step between the lysis of the cell and the nuclear membranes. If at all, there is a higher probability of contamination of the cytoplasmic fraction with the nuclear one if the first lysis is so strong that it would break the nuclear membrane. Our analysis of nucleoplasmic fractionations always includes the immunoblotting for cytoplasm- (GAPDH) and nucleus-

specific (histone H3) proteins to confirm the purity of the corresponding fractions (**Figures 2D, 2F, 6K, S2D**).

11. Figure 4, and matching text part: the authors state that TDP-43 forms 'conformational distinct oligomers', however, they do not apply structural methods to test TDP-43 conformation or oligomer structure. The word 'conformation' refers to protein structure or folding states. The authors do observe specific stoichiometries of oligomers though, which I guess they are referring to. Please correct the wording accordingly.

Our response: The referee raises a valid point. While we do think that our PLA and GFP complementation data suggest that the RNAless droplets have distinct conformations, these methods are indirect and do not give structural information. In our revised manuscript we have replaced the word "conformation" with "orientation" or "spatial organization", which more accurately describe our findings.

12. Instead of referring to 'monomeric GFP-TDP-43' and 'RNA-binding deficient GFP-TDP-43' in the text, it would help the understanding if the authors stick to the abbreviations/nomenclature introduced in the beginning of the manuscript: WT, 6M, RRMm and 6M&RRMm

Our response: We appreciate the referee's feedback on the readability of our manuscript. In order to facilitate the understanding of the mechanistic details of our study, we consider that it is in the reader's best interest to keep the description of the GFP-TDP-43 variants in the main text. Therefore, in the revised manuscript we have added the accompanying mutation names between parentheses when 'monomeric GFP-TDP-43' and 'RNA-binding deficient GFP-TDP-43' are mentioned throughout the *Results* section, to facilitate the visualization and understanding of the corresponding figures. For example:

- Page 6, lines 354-355: "*In contrast, the oligomerization-deficient variants (6M and 6M&RRMm) showed a markedly decreased PLA signal…*"
- Page 9, lines 756-759: "In human neurons treated with the proteasome inhibitor MG132, monomeric TDP-43-HA variants (6M and 6M&RRMm) also predominantly aggregated in the cytoplasm, whereas RNA binding-deficient TDP-43-HA (RRMm) additionally presented nuclear inclusions in >50% of transduced neurons (**Figure 7C-D**)..."

13. Lane 268/269: the oligomerization domain of TDP-43 may catalyze incorporation of TDP-43 into Cajal bodies and paraspeckles, independent of actual oligomerization. The authors should either test this or rephrase the text, since it is not clear whether TDP-43 oligomerization is really necessary for joining these nuclear bodies.

Our response: Unlike previous studies using NTD deletion mutants of TDP-43 (PMIDs: 22473010, 27317832), our results are based on the use of point mutations in the NTD (6M) that specifically disrupt its self-interaction without affecting the folding of the domain (PMID: 28663553). As the NTD remains present in this TDP-43 variant and the only altered variable is the capacity of TDP-43 to oligomerize through this

domain, we consider that it is unlikely that the lack of incorporation into nuclear subcompartments shown for the 6M in **Figure 5** is NTD-mediated but independent of oligomerization, as the referee suggests. The same principle applies for the RNA-binding TDP-43 mutant (RRMm).

14. Lane 272-279: I suggest to speak about TDP-43 foci instead of droplets since the liquid nature of the TDP-43 species is not proven or tested. Furthermore, the unidentified TDP-43 foci containing RRMm are likely to contain RNA - like all known nuclear bodies - which may not bind TDP-43 specifically but still be important for the formation of the formations. Polyanionic RNA can coordinate and recruit proteins into condensates even without specific binding activity.

Our response: We thank the reviewer for their suggestion. However, we consider that the nuclear TDP-43 foci observed in the isogenic lines do present a liquid nature according to the experiments presented in this manuscript, in addition to the large body of literature identifying nuclear TDP-43 punctae as liquid droplets (PMID: 30853299). The condensates formed by GFP-TDP-43 WT and RRMm arise proportionally to their concentration (**Figures 3F, S3E**) (PMID: 30682370), dissolve upon treatment with the aliphatic alcohol 1,6-hexanediol (**Figures 3A-B and S3B**), which disrupts weak hydrophobic interactions mediating LLPS (PMID: 30682370) and are observed to fuse by live cell imaging (**Figure 8B-C**).

Regarding the RNA content of the droplets formed by GFP-TDP-43, we agree with the referee on this point and we have clarified this in our revised manuscript in the sentence (page 5, lines 268-270): "*This observation suggests that nuclear TDP-43 droplets arise independently of specific RNA binding, but does not discard the involvement of RNA in their formation.*"

15. Figures S4E, S5B would benefit from a logarithmic y-axis

Our response: We thank the referee for this suggestion, which we have implemented in the revised version.

16. Figure S2C: why does the RRMm but not the 6M mutant have oligomeric TDP-43? shouldn't that be the opposite way? Is the western blot labeling mixed up?

Our response: Indeed, the labels were mixed up but have now been corrected. We thank the referee for spotting this labeling issue.

Dear Magda,

Thank you for submitting the revised manuscript to The EMBO Journal. The revised version has now been seen by the two referees. As you can see below, both referees appreciate the introduced changes and support publication here.

Referee #1 has a few remaining comments that I would like to ask you to incorporate in a revised version.

When you submit the revised version will you also address the following points:

- Funding info: The grant numbers 182880, 18-IIA-411 are not listed in ms file; Candoc Grant (Forschungskredit) from the University of Zurich not listed in the online submission system.

- Please add 3-5 keywords

- The reference format needs to be adjusted to EJ format.

- Please deposit the proteomics and transcriptome data and add the accession numbers to the Data Availability section.

- The 'Author Contributions' section is replaced by the CRediT contributor roles taxonomy to specify the contributions of each author in the journal submission system. Please use the free text box in the 'author information' section of the manuscript submisssion system to provide more detailed descriptions (e.g., 'X provided intracellular Ca++ measurements in fig Y')

- COI needs to be re-named as Disclosure and competing interests statement.

- The supplemental file should be re-labelled as Appendix file. It should have a ToC as well as page numbers. Supplemental figures should not be uploaded individually, only in Appendix file. Appendix Figure S9 is missing in ToC; Figures and Tables should be renamed with the corresponding callouts - Appendix Figure S1-S9 and Appendix Table S1-S3

- I see that you have not uploaded source data files. My colleague Daniele Viarisio had emailed you back in July with a list of figures that would be good to have source data files for. I have attached the documents again.

-- Figure 3C - Both 6m cells contain no signal image, which I understand why. But please confirm that there are cells there and not simply a black box. Maybe the source data for this data set will also confirm this.

- Appendix figure S5B - Please check highlight box 4. The highlighted figure looks different to the figure selected in the entire figure.

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Take a look at the word file and the comments regarding the figure legends and respond to the issues.

- Please upload a synopsis text => a summary statement plus 3-5 bullet points describing the key findings of the

-We also need a synopsis image => 550 wide by [200-400]

- Emails bounced for Katharina M. Betz - katharina.hembach@uzh.ch, and Chiara Foglieni - chiara.foglieni@eoc.ch

That should be all - congratulations on a nice manuscript!

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

In the revised version, the concerns and specific points raised by the previous reviewers' were all adequately addressed. In particular the title and abstract now much better reflect the main messages of the manuscript. Moreover, the major concern that some of the observed differences come merely from reduced expression levels has now been adequately addressed and excluded. The text revisions have greatly enhanced the readability of the manuscript and it is easier to follow the storyline now.

The following minor revisions are still recommended:
- P. 5, line 72-74: "Since local protein concentration is a known driver of phase separation, this observation further strengthens that indeed the observed droplets in our model are liquid compartments". This sentence should be corrected, as the concentration-dependence does not reveal anything about the material state, i.e. that the compartments are liquid-like. The concentration-dependence does support the view that the compartments arise through phase separation, but NOT in any way that they are liquid-like droplets, as phase separated compartments can also have non-liquid-like properties (see examples listed in PMID: 29602697, e.g. Balbiani body etc.). Therefore, I second the other reviewers' suggestion that it would be more appropriate to speak about "TDP-43 foci" or "TDP-43 condensates" instead of TDP-43 droplets. The concentration argument made is not valid (see above), and no fusion events of TDP-43 WT "droplets are shown, only a static behavior of RRMm is shown in Fig. 8C by live cell imaging. Thus, it is recommended to replace the term "LLPS" simply with "phase separation or PS", and "droplets" with "condensates".

- Choice of data displayed in Fig. 3/Fig. S3: I find the comparison of the variants at equal protein levels (4h after dox induction) much more relevant than the comparison of the variants at vastly different expression levels (48h after dox induction), and it is a bit confusing that different expression timepoints are shown in the same Figure. Therefore, I would recommend to move the quantification of nuclear droplets obtained with the 4h induction into the main Figure 3, i.e. swap Fig. S3D/E with Fig. 3E/F/G. Then all data obtained with a 4h induction would be shown together in the main figure (imaging + biochemical experiments), and all 48h induction data (imaging + biochemistry) would be shown together in the supplementary figure. I also would recommend to point out more explicitly when the variants were compared at equal levels (by choosing a short induction period), this is so far not yet done in the results text describing e.g. Fig. 3H-I and should be highlighted more.

- "Expanded view" document contains wrong Fig. S1 (shown is main Fig. 1, needs to be exchanged for the correct Fig. S1).

Some remarks on Rebuttal Figure 1:
The Western blot analysis of MG132-treated isogenic cell lines (shown in Rebuttal Figure 1) is indeed a but puzzling and unexpected, but I agree that following up the reasons for this unexpected result would go beyond the scope of this manuscript, and the current manuscript already includes a lot of interesting and timely data that should be reported now. The authors could consider whether difficulties in solubilizing the 6M mutant proteins could be the reason why the 6M proteins show lower levels in this experiment, e.g. in case RIPA buffer was used. Potentially much harsher solubilization conditions, e.g. denaturing urea buffer, may be necessary to dissolve the insoluble 6M mutant proteins, so that they can be visualized in a WB?

Referee #2:

In my eyes, the authors addressed all my and other referee concerns. The text changes and added data provide additional support of the findings. I have no more concerns for publishing the work in EMBO Journal.

Pérez-Berlanga et al: Point-by-point response to the referees' comments to the revision

Referee #1:

In the revised version, the concerns and specific points raised by the previous reviewers' were all adequately addressed. In particular the title and abstract now much better reflect the main messages of the manuscript. Moreover, the major concern that some of the observed differences come merely from reduced expression levels has now been adequately addressed and excluded. The text revisions have greatly enhanced the readability of the manuscript and it is easier to follow the storyline now.

We thank the referee for their positive assessment and for all the constructive feedback that helped us improve our manuscript.

The following minor revisions are still recommended:

- P. 5, line 72-74: "Since local protein concentration is a known driver of phase separation, this observation further strengthens that indeed the observed droplets in our model are liquid compartments". This sentence should be corrected, as the concentration-dependence does not reveal anything about the material state, i.e. that the compartments are liquid-like. The concentration-dependence does support the view that the compartments arise through phase separation, but NOT in any way that they are liquid-like droplets, as phase separated compartments can also have non-liquid-like properties (see examples listed in PMID: 29602697, e.g. Balbiani body etc.). Therefore, I second the other reviewers' suggestion that it would be more appropriate to speak about "TDP-43 foci" or "TDP-43 condensates" instead of TDP-43 droplets. The concentration argument made is not valid (see above), and no fusion events of TDP-43 WT "droplets are shown, only a static behavior of RRMm is shown in Fig. 8C by live cell imaging. Thus, it is recommended to replace the term "LLPS" simply with "phase separation or PS", and "droplets" with "condensates".

  We thank the referee for their feedback, and agree that the use of the word "driver" could lead to the misunderstanding that the concentration dependency implies that the protein is in a liquid state. To this end, we have rephrased the sentence as follows (page 6, line 335): "Since local protein concentration modulates phase separation [...]". It is now well established in the field that TDP-43 nuclear "foci" are condensates formed by LLPS (PMID: 30853299, 32649883, 33335017), and we therefore consider that LLPS is the appropriate term to use in this case. However, in agreement with the referee, we have replaced the term 'droplet(s)' with 'condensate(s)'' in the revised manuscript, such as in the following examples:

  - Page 5, lines 253-254: "...a phenomenon visible in the nucleus as small condensates that fuse and split at endogenous protein concentrations."

- Page 5, lines 263-264: “...this indicates that the observed nuclear TDP-43 punctae are LLPS-driven condensates.”
- Page 5, lines 273 274: “...oligomerization-deficient TDP-43 (6M) did not form condensates under the same conditions, suggesting that NTD interactions are essential for TDP-43 LLPS “

- Choice of data displayed in Fig. 3/Fig. S3: I find the comparison of the variants at equal protein levels (4h after dox induction) much more relevant than the comparison of the variants at vastly different expression levels (48h after dox induction), and it is a bit confusing that different expression timepoints are shown in the same Figure. Therefore, I would recommend to move the quantification of nuclear droplets obtained with the 4h induction into the main Figure 3, i.e. swap Fig. S3D/E with Fig. 3E/F/G. Then all data obtained with a 4h induction would be shown together in the main figure (imaging + biochemical experiments), and all 48h induction data (imaging + biochemistry) would be shown together in the supplementary figure. I also would recommend to point out more explicitly when the variants were compared at equal levels (by choosing a short induction period), this is so far not yet done in the results text describing e.g. Fig. 3H-I and should be highlighted more.

  We agree with the reviewer that by looking at Fig.3/Fig. S3 it is not directly clear that different timepoints are shown within the same Figure. Yet, for visualization purposes, we do prefer to keep the panels as they are, since nuclear TDP-43 condensates are more difficult to see by the naked eye at the 4h time point given the lower total amount of protein compared to 48h post-induction. Results obtained at the 48h induction time point completely align with the conclusions obtained at the 4h time point, and this data will be available in the Appendix file. As they are now, the panels in the main figure best visually illustrate the results, which facilitates the understanding of the main text. To further enhance clarity, we have now added the timepoints to the Figure itself. Furthermore, as suggested by the referee, we have now highlighted the comparisons made at equal protein levels in the revised manuscript (page 6, lines 342-344): “[DSG-crosslinking experiments] showed protein complexes at the expected size of GFP-TDP-43 dimers for both WT and RRMm, but not for the monomeric variants (6M and 6M&RRMm) at comparable protein levels (Figures 3H-I and S3F-G).”

- "Expanded view" document contains wrong Fig. S1 (shown is main Fig. 1, needs to be exchanged for the correct Fig. S1).

  We apologize for this mistake and thank the referee for noticing it. Appendix figure S1 has been corrected and now contains the appropriate panels.

Some remarks on Rebuttal Figure 1:

The Western blot analysis of MG132-treated isogenic cell lines (shown in Rebuttal Figure 1) is indeed a but puzzling and unexpected, but I agree that following up the reasons for this unexpected result would go beyond the scope of this manuscript, and the current manuscript already includes a lot of interesting and timely data that should be reported now. The authors could consider whether difficulties in solubilizing the 6M mutant proteins could be the reason why the 6M proteins show lower levels in this experiment, e.g. in case RIPA buffer was used. Potentially much harsher solubilization conditions, e.g. denaturing urea buffer, may be necessary to dissolve the insoluble 6M mutant proteins, so that they can be visualized in a WB?

We thank the reviewer once again for the positive assessment of the results included in this manuscript and for being understanding that this question is currently out of its scope. RIPA buffer was indeed used for solubilization of the lysates analyzed in Rebuttal Figure 1 but no high-molecular weight complexes were detected trapped in the gel wells. We are currently working on understanding the molecular mechanism behind this seemingly contradictory result, for which we will gratefully consider the referee's suggestions.

Referee #2:

In my eyes, the authors addressed all my and other referee concerns. The text changes and added data provide additional support of the findings. I have no more concerns for publishing the work in EMBO

We thank the referee for their favorable evaluation and for all the helpful comments that guided us to improve our manuscript.

2nd Revision - Editorial Decision **12th Jun 2023**

Dear Magda,

Thank you for submitting your revised manuscript. I have now had a chance to take a look at everything and all looks good. I am therefore very pleased to accept the MS for publication here.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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 χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	No restrictions apply

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Table S3

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Tables S1 and S2

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	'Materials and methods', subsection 'Cell culture' 'Supplementary materials and methods', subsection 'Development of Flp-In T-REx lines'
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	'Materials and methods', subsection 'Cell culture'

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	'Supplementary materials and methods', subsection 'TDP-43-MBP expression and purification'

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)

If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	**Yes**	Sample size is detailed in the panel legend when applicable
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	**Yes**	'Supplementary materials and methods', subsection 'Statitical analysis'

Sample definition and in-laboratory replication	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	**Yes**	In every figure legend, according to the panels showed
In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	In every figure legend, according to the panels showed

Ethics

Ethics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	