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Cell-to-cell transmission of *C9orf72* poly-(Gly-Ala) triggers key features of ALS/FTD

Bahram Khosravi, Kathrine D. LaClair, Henrick Riemenschneider, Qihui Zhou, Frédéric Frottin, Nikola Mareljic, Mareike Czuppa, Daniel Farny, Hannelore Hartmann, Meike Michaelsen, Thomas Arzberger, F. Ulrich Hartl, Mark S. Hipp, Dieter Edbauer

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 19 August 2019

Thanks for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from these comments, the referees find the analysis interesting and insightful. They raise a number of constructive comments that I would like to ask you to address in a revised version. Let me know if we need to discuss any of them in detail - happy to do so.

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

We generally allow three months as standard revision time. 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, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

Summary: In this manuscript, Khosravi and co-authors have focused on understanding the correlation of a C9 RAN species (polyGA) and TDP-43 pathology. TDP-43 pathology is a

characteristic of a number of fALS and sALS cases and dipeptide repeat protein pathology is a distinct feature of C9orf72 ALS/FTD. There are several studies showing that C9 BAC mouse models did not develop or developed only modest TDP-43 pathology and expression of polyGA results in a subtle TDP-43 mislocalization and phosphorylation. In this manuscript, the authors show that polyGA triggers TDP-43 mislocalization in both a cell-autonomous and non-cell-autonomous fashion, possibly by inhibiting proteasome activity. Specifically, using primary neuron or HeLa cell co-cultures, they show that polyGA expression in donor cells inhibits proteasome activity and enhances TDP-43 mislocalization in receiver cells. Treating these cells with anti-GA antibody or enhancing proteasome activity using rolipram rescues the effects of polyGA on TDP43 pathology. In another mechanistic study, Khosravi and coauthors show expression of polyGA is linked with ubiquitination within the TDP-43 nuclear localization signal. Overall, the results from this study provide possible explanations for a poor correlation of TDP-43 and C9 dipeptide protein pathology in patient brain tissue by demonstrating that polyGA can trigger TDP-43 pathology in neighboring cells which do not contain GA aggregates. Specific suggestions are below.
1) In Fig 1EVD/E, please clarify which part of spinal cord was used to quantify cytoplasmic TDP-43. Also, if cytoplasmic TDP-43 accumulates in any specific cell type?
2) Fig EVD1A - the aggregate area should also be quantified as it looks like the aggregate area in the GFP-GR treated cells is higher than in the GA treated cells. If this is true, the authors need to discuss this.
3) EV3E is not mentioned in the text - please correct
4) Fig 1EVD3 - the results of this figure need to be explained in the text. As written, it is not clear what this gel is supposed to show.
5) Page 5, paragraph 2, the authors should include image/data showing that cytoplasmic TDP43 inclusions are present in neighboring cells that do not have clear GA aggregates to support their conclusions.

6) Fig EV2A/B, statistical comparisons between IgG and anti-GA antibody treatment groups is needed, instead of the comparisons that are shown are needed to support their statements.

7) In Fig. 2C/D, since 5F2 antibody was used in both treatment experiment and MSD, it is necessary to show that antibody used in treatment does not interfere with MSD detection of polyGA.

8) In the section stating that 'boosting proteasome activity restores nuclear import via the TDP-43 NLS', page 8, it is not clear if the effects of enhanced proteasome activity resulting in decreased cytoplasmic TDP-43 aggregates is due to improved cytoplasmic TDP-43 clearance or improved TDP-43 nuclear import. The authors should test this directly by blocking nuclear import under conditions that enhance proteasome activity to determine if increased proteasome function has the same effect.

9) RNA levels need to be measured and provided as supplemental data for transfection experiments that compare outcomes to ensure comparable expression.

10) Figure 6. In addition, experiments to compare protein turnover of RFP-NLSwt and mutant proteins should be done to compare their stability to ensure that the apparent decreased in GFP-K95A protein is not simply due to differences in protein stability understand the role of lysine 95 for protein clearance and nuclear import.

Referee #2:

This manuscript addresses a fundamental question that has remained largely unanswered since the identification of C9orf72 mutations underlying ALS/FTD in 2011: how does TDP43 pathology arise in C9orf72 mutation carriers? Here, Khosravi et al. provide intriguing evidence demonstrating that polyGR proteins, produced by repeat associated non-AUG (RAN) translation of the C9orf72 repeat, induce cytoplasmic TDP43 mislocalization. They convincingly show that polyGA overexpression results in cytoplasmic TDP43 mislocalization not only in cells expressing polyGA, but also in neighboring or recipient cells. polyGA expression causes proteasomal inhibition, and similarly TDP43 mislocalization is reproduced and exacerbated by proteasomal inhibition, but attenuated by proteasomal stimulation with rolipram. Engineered mutations of the TDP43 NLS that block ubiquitination at K95 also prevent polyGA-induced mislocalization, suggesting that abnormal

ubiquitination of this residue (due to a block in the UPS) is responsible for TDP43 mislocalization in response to polyGA. Not only does the work provide an interesting mechanism for TDP43 pathology in C9orf72 ALS/FTD, but it also illustrates that TDP43 nuclear transport may be regulated in part by ubiquitination of the NLS, a novel and important finding.

The manuscript is well written and most of the figures are very clear and easily interpretable (with some exception, see below). I have only a few concerns and suggestions, intended primarily to strengthen the manuscript further:

- One potential caveat for these studies, and in particular the non cell autonomous phenotypes observed, is the association with protein overexpression. In contrast to endogenously expressed genes, transgenes tend to create higher protein loads that can be externalized from the cell at a higher rate (PMID: 27295555), contributing to the pool of material in extracellular media and potentially underlying the extrinsic phenomena noted in receiver cells. To exclude this possibility, the authors could determine if co-culture with cells isolated from patients carrying the C9orf72 mutation (i.e. iPSCs or iPSC-derived neurons) are capable of supporting non cell autonomous TDP43 mislocalization and other phenomena observed in this manuscript.

- Another possible concern is that the TDP43 mislocalization observed in cells expressing or exposed to GA-GFP may be a nonspecific effect from dead/dying or dysfunctional cells. The antibody experiments in Fig. 2 help address this possibility, but to establish specificity, the authors could repeat the experiments in Fig. 1 using a separate construct such as mSOD1 or FUS (or GR- or PR-GFP) that elicits toxicity but would not be predicted to result in TDP43 mislocalization.

- The data in Fig. 6 showing the effects of the K95A and K95R mutants are impressive. However, ubiquitination of the NLS would not be expected to affected the transport of TDP43-dNLS (Fig. 1) or TDP-CTF (Fig. EV1). There is likely to be a separate explanation for the effects on these constructs.

- Related to this, but in addition, it would be important to determine the impact of the K95A and K95R mutants on full-length TDP43, rather than a reporter containing the TDP43 NLS.

- TDP43 is a substrate of the UPS (Igaz et al. 2009; Lokireddy et al., 2015 (both referenced in the manuscript); Flores et al. 2019 (PMC6499398)). It is therefore possible that UPS inhibition (via polyGA or MG132) may result in cytoplasmic mislocalization due to TDP43 accumulation, rather than changes in transport per se. The authors could discriminate among these possibilities by examining TDP43 clearance.

- As an additional control, the authors could investigate the effects of polyGR and/or polyPR on proteasome function to determine if proteasomal inhibition is specific to polyGA.

Minor concerns:

Nuclear export of TDP43 is not solely passive; see for example Archbold et al. 2018 (PMC5854632) and Aksu et al. 2018 (PMC6028547).

Additional data supporting the potential toxicity of polyGA come from in vitro studies using purified DPRs - Chang et al. 2015 (PMC4777828) and Flores et al. 2016 (PMC5077081).

In Fig. EV1A-C, TDP-CTF should be labeled as RFP-TDP-CTF, and in C the figure should some indication that (-) lanes contain lysates from cells transfected with RFP alone.

In EV1C, there appears to be reduction in RFP-TDP-CTF in cells transfected with PR-GFP - is this consistent?

As currently organized, Fig. 1C is somewhat confusing. This panel and experiments like it are important for the manuscript, making it important to present the results in a more intuitive fashion.

In EV3, for polyGA-transfected cells it is difficult to determine if the cells pictured are alive, or simply carcasses with inclusions.

In. Fig 5, if TDP43-NLS localization is affected by proteasomal inhibition, then MG132 have the same effect as polyGA expression. This is shown in bargraph format in Fig 6D, but images within Fig. 5 would also help drive home this point.

Even with a paracrine mode of action for DPRs, as indicated by the authors in the discussion, there should be an area surrounding DPR-producing cells that is rich in neurons demonstrating TDP43 mislocalization. This is not apparent from neuropathological studies.

Referee #3:

In this manuscript, the authors investigate how C9orf72 repeat expansion influences TDP-43 pathology in C9orf72-assoicated frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) (c9FTD/ALS). The authors report that C9orf72 poly(GA) proteins inhibit proteasome activity, which consequently causes TDP-43 cytoplasmic accumulation and aggregation in poly(GA)-infected cells. Moreover, poly(GA) also causes TDP-43 abnormalities in the neighbor cells via cell-to-cell transmission. Treatment with anti-GA antibodies or proteasomal activation ameliorates poly(GA)-induced TDP-43 abnormalities. Further mechanistic studies reveal that proteasomal inhibition results in TDP-43 ubiquitination at lysine 95, which is within in a nuclear localization signal (NLS) of TDP-43. Importantly, ubiquitination of lysine 95 reduces its binding to nuclear import receptor, and then blocks its nuclear import. While this study is interesting, the data in its current format is sufficient to support the conclusions.
The specific points are listed below.
1. To evaluate the pathological significance, the authors should examine the correlation of poly(GA) and TDP-43 pathology in c9FTD/ALS patients.
2. In Figure 1A-C, the authors show poly(GA) proteins induce cytoplasmic accumulation of endogenous TDP-43. The authors should clarify whether TDP-43 aggregates are observed under this conditions. Moreover, the authors should perform the filter trap assay to determine whether poly(GA) affects solubility of endogenous TDP-43. Western blot analysis should also be performed to determine whether TDP-43 cleavage occurs in culture cells and mouse model expressing poly(GA). Lastly, the authors should examine whether other dipeptide repeat (DPR) proteins also cause endogenous TDP-43 abnormalities.
3. In Figure 1D, the representative figures indicate that a subset of inclusions contains both poly(GA) and TDP-43. If this is case, the authors should quantify the percentage of inclusions contains both proteins. In Figure 1E, the authors should quantify TDP-43 inclusions in poly(GA)-positive and poly(GA)-negative cells as did in Figure 1C.
4. In Figure 3C, the authors should perform double or triple labelling of UbG76V-GFP, GA-iRFP and TDP-43 to determine the correlation of GA, proteasome inhibition and TDP-43 cytoplasmic accumulation in poly(GA)-positive and poly(GA)-negative cells. In addition, RNA levels of UbG76V-GFP should be examined.
5. In Figure 4F, the authors show Roliprgam treatment reduces the levels of insoluble TDP-43 C-terminal fragment (CTF). The authors should examine whether Roliprgam can also influence the solubility of endogenous TDP-43 and TDP-43ΔNLS.
6. In Figure 5, the authors state that 'Boosting proteasomal activity restores nuclear import via the TDP-43 NLS'. However, the data in Figure 6 and 7 indicate that proteasome activation promotes the degradation of ubiquinated TDP-43 at lysine 95, rather than influence TDP-43 nuclear import. The authors should reconcile this point.
7. In Figure 6 and 7, The authors used GFP-NLS or RFP-NLS reporter constructs; however TDP-43 wild-type and various NLS mutants should be included to verify the critical results shown in Figure 6C-F, and Figure 7.
8. Previous studies have reported that proteasomal inhibition results in stress granule formation. Since stress granule pathway also regulates TDP-43 cytoplasmic mislocalizaiton and aggregation, the authors should examine whether stress granules markers such as G3BP1 and TIA-1 are present in cell expressing poly(GA) or treated with MG-132.
9. In the abstract, the authors state that 'Poly-GA promoted cytoplasmic mislocalization and aggregation of TDP-43 non-cell-autonomously'. However, cytoplasmic mislocalization and aggregation of TDP-43 are also observed in poly(GA)-positive cells, indicating involvement of cell-autonomous pathway. The authors should clarify this point.

1st Revision - authors' response 11 December 2019

Referee #1:

Summary: In this manuscript, Khosravi and co-authors have focused on understanding the correlation of a C9 RAN species (polyGA) and TDP-43 pathology. TDP-43 pathology is a characteristic of a number of fALS and sALS cases and dipeptide repeat protein pathology is a distinct feature of C9orf72 ALS/FTD. There are several studies showing that C9 BAC mouse models did not develop or developed only modest TDP-43 pathology and expression of polyGA results in a subtle TDP-43 mislocalization and phosphorylation. In this manuscript, the authors show that polyGA triggers TDP-43 mislocalization in both a cell-autonomous and non-cell-autonomous fashion, possibly by inhibiting proteasome activity. Specifically, using primary neuron or HeLa cell co-cultures, they show that polyGA expression in donor cells inhibits proteasome activity and enhances TDP-43 mislocalization in receiver cells. Treating these cells with anti-GA antibody or enhancing proteasome activity using rolipram rescues the effects of polyGA on TDP43 pathology. In another mechanistic study, Khosravi and coauthors show expression of polyGA is linked with ubiquitination within the TDP-43 nuclear localization signal. Overall, the results from this study provide possible explanations for a poor correlation of TDP-43 and C9 dipeptide protein pathology in patient brain tissue by demonstrating that polyGA can trigger TDP-43 pathology in neighboring cells which do not contain GA aggregates. Specific suggestions are below.

1) In Fig 1EVD/E, please clarify which part of spinal cord was used to quantify cytoplasmic TDP-43. Also, if cytoplasmic TDP-43 accumulates in any specific cell type?

We apologize for imprecise description. In the revised manuscript we clearly state that we had analyzed TDP-43 mislocalization in large neurons of the anterior horn, because Thy1 drives poly-GA expression in this mouse model predominantly in motor neurons (Schludi et al, Acta Neuropathol 2017). Additionally, we performed co-staining of Choline Acetyltransferase (ChAT), TDP-43 and poly-GA in anterior and posterior horn of the spinal cord. TDP-43 mislocalization is mainly observed in ChAT positive motor neurons of the anterior horn and not in the posterior horn (new Fig EV1D).

2) Fig EVD1A - the aggregate area should also be quantified as it looks like the aggregate area in the GFP-GR treated cells is higher than in the GA treated cells. If this is true, the authors need to discuss this.

We followed the reviewer's suggestion and quantified the aggregated area from the existing images (new Appendix Fig S1C). TDP-CTF aggregate size was not significantly affected by expression of poly-GA or the other DPRs.

3) EV3E is not mentioned in the text - please correct

We corrected this omission.

4) Fig 1EVD3 - the results of this figure need to be explained in the text. As written, it is not clear what this gel is supposed to show.

Since there is no Fig 1EVD3 we think this comment refers most likely to Fig EV1C (now Appendix Fig. S1D) and improved the legend for this panel in the revised manuscript.

5) Page 5, paragraph 2, the authors should include image/data showing that cytoplasmic TDP43 inclusions are present in neighboring cells that do not have clear GA aggregates to support their conclusions.

We now provide larger fields of view for Fig. 1B/D in the new Appendix Fig S2A/E showing more examples of cell-autonomous and non-cell-autonomous effects.

6) Fig EV2A/B, statistical comparisons between IgG and anti-GA antibody treatment groups is needed, instead of the comparisons that are shown are needed to support their statements.

The reviewer is correct and we apologize for the omission. We added the crucial statistical comparison of anti-GA and control IgG treated conditions (revised Fig EV2B). The additional statistics fully confirm the conclusion that anti-GA treatment ameliorates poly-GA-induced non-cell autonomous TDP-43 mislocalization.

7) In Fig 2C/D, since 5F2 antibody was used in both treatment experiment and MSD, it is necessary to show that antibody used in treatment does not interfere with MSD detection of polyGA.
This is a very reasonable comment. We repeated the experiment and analyzed the supernatant before and after anti-GA depletion using western blotting. After immunodepletion no residual anti-GA antibody was detected by western blot (revised Fig 2C). This new data strongly supports the validity of our immunoassay results in Fig 2D.

8) In the section stating that 'boosting proteasome activity restores nuclear import via the TDP-43 NLS', page 8, it is not clear if the effects of enhanced proteasome activity resulting in decreased cytoplasmic TDP-43 aggregates is due to improved cytoplasmic TDP-43 clearance or improved TDP-43 nuclear import. The authors should test this directly by blocking nuclear import under conditions that enhance proteasome activity to determine if increased proteasome function has the same effect.
We followed this insightful suggestion using combination treatment of an importin-α/β pathway inhibitor (10 μM ivermectin) and 30μM rolipram to activate the proteasome (new Appendix Fig S4). Interestingly, rolipram reduced even ivermectin-induced RFP-NLS reporter mislocalization suggesting that proteasome induction enhances degradation of the cytoplasmic TDP-43 reporters. Conversely, poly-GA induced proteasome impairment results in accumulation of TDP-43 ubiquitinated within its NLS leading to a specific import deficit due to impaired importin-α binding (Fig 6E/F and new EV4).

9) RNA levels need to be measured and provided as supplemental data for transfection experiments that compare outcomes to ensure comparable expression.
Excluding expression changes due to poly-GA is indeed an important control. We now provide qPCR data for the experiments in Fig 1D-F (new panel Fig 1G) and for experiment in Fig 5 (new panels 5C/F/I). Together these data fully support our conclusion that poly-GA affects TDP-43 localization via ubiquitination at K95.

10) Figure 6. In addition, experiments to compare protein turnover of RFP-NLSwt and mutant proteins should be done to compare their stability to ensure that the apparent decreased in GFP-K95A protein is not simply due to differences in protein stability understand the role of lysine 95 for protein clearance and nuclear import.
We followed this excellent suggestion and analyzed the turn-over of the critical RFP-TDP-43-NLS reporter constructs and the respective full-length mutants (wt, K84A, K95A). Blocking new protein translation using cycloheximide resulted in similar decay of all proteins at 4, 8 and 24 hours (new Appendix Fig S5A/B and Appendix Fig S5E/F).

Referee #2:

This manuscript addresses a fundamental question that has remained largely unanswered since the identification of C9orf72 mutations underlying ALS/FTD in 2011: how does TDP43 pathology arise in C9orf72 mutation carriers? Here, Khosravi et al. provide intriguing evidence demonstrating that polyGR proteins, produced by repeat associated non-AUG (RAN) translation of the C9orf72 repeat, induce cytoplasmic TDP43 mislocalization. They convincingly show that polyGA overexpression results in cytoplasmic TDP43 mislocalization not only in cells expressing polyGA, but also in neighboring or recipient cells. polyGA expression causes proteasomal inhibition, and similarly TDP43 mislocalization is reproduced and exacerbated by proteasomal inhibition, but attenuated by proteasomal stimulation with rolipram. Engineered mutations of the TDP43 NLS that block ubiquitination at K95 also prevent polyGA-induced mislocalization, suggesting that abnormal ubiquitination of this residue (due to a block in the UPS) is responsible for TDP43 mislocalization in response to polyGA. Not only does the work provide an interesting mechanism for TDP43 pathology in C9orf72 ALS/FTD, but it also illustrates that TDP43 nuclear transport may be regulated in part by ubiquitination of the NLS, a novel and important finding.

The manuscript is well written and most of the figures are very clear and easily interpretable (with some exception, see below). I have only a few concerns and suggestions, intended primarily to strengthen the manuscript further:

- One potential caveat for these studies, and in particular the non cell autonomous phenotypes

observed, is the association with protein overexpression. In contrast to endogenously expressed genes, transgenes tend to create higher protein loads that can be externalized from the cell at a higher rate (PMID: 27295555), contributing to the pool of material in extracellular media and potentially underlying the extrinsic phenomena noted in receiver cells. To exclude this possibility, the authors could determine if co-culture with cells isolated from patients carrying the C9orf72 mutation (i.e. iPSCs or iPSC-derived neurons) are capable of supporting non cell autonomous TDP43 mislocalization and other phenomena observed in this manuscript.
Overexpression effects are a limitation that is inherent to all gain-of-function models. However, we had shown already in May et al. Acta Neuropathologica 2014 that lentiviral poly-GA expression in primary neurons results in poly-GA levels similar to patient tissue suggesting the observed phenomena are biologically relevant.
Unfortunately, iPSC derived neurons develop neither visible DPR nor TDP-43 inclusions. Only a few weeks ago did the first group succeed to detect DPR species other than poly-GP in iPSC derived neurons (PMID: 31624870). Therefore, the experiment was not feasible as suggested.
While we cannot directly probe the non-cell autonomous component in patients, we now show that poly-GA bearing neurons in *C9orf72* FTLD patients are more likely to show cytoplasmic TDP-43 than neurons without detectable poly-GA using an automated image analysis pipeline (new Fig EV1E/F).

- Another possible concern is that the TDP43 mislocalization observed in cells expressing or exposed to GA-GFP may be a nonspecific effect from dead/dying or dysfunctional cells. The antibody experiments in Fig 2 help address this possibility, but to establish specificity, the authors could repeat the experiments in Fig 1 using a separate construct such as mSOD1 or FUS (or GR- or PR-GFP) that elicits toxicity but would not be predicted to result in TDP43 mislocalization.
Other secreted factors are a reasonable explanation for non-cell autonomous effects, which we aimed to rule out using immunodepletion as acknowledged by the reviewer (Fig 2 and EV2). To address residual concerns, we analyzed TDP-43 localization in receiver cells co-cultured with poly-PR and poly-GR expressing neurons as suggested (new Appendix Fig S2C/D). Although poly-PR expression is considerably more toxic than poly-GA expression (e.g. Wen et al, Neuron 2014), poly-GR/PR expression had no statistically significant effect on TDP-43 localization in donor or receiver cells. These results support our conclusion that poly-GA itself and not other released factors induce TDP-43 mislocalization.

- The data in Fig 6 showing the effects of the K95A and K95R mutants are impressive. However, ubiquitination of the NLS would not be expected to affected the transport of TDP43-dNLS (Fig 1) or TDP-CTF (Fig EV1). There is likely to be a separate explanation for the effects on these constructs.
We thank the reviewer for this thoughtful comment. Indeed, TDP-43ΔNLS and TDP-CTF lack the critical K95 residue. Strikingly, the turn-over of TDP-CTF is not affected by poly-GA expression in a cycloheximide experiment suggesting poly-GA mainly promotes its cytoplasmic aggregation of TDP-CTF (new data in Appendix Fig S1E/F) consistent with the filter trap data (Fig 1F). Enhanced ubiquitination or other post-translational modification within the TDP-CTF may promote inclusion formation. This important point is now mentioned in the revised discussion.

- Related to this, but in addition, it would be important to determine the impact of the K95A and K95R mutants on full-length TDP43, rather than a reporter containing the TDP43 NLS.
We had shown that in original Fig EV5 (now new Appendix Fig S5C/D) that poly-GA also affects localization of full-length TDP-43, which can be blocked by the K95A and K95R mutations. In addition, we now show that the K95A mutation reduces the effects of poly-GA on importin-β binding (new Fig EV4). Finally, total ubiquitination of TDP-43 full length with a K95A mutation is hardly affected by poly-GA expression (new Fig EV5). These experiments fully validate our findings in the context of full-length TDP-43 and highlight the importance of K95 ubiquitination.

- TDP43 is a substrate of the UPS (Igaz et al. 2009; Lokireddy et al., 2015 (both referenced in the manuscript); Flores et al. 2019 (PMC6499398)). It is therefore possible that UPS inhibition (via polyGA or MG132) may result in cytoplasmic mislocalization due to TDP43 accumulation, rather than changes in transport per se. The authors could discriminate among these possibilities by examining TDP43 clearance.
We followed this suggestion and analyzed the clearance of TDP-43 variants using cycloheximide experiments. The turn-over of full length TDP-43 was not affected by K84A and K95A mutations (new data in Appendix Fig S5A/B, E/F. Moreover, poly-GA expression did not alter the turn-over of

TDP-CTF as mentioned above (new Appendix Fig S1E/F). Thus, we assume that UPS inhibition results in accumulation of cytoplasmic TDP-43 ubiquitinated at K95, which blocks nuclear import and gradually results in cytoplasmic aggregation.

- As an additional control, the authors could investigate the effects of polyGR and/or polyPR on proteasome function to determine if proteasomal inhibition is specific to polyGA.
We addressed this important question by analyzing high-molecular weight ubiquitin levels in DPR-GFP expressing cells. Only poly-GA, but not the other DPR species, resulted in accumulation of ubiquitinated proteins. This new data is shown in the new Appendix Fig S2C/D of the revised manuscript.

Minor concerns:

Nuclear export of TDP43 is not solely passive; see for example Archbold et al. 2018 (PMC5854632) and Aksu et al. 2018 (PMC6028547).
We added this important information and the two references to the introduction.

Additional data supporting the potential toxicity of polyGA come from in vitro studies using purified DPRs - Chang et al. 2015 (PMC4777828) and Flores et al. 2016 (PMC5077081).
We added these helpful references to the discussion.

In Fig EV1A-C, TDP-CTF should be labeled as RFP-TDP-CTF, and in C the figure should some indication that (-) lanes contain lysates from cells transfected with RFP alone.
We corrected the mistake in the revised figure.

In EV1C, there appears to be reduction in RFP-TDP-CTF in cells transfected with PR-GFP - is this consistent?
We addressed this question by checking the other replicates of the same experiment, and PR-GFP does not show reduction in RFP-TDP-CTF levels in other experiments. We replaced the immunoblots with a more representative example (revised Appendix Fig S1D).

As currently organized, Fig 1C is somewhat confusing. This panel and experiments like it are important for the manuscript, making it important to present the results in a more intuitive fashion.
We change "GFP" to "GFP staining" to show this row refers to the GFP level in each analyzed cell and not to the transduction condition.

In EV3, for polyGA-transfected cells it is difficult to determine if the cells pictured are alive, or simply carcasses with inclusions.
We repeated the experiment with additional co-staining of cytosol (HCS CellMask™ Deep Red Stain) in HeLa cells and dendrites (MAP2) in neurons to show overall cell viability. DAPI staining also shows non-apoptotic nuclei. This new data is shown in the revised Fig. EV3A/B.

In. Fig 5, if TDP43-NLS localization is affected by proteasomal inhibition, then MG132 have the same effect as polyGA expression. This is shown in bargraph format in Fig 6D, but images within Fig 5 would also help drive home this point.
Images in Fig 6c show the effect of MG132 alongside GA-GFP and allow side by side comparison of their effects.

Even with a paracrine mode of action for DPRs, as indicated by the authors in the discussion, there should be an area surrounding DPR-producing cells that is rich in neurons demonstrating TDP43 mislocalization. This is not apparent from neuropathological studies.
While regional clustering of DPRs has been reported (Zhu et al, PNAS 2013), clustering of TDP-43 has not been analyzed to our knowledge. Since rigorous analysis would require 3D reconstruction, we decided to investigate the frequency of cytoplasmic TDP-43 mislocalization in frontal cortex of *C9orf72* FTD cases (new Fig EV1E/F). Poly-GA expressing neurons have a higher abundance of TDP-43 mislocalization than poly-GA negative neurons. We aim to optimize the automated image analysis pipeline further to also address the non-cell-autonomous effect specifically in a larger series of cases in the future.

Referee #3:

In this manuscript, the authors investigate how C9orf72 repeat expansion influences TDP-43 pathology in C9orf72-assoicated frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) (c9FTD/ALS). The authors report that C9orf72 poly(GA) proteins inhibit proteasome activity, which consequently causes TDP-43 cytoplasmic accumulation and aggregation in poly(GA)-infected cells. Moreover, poly(GA) also causes TDP-43 abnormalities in the neighbor cells via cell-to-cell transmission. Treatment with anti-GA antibodies or proteasomal activation ameliorates poly(GA)-induced TDP-43 abnormalities. Further mechanistic studies reveal that proteasomal inhibition results in TDP-43 ubiquitination at lysine 95, which is within in a nuclear localization signal (NLS) of TDP-43. Importantly, ubiquitination of lysine 95 reduces its binding to nuclear import receptor, and then blocks its nuclear import. While this study is interesting, the data in its current format is sufficient to support the conclusions.
We followed the reviewer's helpful suggestions and are confident that the data now fully support our conclusions.

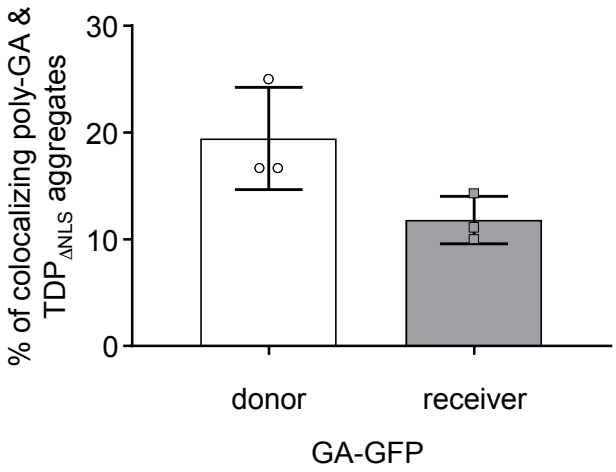
The specific points are listed below.
1. To evaluate the pathological significance, the authors should examine the correlation of poly(GA) and TDP-43 pathology in c9FTD/ALS patients.
We followed this excellent suggestion and analyzed cytoplasmic TDP-43 mislocalization in the frontal cortex of 8 *C9orf72*-FTLD cases. Strikingly, cytoplasmic TDP-43 was significantly more common in neurons containing poly-GA inclusions, which fully supports our conclusion that poly-GA is a critical driver of TDP-43 pathology (new Fig EV1E/F).

2. In Figure 1A-C, the authors show poly(GA) proteins induce cytoplasmic accumulation of endogenous TDP-43. The authors should clarify whether TDP-43 aggregates are observed under this conditions.
Unfortunately, there are no mature TDP-43 aggregates visible under these conditions. We mentioned this in the revised result section.

Moreover, the authors should perform the filter trap assay to determine whether poly(GA) affects solubility of endogenous TDP-43. Western blot analysis should also be performed to determine whether TDP-43 cleavage occurs in culture cells and mouse model expressing poly(GA).
While filter trap is very sensitive for poly-Q and poly-GA, it has to our knowledge not been used on endogenous TDP-43 even in patient tissue. We also only detected filter-trap signal with TDP-CTF (Fig 4F/G) or TDP-43ΔNLS (Fig 1F and new Fig 4H/I), but not with endogenous TDP-43 (not shown). Thus, we analyzed TDP-43 by western blot, but found no evidence for poly-GA induced cleavage in rat neurons (new Appendix Fig S2B) or GA-CFP mice (new Fig EV1C).

Lastly, the authors should examine whether other dipeptide repeat (DPR) proteins also cause endogenous TDP-43 abnormalities.
Although individual expression of poly-GR or -PR is more toxic than poly-GA in most systems, TDP-43 aggregates have not reported. We analyzed TDP-43 mislocalization in donor and receiver cells similar to Fig 1, but found no effect of poly-GR/PR on TDP-43 (new Appendix Fig S2C/D), which is consistent with our previous report (Khosravi et al HMG 2017).

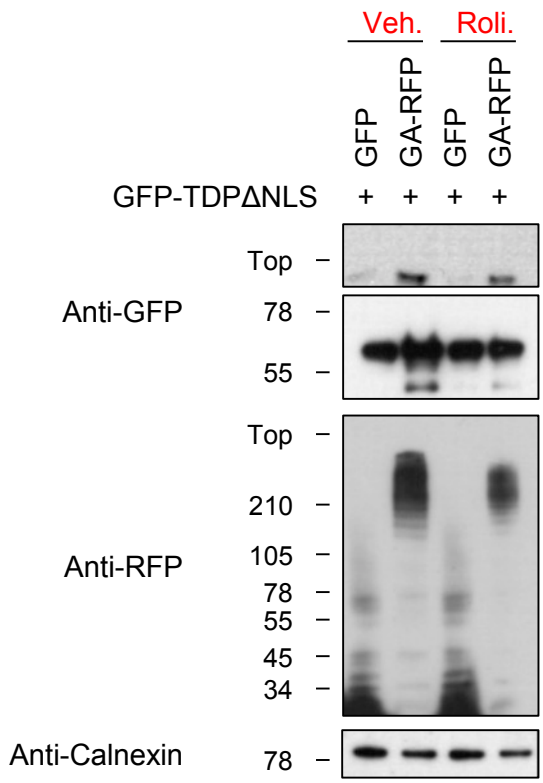
3. In Figure 1D, the representative figures indicate that a subset of inclusions contains both poly(GA) and TDP-43. If this is case, the authors should quantify the percentage of inclusions contains both proteins. In Figure 1E, the authors should quantify TDP-43 inclusions in poly(GA)-positive and poly(GA)-negative cells as did in Figure 1C.
We followed this suggestion and reanalyzed our images to separate TDPΔNLS aggregation in GA-iRFP positive and negative cell. As in the other experiments, poly-GA donor cells enhanced TDPΔNLS aggregation in neighboring cells even without detectable poly-GA uptake (new Fig 1E). Moreover, about 10-20% of poly-GA inclusions also contained TDPΔNLS, which is consistent with patient data (e.g. Mori et al, Science 2013 and Mackenzie et al, ANP 2013). This is mentioned in the revised results and a quantification of three independent biological experiments is shown below.



4. In Figure 3C, the authors should perform double or triple labelling of UbG76V-GFP, GA-iRFP and TDP-43 to determine the correlation of GA, proteasome inhibition and TDP-43 cytoplasmic accumulation in poly(GA)-positive and poly(GA)-negative cells.
We assume this question refers to the flow cytometry data in Fig 3/EV3 as one cannot easily separate poly-GA positive and negative cells in an immunoblot experiment with unsorted cells. Thus, we reanalyzed our flow cytometry data to group cells by poly-GA uptake (new Fig EV3F). Likely due to the greater sensitivity of flow cytometry, the non-cell autonomous proteasome inhibition is only seen in receiver cells with detectable poly-GA uptake. Unfortunately, it is not possible to quantify subcellular localization of TDP-43 by flow cytometry (or by western blotting).

In addition, RNA levels of UbG76V-GFP should be examined.
We followed this suggestion and excluded transcriptional effects of poly-GA on UbG76V-GFP mRNA expression (new Fig 3E).

5. In Figure 4F, the authors show Rolipram treatment reduces the levels of insoluble TDP-43 C-terminal fragment (CTF). The authors should examine whether Rolipram can also influence the solubility of endogenous TDP-43 and TDP-43ΔNLS.
We attempted filter trap experiments for endogenous TDP-43, but could not detect aggregation even upon poly-GA expression (data not shown). However, poly-GA expression resulted in detectable aggregation of GFP-TDPΔNLS, which was rescued by rolipram treatment (new Fig 4H/I).



6. In Figure 5, the authors state that 'Boosting proteasomal activity restores nuclear import via the TDP-43 NLS'. However, the data in Figure 6 and 7 indicate that proteasome activation promotes the degradation of ubiquinated TDP-43 at lysine 95, rather than influence TDP-43 nuclear import. The authors should reconcile this point.

We followed the suggestions of referee #2, who raised a similar concern (8.) to address this point. We used a double treatment with an importin-α/β pathway inhibitor ivermectin and rolipram (new Appendix Fig S4). Interestingly, rolipram reduced reporter mislocalization even under these conditions suggesting the main effect of proteasome induction is on enhancing degradation of cytoplasmic TDP-43. Conversely, poly-GA induced proteasome impairment results in accumulation of TDP-43 ubiquitinated within its NLS leading to a specific import deficit due to impaired binding to importins (Fig 6). We changed the title of this section accordingly.

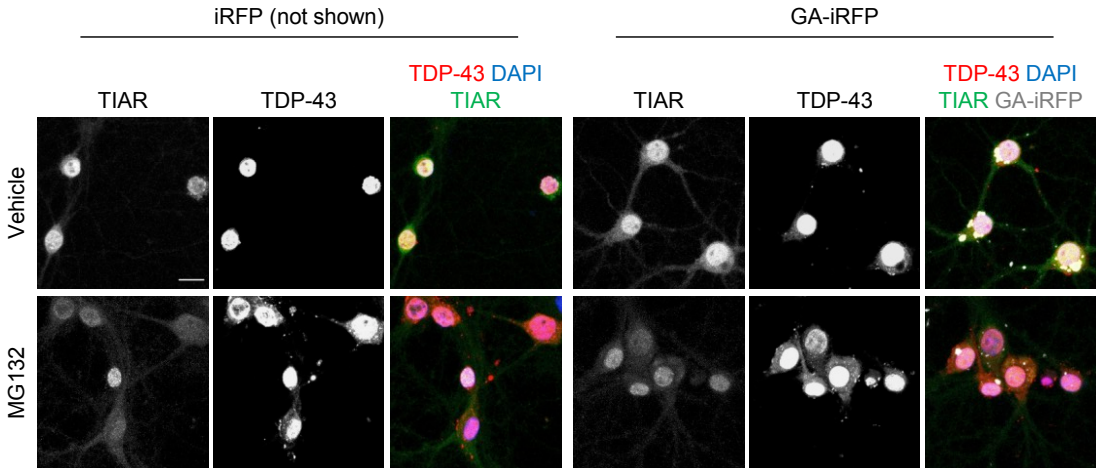
7. In Figure 6 and 7, The authors used GFP-NLS or RFP-NLS reporter constructs; however TDP-43 wild-type and various NLS mutants should be included to verify the critical results shown in Figure 6C-F, and Figure 7.

We followed this excellent suggestion and replicated the key findings with the TDP-43 NLS reporter with full length TDP-43. Already in the original submission, we had analyzed poly-GA induced mislocalization of TDP-43 full length wt, K95A and K95R in figure EV5 (now Appendix Fig S5C/D) validating figure 6C/D. Replication of Fig 6E/F with full length constructs (wt, K95A, K84A) is shown in the new Fig EV4 and fully confirms that the effect of poly-GA on TDP-43/KPNA1 interaction depends on K95. Moreover, replication of Fig 7 with full length TDP-43 (wt, K95A, K84A) confirms that poly-GA leads to accumulation of TDP-43 predominantly ubiquitinated at K95 (new Fig EV5).

8. Previous studies have reported that proteasomal inhibition results in stress granule formation. Since stress granule pathway also regulates TDP-43 cytoplasmic mislocalizaiton and aggregation, the authors should examine whether stress granules markers such as G3BP1 and TIA-1 are present in cell expressing poly(GA) or treated with MG-132.

We had previously shown that the poly-GA induced cytoplasmic TDP-43 punctae mainly colocalize with lysosomal markers but not with stress granules (Khosravi et al, HMG 2017). Even additional proteasome inhibition did not result in co-localization with the stress granule marker (see below). Since the revised manuscript is already pretty dense, we would prefer to not include this negative

data.



9. In the abstract, the authors state that 'Poly-GA promoted cytoplasmic mislocalization and aggregation of TDP-43 non-cell-autonomously'. However, cytoplasmic mislocalization and aggregation of TDP-43 are also observed in poly(GA)-positive cells, indicating involvement of cell-autonomous pathway. The authors should clarify this point.
We agree and changed the abstract accordingly.

2nd Editorial Decision 3 February 2020

Thank you for submitting your revised manuscript to The EMBO Journal. I am sorry for the slight delay in getting back to you, but I have now received the comments back from the three referees. As you can see from the comments below, the referees appreciate the added data and support publication. They raise a few concerns that should be fairly easy to address.

Regarding the points raised by referee #2 "...The new data were much appreciated. Some errors were noted in these data however - S5A (Anti-Calnexin, +CHX, no bands and edge of gel?), S5E (no bands). S3E (no bands), S1E (no bands, and top of gel?), S1D (Anti-Calnexin), S3C (anti-HA, top of gel?)." I queried the raised issues with the referee as everything looks fine when you look at the PDF. The referee looked at the word file and there things look off. Just delete the word file to avoid any possible problems down the road

When you re-submit the revised version would you also take care of the following points:

There is no author contribution listed for Mareike Czuppa

There is a callout for Appendix Fig S23A/E in the figure legend Fig. 1G. Please fix

The scale bar in Fig 2A is very small and hardly visible.

Scale bars are missing in Fig EV1A, D, E

Please provide the excel source data as 1 file per figure.

Please note that the grants "Deutsche Forschungsgemeinschaft EXC 2145 and EXC 1010" are not mentioned in the acknowledgements, but only in the online system. Please check and include in acknowledgements if needed.

I have asked our publisher to do their pre-publication checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

We encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you

could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

That should be all let me know if we need to discuss anything further

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REFEREE REPORTS

Referee #1:

In general I am satisfied with the revisions. I have a few changes that I feel should be made before the manuscript is accepted.

Page 4 It is not correct to say that CT TDP43 tagged with RFP is the most common aggregating form since there is no RFP tagged protein in humans. The authors should reword to correct the intended meaning.

The authors should discuss the recent publications by both Zhou et al and Nguyen et al. to put the current results in context.

In Nguyen et al (online and citable) the observation that proteasome function was restored when GA proteins are targeted with a anti-GA antibody is similar to results described here and should be discussed and cited.

There is text repeated on the bottom of page 3.

Referee #2:

The authors have added thoughtful experiments and addressed nearly all of my concerns. Protein overexpression is a difficult issue, and as long as the potential caveats are discussed adequately there is little else that can be done for the time being.

The new data were much appreciated. Some errors were noted in these data however - S5A (Anti-Calnexin, +CHX, no bands and edge of gel?), S5E (no bands). S3E (no bands), S1E (no bands, and top of gel?), S1D (Anti-Calnexin), S3C (anti-HA, top of gel?).

Referee #3:

The revised manuscript is markedly improved. All previous concerns have been adequately addressed by the authors. No additional comments are noted.

2nd Revision - authors' response 12 February 2020

Point-by-point response

Referee #1:

In general I am satisfied with the revisions. I have a few changes that I feel should be made before the manuscript is accepted.

Page 4 It is not correct to say that CT TDP43 tagged with RFP is the most common aggregating

form since there is no RFP tagged protein in humans. The authors should reword to correct the intended meaning.
The reviewer is correct. We changed the text accordingly.

The authors should discuss the recent publications by both Zhou et al and Nguyen et al. to put the current results in context.
In Nguyen et al (online and citable) the observation that proteasome function was restored when GA proteins are targeted with a anti-GA antibody is similar to results described here and should be discussed and cited.
We had mentioned both papers already and now specifically mention the proteasome data by Nguyen et al and provide the proper references.

There is text repeated on the bottom of page 3.

We corrected this mistake.

Referee #2:

The authors have added thoughtful experiments and addressed nearly all of my concerns. Protein overexpression is a difficult issue, and as long as the potential caveats are discussed adequately there is little else that can be done for the time being.

The new data were much appreciated. Some errors were noted in these data however - S5A (Anti-Calnexin, +CHX, no bands and edge of gel?), S5E (no bands). S3E (no bands), S1E (no bands, and top of gel?), S1D (Anti-Calnexin), S3C (anti-HA, top of gel?).

This problem must have been due to incompatibility of different MS Word versions. We now include only the properly converted PDF file in the resubmission.

Referee #3:

The revised manuscript is markedly improved. All previous concerns have been adequately addressed by the authors. No additional comments are noted.

THE EMBO JOURNAL

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

Corresponding Author Name: Dieter Edbauer
Manusript Number: EMBOJ-2019-102811

Reporting Checklist For Life Sciences Articles

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars only for independent experiments and sample sizes where the application of statistical tests is warranted (error bars should not be shown for technical replicates)
- when n is small (n < 5), the individual data points from each experiment should be plotted alongside an error bar.
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation (see link list at top right).

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Please fill out these boxes

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen according to experience and studies with comparable samples. All experiments were done at least 3 times.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Only figure EV1A-D shows animal data. Sample size was chosen without formal power analysis based on experience.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For the analysis of human C9orf72 tissue, one case was excluded from analysis due to extremely poor DAPI staining that precluded quantification of the frequency of poly-GA and cytoplasmic TDP-43. No other samples were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Image acquisition was done blinded and for most experiments image analysis was fully automated
For animal studies, include a statement about randomization even if no randomization was used.	we used transgenic animals and their littermates
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Image acquisition was done blinded and for most experiments image analysis was fully automated
4.b. For animal studies, include a statement about blinding even if no blinding was done	Investigators were blinded to genotype during data collection by the use of a number code for each animal.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, all samples were tested by GraphPad Prism Shapiro-Wilk normality test for normal distribution before applying t-test, one-way or two-way ANOVA analysis
Is there an estimate of variation within each group of data?	SD is shown
Is the variance similar between the groups that are being statistically compared?	all compared groups show similar variance as analyzed by GraphPad Prism (F-test).

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	TDP-43 (Cosmo Bio Co, TIP-TD-P09), TDP-43 (Proteintech 10782-2-AP), TDP-43 phospho-S409/410 (Cosmo Bio Co.,LTD TIP-PTD-P02), TDP-43 (C-Terminal) (Proteintech, 12892-1-AP), ChAT (Merck, AB144P), GFP (UC Davis/NIH NeuroMab Facility N86/8 and N86/38), PSMC4 (Bethyl Laboratories A303-850A and A303-849A), tagRFP (ThermoFisher Scientific R10367), KPNA1 clone 114-E12 (ThermoFisher Scientific 37-0800), Calnexin (Enzo Life Sciences ADI-SPA-860-F), HA 3F10 (Merck, 11867423001), GA 5F2 (Mackenzie et al., 2013), control IgG from mouse serum (Merck I5381), HCS CellMask™ Deep Red Stain (ThermoFisher Scientific H32721). Homemade anti-GA 5F2 is extensively validated (Mackenzie et al., 2013). TDP-43 antibodies have been validated by shRNA knockdown (Schwenk et al., EMBO J 2016).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HeLa cells were a gift from Dr. Rainer Pepperkok (EMBL Heidelberg, Germany). HEK293FT cells were purchased from ThermoFisher. Cells lines were regularly checked for mycoplasma contamination and always tested negative.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Generation and characterization of Thy1-GA149-CFP (abbreviated as GA-CFP) mice was reported previously (Schludi et al, 2017). Expression of GA149-CFP was driven by Thy1.2 promoter. GA-CFP transgenic mice were kept in the C57BL/6N background. Animals handling were performed in accordance to animal law of the Government of Upper Bavaria, Germany. Animals were housed in standard cages with ad libitum access to food and water in pathogen-free facility on a 12 h day/night cycle. Six transgenic (4 male, 2 female) mice and three littermates (2 male and 1 female) were analyzed.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	not applicable
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines' (see link list at top right). See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	not applicable

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	not applicable
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	not applicable
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines' (see link list at top right).	not applicable
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines' (see link list at top right).	not applicable

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition' (see link list at top right). Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	not applicable
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	not applicable
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	not applicable
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section: Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	not applicable
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	not applicable

G- Dual use research of concern

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