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FTLD-TDP assemblies seed neoaggregates with subtype-specific features via a prion-like cascade

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Dear Magda,

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is pasted below.

As you will see, referees 1 and 2 support the publication of your study with some more minor revisions, however, referee 3 is more critical. I would like to know whether you can address any of referee 3's and referee 1's concerns in a timely manner. All of referee 2's comments must be addressed. Given the related and already published paper, we can only offer to publish your manuscript if it can be accepted before the end of October. This means that we will need to receive your revised manuscript before the 10th of October. We can also video chat about the revisions, if you like.

If we can agree on the revision requirements, I would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

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2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

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4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

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If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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I look forward to seeing a revised version of your manuscript as soon as possible. Please let me know if you have questions or comments regarding the revision.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In this work, De Rossi et al. have investigated the differences in aggregation induced by TDP-43 extracts from two different FTD types, FTLD-TDP-A and FTLD-TDP-C which have different disease severity. Using several state-of-the-art techniques the authors have shown that there is a clear difference between the aggregates induced by Type A compared to Type C. In particular, the authors have focused on the type of fibrils composition, more compact for Type A compared to more heterogeneous for Type C. In general, the study has been conducted in a very careful and well thought-out manner, and the conclusions will certainly be of interest to the field.

Nonetheless, a few general and more specific issues could be considered by the authors to improve the significance of the

bindings.

Although addressed in Figure 3, the manuscript could have also addressed what could be the general cellular consequences, if any, of Type-A vs. Type-C fibril formation in the cellular environment. For example, in the case of Figure 1 could it have been possible to measure some specific cellular features such as shape, nuclear size, etc. of the aggregate-containing cells in order to see if in these cells there are differences in these features compared to cells not containing aggregates and if they differ from one type of aggregate to the other?.

Secondly, regarding the phosphorylation studies in Figure 3, an interesting experiment to check for the importance of the 403/404 and 409/410 events in the experimental system would have been to knock out Casein Kinase I before seeding with the Type A and Type C aggregates. Has this been tried to see what happens to aggregate formation?.

Referee #2:

De Rossi et al analyzed the seeding properties of FTLD-TDP subtype A vs C and the nature of the inclusions in patients. There is significant overlap with a recent publication (Porta et al 2021) on the seeding data, but De Rossi et al use sophisticated imaging techniques (CLEM and dSTORM) in the seeding assay and in patient tissue for significant conceptual advance. Spreading and seeding is firmly established for Tau and a-synuclein, and much less so for TDP-43. The new data is important to the field given the high variability between patients and inherent limitation in patient numbers for such studies. De Rossi clearly used the better model system (TDP-43 WT vs TDP-43 deltaNLS). They provide novel evidence for actual formation of neoaggregates and data to support a model of sequential phosphorylation (403/404 to 409/410) in the seeding experiments and with overexpression of phosphomimicking mutations. The different ultrastructure of type A and type C neoaggregates is stunning (Fig 4), although both subtypes show similar fibrils in patients (Fig 5). Maybe a neuronal model system would be more meaningful to replicate the neuritic pathology in type C. With some revisions the manuscript seems suitable for EMBO Reports.
Specific points:
-It should be reiterated in the discussion that all experiments are done using liposome-based transfection of TDP-43 patient material, which greatly facilitates uptake and probably accelerates and maybe also exaggerates the effects.
-The seeding assay shows significant patient to patient variability. In figure 1C only FTLD-A at day 5 reaches the traditional $p<0.05$ cutoff although the overall time course looks convincing. Maybe showing data integrated over the 4 time points would emphasize the differences between groups.
-Fig 1E: add p-values for the correlation analysis
-Fig 1F/G is a new control for specificity. How were the n=3 patients selected from the n=7 in 1C? Is it possible to add date for the other 4 patients?
-Fig 2D/3G/3H: the data clearly does not follow a normal distribution. Non-parametric statistics would be more appropriate.
-Fig 3A-C: some quantification should be provided. Are these single z-sections? The tubulin staining does not look like cytoskeletal staining.
-The legend for Fig 3 does not fit the actual figure.
-Fig 4: The different morphology between type A and C seeding looks impressive. How many such inclusions were analyzed? FRAP assays to show fibrillar vs gel or liquid-like behavior would be interesting but is probably not possible with the HA-tagged system.
-Fig 5: The data TDP-43 fibrils in patient material is exciting, although the authors find no clear differences between type A and C fibrils. Please specify the estimated resolutions from model 5E. What does the parameter in Fig 5G actually measure - the density of fibrils?
-Overall, the manuscript is a bit wordy and would benefit from shortening the text.

Referee #3:

The manuscript by de Rossi et al. reports distinct seeding properties of different TDP-43 aggregate forms from FTLD-TDP subtypes, reinforcing the current thinking that a templated-based mechanism akin to prion strains underlies the heterogeneity of the disease. Whereas the manuscript inform interesting and important results, its main message is complementary to existing literature and therefore does not represent a significant step forward in the field.
The manuscript contains two main messages; namely, (1) that different structures propagate as different prion strains, with specific velocities, localization and distinguishing features; and (2) that the different structures are subject of distinctive phosphorylation events.
Whereas the first such message is well achieved and demonstrated, this was already proven by Lee and Trojanowski in a more physiologically relevant context. Therefore, while it is acknowledged that the findings indicate that such different structures could distinguish among pathology states, the authors could unfortunately not advance this aspect sufficiently and rather pointed to the potential of higher-resolution methods to accomplish this task in the future. The same is true for the second message, which relates to phosphorylation. It is noticeable that this is a very stimulating result, as the role of this post-translational modification in TDP-43 proteinopathies is a subject of debate in which some people argue that phosphorylation is neuroprotective while others point to their detrimental effects. Unfortunately, very limited information is provided if one considers that the C-terminal region of TDP-43 contains a large number of serine residues that can be phosphorylated within throughout the entire stretch comprising

residues 267-402 and that the authors exclude by focusing on the latest 403-414 few amino acid residues of the sequence when they focus on S403/404 and S409/410. Although this is in part justified due to the availability of antibodies to identify TDP-43 phosphorylated at these positions, the strategy of using phosphomimics used in this manuscript could have well been used to address the potential effect of phosphorylation in other serines throughout the sequence. Similarly, using other approaches such as proteolysis and mass spectrometry to identify the resulting fragments showed very powerful to identify post-translational modifications in patient-derived aggregates (see, e.g. PMID 26980269 as shown specifically for TDP-43). Addressing, at least in part, these two aspects (structure- and phosphorylation-wise) will significantly improve the two main messages of the manuscript that one expects from the abstract.

A minor concern relates to the structure of the manuscript. The Introduction section already contains many results, which should not be placed there as this bias the reader. Similarly, in the Discussion section the authors do a good job by repeating the main results of the manuscript, which is good for clarity. Having written that, it is also true that the Discussion section repeats what is said in the Results too much -especially in the first two paragraphs-. Nevertheless, the manuscript is very well-written and the authors may consider reorganizing the content of the sections and to check for some minor English style correction (e.g. "overtime" instead of "over time" is used consistently in the text, which has a completely different meaning in English).

Point-by-point response to the referees

We thank the referees for their evaluation of our work and their constructive feedback, which we have carefully considered. We note that all three referees found our data interesting and important and appreciated the value of our work for the field. The comments of the referees prompted us to perform additional experimentation to address their criticism and we feel that the revision has strengthened our work. Nevertheless, we note that the tight timeline for the revision limited the possibilities for more extensive experimentation. However, we are planning to undertake several of the suggestions of the referees that could not be included in this revision, as a part of a follow-up study. Below is our point-by-point response (in blue) to the referees' critique.

Referee #1:

In this work, De Rossi et al. have investigated the differences in aggregation induced by TDP-43 extracts from two different FTD types, FTLD-TDP-A and FTLD-TDP-C which have different disease severity. Using several state-of-the-art techniques the authors have shown that there is a clear difference between the aggregates induced by Type A compared to Type C. In particular, the authors have focused on the type of fibrils composition, more compact for Type A compared to more heterogeneous for Type C. In general, the study has been conducted in a very careful and well thought-out manner, and the conclusions will certainly be of interest to the field.

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

Nonetheless, a few general and more specific issues could be considered by the authors to improve the significance of the bindings.

- Although addressed in Figure 3, the manuscript could have also addressed what could be the general cellular consequences, if any, of Type-A vs. Type-C fibril formation in the cellular environment. For example, in the case of Figure 1 could it have been possible to measure some specific cellular features such as shape, nuclear size, etc. of the aggregate-containing cells in order to see if in these cells there are differences in these features compared to cells not containing aggregates and if they differ from one type of aggregate to the other?

We would like to thank the reviewer for this suggestion, which we have followed. In our new Fig.1 EVG-J, we now show the comparison of the nuclear morphology between the different experimental conditions. Analysis of different nuclear parameters revealed that cells which have been exposed to purified pathological TDP-43 for an extended period of time show alterations in ellipticity and sphericity of their nuclei after 6 days.

The following text was added in the results: "*Nuclear clearance of TDP-43 was accompanied by changes in the nuclear morphology. Nuclei of both FTLD-TDP-A and FTLD-TDP-C seeded cells became more round compared to control cells, as evidenced by the significant increase of their sphericity and the reduction of their oblate axis, while the prolate axis remained unchanged (Fig. EV1G-J).*"

- Secondly, regarding the phosphorylation studies in Figure 3, an interesting experiment to check for the importance of the 403/404 and 409/410 events in the experimental system would have been to knock out Casein Kinase I before seeding with the Type A and Type C aggregates. Has this been tried to see what happens to aggregate formation?

The role of phosphorylation in aggregate formation is an important and interesting point that we have carefully considered. Referee #3 also raised the possibility of using our phosphomutants to address the differences that phosphorylation may impose on the neoaggregate structures. We reasoned that altering TDP-43 phosphorylation might be a more targeted way to address the question than altering the levels of Casein Kinase I, which has many different substrates in cells and its depletion will certainly cause changes that are independent of TDP-43, complicating the interpretation of the data.

Nevertheless, to properly address the point, we would need more time. Specifically, we need to generate stable lines with our phosphomutants (~3 months to complete the line generation and characterization) and then perform seeding experiments and quantification, plus structural analysis (a minimum of additional 3 months). Due to this prohibiting timeline, we took an alternative approach, namely we overexpressed the mutants in HEK cells, which we know leads to aggregation in some cells because of the increased protein concentration. However, we noticed that upon overexpression the aggregates are much more diffused without detectable foci or fibril formation, suggesting that seeding is needed to generate patient-like assemblies. While this precludes this approach for understanding the role of phosphorylation in fibril formation, it does highlight the value of our seeding model. With this knowledge, we are now planning to generate the additional lines with the phosphomutants and perform seeding experiments, as a part of a follow-up study.

Referee #2:

De Rossi et al analyzed the seeding properties of FTLD-TDP subtype A vs C and the nature of the inclusions in patients. There is significant overlap with a recent publication (Porta et al 2021) on the seeding data, but De Rossi et al use sophisticated imaging techniques (CLEM and dSTORM) in the seeding assay and in patient tissue for significant conceptual advance. Spreading and seeding is firmly established for Tau and a-synuclein, and much less so for TDP-43. The new data is important to the field given the high variability between patients and inherent limitation in patient numbers for such studies. De Rossi clearly used the better model system (TDP-43 WT vs TDP-43 deltaNLS). They provide novel evidence for actual formation of neoaggregates and data to support a model of sequential phosphorylation (403/404 to 409/410) in the seeding experiments and with overexpression of phosphomimicking mutations. The different ultrastructure of type A and type C neoaggregates is stunning (Fig 4), although both subtypes show similar fibrils in patients (Fig 5). Maybe a neuronal model system would be more meaningful to replicate the neuritic pathology in type C. With some revisions the manuscript seems suitable for EMBO Reports.

We thank the referee for the accurate summary of our results and for his/her appreciation of the advantages of our approach.

Specific points:

- -It should be reiterated in the discussion that all experiments are done using liposome-based transfection of TDP-43 patient material, which greatly facilitates uptake and probably accelerates and maybe also exaggerates the effects.

We thank the referee for noting this point. Indeed, our study was designed to understand the seeding properties of the patient-derived aggregates once they enter the cells and not the uptake of the aggregates by the cells, which is an important, but independent question that we think, must be addressed in the context of neurons. To clarify this point, we have now added a sentence at the beginning of the second paragraph entitled "*FTLD-TDP-A and C brain-derived aggregates exhibit distinct seeding properties*": "*In this study entrance of pathological aggregates in the cells was facilitated by liposome- based transfection*.""

- -The seeding assay shows significant patient to patient variability. In figure 1C only FTLD-A at day 5 reaches the traditional $p<0.05$ cutoff although the overall time course looks convincing. Maybe showing data integrated over the 4 time points would emphasize the differences between groups.

We thank the reviewer for this suggestion. That was also our initial idea and representation. However, after consultation with a biostatistician at the University of Zurich, we were advised to represent the data as shown in Fig 1C-D. We think these representations are the most statistically appropriate in regard to our data. Our analysis is a repeated measurement of the neoaggregation within HEK cells over time, which statistically is best represented by a linear regression analysis (Fig 1D). However, we wanted to show the variability between patients, for which we used the box and whiskers representation (Fig 1C). Considering the limited number of patients and the variability, integration of the data would misrepresent these data and be statistically incorrect.

- -Fig 1E: add p-values for the correlation analysis

P values were added, as suggested.

- -Fig 1F/G is a new control for specificity. How were the n=3 patients selected from the n=7 in 1C? Is it possible to add data for the other 4 patients?

Patients were selected based on tissue availability in the lab. Unfortunately tissues from some of the patients used in 1C were not available at the time of the experiment. One FTLD-TDP-A patient was used for both Fig 1 and Fig 2 experiments.

- -Fig 2D/3G/3H: the data clearly does not follow a normal distribution. Non-parametric statistics would be more appropriate.

We thank the reviewer for this comment. We double checked all our statistical analyses. The tests included in Figure 3 were already non-parametric and the rest have now been adapted as such. All statistical analyses were performed with Graphpad Prism. Our data are repeated measures (patients) over time, for which Kruskall-Wallis or Friedman do not allow for 2 way-analysis (here our two ways are patient subtype and day post transfection). We consulted with a statistical expert at the University of Zurich who recommended using the mixed effect model for our data, which we followed using two-way ANOVA with repeated measurements. We applied the Geisser-Greenhouse correction and did not assume the sphericity of our data. We used the Fisher LSD

post hoc comparison, which was suggested to be the best non-parametric test (Lin and Haseman, 2007). We precise in the figure legend which test was used to make it unambiguous.

- -Fig 3A-C: some quantification should be provided. Are these single z-sections? The tubulin staining does not look like cytoskeletal staining.

We thank the referee for this suggestion, which we followed. We added the requested quantification of the different markers in Appendix Figure S4B.
Pictures were acquired in 2D and represent only one plane. HEK cells are rather rounded cells, which are hard to image in SRM. The planes were selected based on the aggregates for FTLD-TDP-A and FTLD-TDP-C. For control, the focus plane was selected based on the nucleus. This setup did not authorize for perfect imaging of the cytoskeleton, and here, Tubulin was only used as a marker for cytoplasmic proteins. Moreover, it is known that AraC confers toxicity that leads to changes in cellular morphology (Lengsfeld and Maurer-Schultze, 1986) that may account for the appearance of the Tubulin in this experiment. In our revised Fig. 3C, we have now added a negative control, a marker of the nucleolus, to clarify the point that not all proteins accumulate within the neoaggregates. Accordingly, Tubulin was transferred to Extended View Figure 2.

- -The legend for Fig 3 does not fit the actual figure.

We thank the reviewer for catching this mistake, which was due to a last minute change of the figure arrangement. We apologize for this mistake. We have updated figure 3 legend and Extended View Figure 2 as well in the text:

Figure 3: **A-C)** dSTORM images of cells seeded for 6 days with non-neurodegenerative control (Non-ND control), FTLD-TDP-A and FTLD-TDP-C seeds extracted from post-mortem brain tissue. Staining shows localization of TDP-43 (HA tag, cyan) along with LaminB1 (yellow, A), nucleopore complexes (nucleopores, yellow, B) and nucleolus (yellow, C). Cells seeded with FTLD-TDP seeds show coaggregation of nuclear membrane proteins upon neoaggregates formation. **D)** dSTORM images of cells containing aggregates after 6 days of seeding with SarkoSpin fractions of postmortem brain from patients with FTLD-TDP-A or FTLD-TDP-C. Red arrows point to cytoplasmic aggregates. Cells were labelled with TDP-43 (HA-tag, cyan) and phosphorylated TDP-43 pS403/404 (pTDP-43, magenta) **E)** Bar graph showing the number of neoaggregates per cells. Non-parametric unpaired t test FTLD-TDP-A vs FTLD-TDP-C, p=0.0003. **F)** Heatmap representation of the density of TDP-43-HA and pTDP-43 particles for four representative neoaggregates of FTLD-TDP-A and FTLD-TDP-C seeded cells. **G)** Violin plots showing the density of particles TDP-43 per µm2. Non-parametric unpaired t test FTLD-TDP-A vs FTLD-TDP-C, p=0.0083 **H)** Violin plots showing the size of analyzed neoaggregates using TDP-43-HA area. Non-parametric unpaired t test FTLD-TDP-A vs FTLD-TDP-C, p<0.0001

Extended View Figure 2: Super resolution analysis of the neoaggregates: A) dSTORM images of cells seeded for 6 days with non-neurodegenerative control (non-ND control), FTLD-TDP-A and FTLD-TDP-C seeds extracted from post-mortem brain tissue. Staining shows localization of TDP-43 (HA tag, cyan) along with tubulin (yellow). B) Violin graph representing the ratio of (marker particle number / TDP-43 particle number) within TDP-43 neoaggregates. Non-parametric unpaired t-test FTLD-TDP-A vs FTLD-TDP-C, LaminB1, p=0.0096, tubulin, p=0.0615. C) Violin

plot showing the size of analyzed neoaggregates using pTDP-43 area. Non-parametric unpaired t-test FTLD-TDP-A vs FTLD-TDP-C, p=0.0007. D) Violin plot showing the density of particles pTDP-43 per um2. Non-parametric unpaired t-test FTLD-TDP-A vs FTLD-TDP-C, p=0.3611

- -Fig 4: The different morphology between type A and C seeding looks impressive. How many such inclusions were analyzed?

For FTLD-TDP-A, we analyzed four inclusions by EM, as shown in Fig. 4A. While many more aggregates were observed by LM, not all aggregates could be localized by EM due to presence of grid bars, tissue folds or being too far in distance from the LM section. For FTLD-TDP-C, multiple aggregates were also seen by LM, but as stated in the results section "*there was no characteristic ultrastructure that could distinguish the immunopositive area from the surrounding cellular material. As such only a single C neoaggregate could be correlated with an ultrastructure, as shown in Fig 4B*".
We have modified the text to make it clearer how many aggregates that were localised by EM. The text now reads: "*In FTLD-TDP-A-seeded cells, we correlated four HA-immunopositive aggregates by EM that corresponded to accumulated fibrils (Fig. 4A, Fig. EV4A).*" For FTLD-TDP-C, we had already stated in the text: "*...only a single FTLD-TDP-C seeded neoaggregate could be successfully correlated with an ultrastructure via EM.***"**

- FRAP assays to show fibrillar vs gel or liquid-like behavior would be interesting but is probably not possible with the HA-tagged system.

While we agree that assays to show fibrillar vs gel or liquid-like behavior would be interesting, the FRAP experiment is not possible in HA cells, as the referee correctly notes. To do this, we would require a GFP-TDP-43 cell line. However, we tried seeding in the GFP-TDP-43 cell line, and observed that cellular stress induced by arresting the cells (AraC) and lipofectamine transfection triggered significant protein aggregation which skewed the seeding effect. Our observations are in line with similar experiments performed by the Cleveland lab, and recently showed that once aggregated, the dynamics of the proteins change, and are not liquid like anymore (Gasset-Rosa *et al.*, 2019; Yu *et al.*, 2021). Due to these observations with GFP-TDP-43, we concluded that the use of the minimally invasive HA tag in our current setting with the TDP-43-HA cell line is the best for studying protein seeding effects.

- -Fig 5: The data TDP-43 fibrils in patient material is exciting, although the authors find no clear differences between type A and C fibrils. Please specify the estimated resolutions from model 5E. What does the parameter in Fig 5G actually measure - the density of fibrils?

The estimated resolution from the models in 5E was ~40Å and we have now included this in the results section. The text now reads: "*The resulting 3D averages had an optimal resolution of ~40Å and showed a rod-like signature typical of fibrils, however there were no visually obvious features that differed between the two subtypes (Fig. 5E).***"** We also included the description of the calculation in the methods section" Subtomogram averaging of fibrils". The text now reads: "*The resolution was calculated using gold standard methodology and used for bandpass filtering of the final maps.***"**

The parameter in Fig 5G measures the distance between each fibril, equating to the density of fibril packing within the aggregates. We have updated the text describing this panel to make this clearer. The text now reads: *"We measured the density of fibrils in each tomogram by calculating the distance between the backbones of adjacent fibrils."* We have similarly updated the figure legend for simplicity. The text now reads: The average distance between fibrils within each aggregate for FTLD-TDP-A and FTLD-TDP-C is shown.

-Overall, the manuscript is a bit wordy and would benefit from shortening the text.

We have shortened the text wherever possible.

Referee #3:

The manuscript by de Rossi et al. reports distinct seeding properties of different TDP-43 aggregate forms from FTLD-TDP subtypes, reinforcing the current thinking that a templated-based mechanism akin to prion strains underlies the heterogeneity of the disease. Whereas the manuscript inform interesting and important results, its main message is complementary to existing literature and therefore does not represent a significant step forward in the field.

We thank the referee for noting that our manuscript includes interesting and important results.

The manuscript contains two main messages; namely, (1) that different structures propagate as different prion strains, with specific velocities, localization and distinguishing features; and (2) that the different structures are subject of distinctive phosphorylation events.

- Whereas the first such message is well achieved and demonstrated, this was already proven by Lee and Trojanowski in a more physiologically relevant context.

We agree with the referee that the very recent work from the Lee and Trojanowski teams (Porta *et al.*, 2021) reports data that are very much in line with our current work. However, we maintain that our current study represents an important contribution to the field for the following reasons:

1. It provides a rapid and fully independent confirmation of the, still controversial concept, of prion-like and strain behavior of TDP-43 aggregates,
2. It establishes TDP-43 seeding in a more physiological cellular environment, namely in cells expressing normal and nuclear localized TDP-43 (TDP-43-HA), as opposed to TDP-43 abnormally located in the cytosol due to the deletion of its nuclear localization signal (Porta et al 2021),
3. It provides a longer timeline of TDP-43 seeding in cells and clear amplification with quantification of neoaggregates,
4. It shows the variability of patient samples in this assay due to inherent differences in the load of TDP-43 seeds in the tissue,
5. It shows that FTLD-TDP-C seeds trigger neoaggregates with lower potency than FTLD-TDP-A,
6. It provides evidence for the template-directed misfolding of TDP-43 that has been assumed in the field, but was not experimentally validated until now,
7. It includes superresolution and CLEM analyses of both patient brains and seeded cells and delineates the parallels between the two systems, and finally

8. It shows that phosphorylation occurs in a N-to-C-terminal fashion in cells and that it is distinct in the different subtypes, suggesting that phosphorylation may impact fibril formation.

- Therefore, while it is acknowledged that the findings indicate that such different structures could distinguish among pathology states, the authors could unfortunately not advance this aspect sufficiently and rather pointed to the potential of higher-resolution methods to accomplish this task in the future.

There are currently no established protocols for the atomic resolution structural analysis of pathological aggregates in post-mortem human brains, due to the unique challenges of working with brain samples and the technical complexity of current gold-standard atomic resolution analysis of structures *in situ*. The Stahlberg lab is currently developing such pipelines to combine cryoCLEM with cryoFIB-SEM and cryo-electron tomography for exactly this purpose. We fully intend to continue our collaborative effort along these lines. However establishing such complex methodologies are outside the scope of this manuscript within an achievable time-frame.

- The same is true for the second message, which relates to phosphorylation. It is noticeable that this is a very stimulating result, as the role of this post-translational modification in TDP-43 proteinopathies is a subject of debate in which some people argue that phosphorylation is neuroprotective while others point to their detrimental effects. Unfortunately, very limited information is provided if one considers that the C-terminal region of TDP-43 contains a large number of serine residues that can be phosphorylated within throughout the entire stretch comprising residues 267-402 and that the authors exclude by focusing on the latest 403-414 few amino acid residues of the sequence when they focus on S403/404 and S409/410. Although this is in part justified due to the availability of antibodies to identify TDP-43 phosphorylated at these positions, the strategy of using phosphomimics used in this manuscript could have well been used to address the potential effect of phosphorylation in other serines throughout the sequence.

We agree with the referee that the phosphorylation data that we show are extremely interesting. In our new Figure 2, we added a more detailed phosphorylation timeline, which now includes quantification at days 3, 4, 5 and 6 post seeding, to better understand the differences between the subtypes. This new data further strengthens the distinct timelines of seeding triggered by the two FTLD subtypes. Moreover, it suggests that phosphorylation, and specifically at site 403/404 may be an initial cellular response to stress that rapidly resolves in control cells, but not in cells challenged with patient-derived aggregates. Please see also point 2 of Referee #1 for our response on this point.

- Similarly, using other approaches such as proteolysis and mass spectrometry to identify the resulting fragments showed very powerful to identify post-translational modifications in patient-derived aggregates (see, e.g. PMID 26980269 as shown specifically for TDP-43). Addressing, at least in part, these two aspects (structure- and phosphorylation-wise)

will significantly improve the two main messages of the manuscript that one expects from the abstract.

We thank the referee for suggesting this new line of investigation, which we have attempted to undertake during our revision. The figure below shows our approach and initial results, which are encouraging. In summary we did the following:

- Production and purification of recombinant TDP-43-MBP
- Optimization of *in vitro* phosphorylation and validation by Western blot
- Digestion trials with recombinant TDP-43-MBP to find optimal sequence coverage
- SaskoSpin preparation and digestion of neoaggregates and recombinant controls for Mass spectrometry analysis
- Data analysis and interpretation using MASCOT and Scaffold

The obtained data are provided as an amendment to this letter to the referees. Our conclusion, which was reiterated after consulting with a mass spectrometry expert (Dr. Alexander Leitner from ETH, Zurich), was that it was unfortunately impossible to acquire conclusive results in the available time for revision, due to a number of critical points that we list below. We think that our approach has the potential to thoroughly address this important point in the future and to compare patient-derived aggregates as well as neoaggregates of different disease subtypes. Nevertheless, we strongly believe that this analysis should not delay publication of the current piece of work and would be best suited as a separate study in the future.

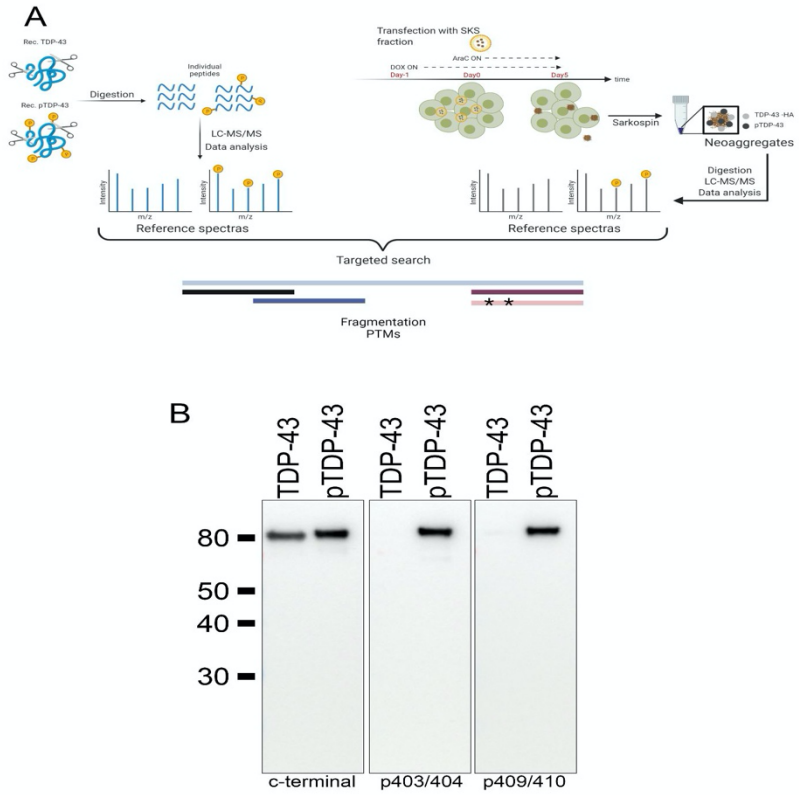

Figure: A) Schematic represents the pipeline used for the mass spectrometry. Recombinant TDP-43 and *in vitro* phosphorylated TDP-43 were digested and analyzed to obtain reference spectra for the sites of phosphorylation. HEK cells were then seeded for 5 days (as described in Fig. 1, EV1) and processed using SarkoSspin purification (Laferrière *et al.*, 2019; Pérez-Berlanga, Laferrière and Polymenidou, 2019). Aggregated fractions were then digested and analyzed by mass spectrophotometry. The obtained results are included in the amended excel file. **B)** Western blots show the specific phosphorylation of TDP-43 on pS403/404 and pS409/410 sites, after *in vitro* phosphorylation with Casein Kinase.

Critical considerations and current limitations for phosphoproteomic analysis of TDP-43 aggregates derived from seeded cells or patient brains:

1. The most commonly used proteomic approach is based on protein fragmentation using Trypsin. However, Trypsin is not suited for digesting the low complexity region (LCR) of TDP-43 and very few trypsin-generated peptides are detectable from the LCR of TDP-43 by this analysis. To overcome this limitation, we have identified an alternative enzyme (ProAlanase) that results in a coverage of ~80% of our recombinant full length TDP-43 (please see the excel file with our obtained results).
2. While ProAlanase digestion offers significant advantages for TDP-43 coverage, its use poses additional challenges, since it is a new enzyme that is not well characterized in the context of mass spectrometry analysis, especially for low sample amounts.
3. Furthermore, we have now determined that ProAlanase digestion generates peptides that do not ionize and fragment well, resulting in generally unfavorable properties for mass spectrometry analysis compared to peptides generated with trypsin as the protease.
4. Nevertheless, *in vitro* phosphorylation with Casein Kinase allowed us to generate a reference of detectable phosphorylatable peptides, with good confidence. This included a high (80%) coverage of the low complexity region of TDP-43.
5. While we enriched phosphorylated TDP-43 from seeded cells with SarkoSpin (Laferrière *et al.*, 2019), the obtained sample is still very complex, as it includes additional insoluble proteins. This complexity poses additional challenges for the robust detection of any phosphorylated peptides. Moving forward we intend to upscale our sample preparation and add an additional enrichment step of the obtained phosphopeptides.

- A minor concern relates to the structure of the manuscript. The Introduction section already contains many results, which should not be placed there as this bias the reader.

We have now shortened the description of the results that were included at the end of the introduction.

- Similarly, in the Discussion section the authors do a good job by repeating the main results of the manuscript, which is good for clarity. Having written that, it is also true that the Discussion section repeats what is said in the Results too much -especially in the first two paragraphs-. Nevertheless, the manuscript is very well-written and the authors may consider reorganizing the content of the sections and to check for some minor English style correction (e.g. "overtime" instead of "over time" is used consistently in the text, which has a completely different meaning in English).

We thank the referee for his/her assessment of our manuscript. We have followed all suggestions and corrected the minor English style errors that we identified, including replacing all instances of “overtime” with the correct “over time”.

References:

Gasset-Rosa, F. *et al.* (2019) 'Cytoplasmic TDP-43 De-mixing Independent of Stress Granules Drives Inhibition of Nuclear Import, Loss of Nuclear TDP-43, and Cell Death', *Neuron*, 102(2). doi: 10.1016/j.neuron.2019.02.038.

Laferrière, F. *et al.* (2019) 'TDP-43 extracted from frontotemporal lobar degeneration subject brains displays distinct aggregate assemblies and neurotoxic effects reflecting disease progression rates', *Nature Neuroscience*, 22(1). doi: 10.1038/s41593-018-0294-y.

Lengsfeld, A. M. and Maurer-Schultze, B. (1986) 'The effect of ara-C on survival and proliferation of HeLa cells', *Journal of Cancer Research and Clinical Oncology 1986 111:3*. Springer, 111(3), pp. 220–228. doi: 10.1007/BF00389237.

Lin, F. A. and Haseman, J. K. (2007) 'An evaluation of some nonparametric multiple comparison procedures by Monte Carlo methods', *http://dx.doi.org/10.1080/03610917808812065*. Marcel Dekker, Inc. , 7(2), pp. 117–128. doi: 10.1080/03610917808812065.

Pérez-Berlanga, M., Laferrière, F. and Polymenidou, M. (2019) 'SarkoSpin: A Technique for Biochemical Isolation and Characterization of Pathological TDP-43 Aggregates', *BIO-PROTOCOL*. Bio-protocol, LLC, 9(22), p. e3424. doi: 10.21769/bioprotoc.3424.

Porta, S. *et al.* (2021) 'Distinct brain-derived TDP-43 strains from FTLD-TDP subtypes induce diverse morphological TDP-43 aggregates and spreading patterns *in vitro* and *in vivo*', *Neuropathology and Applied Neurobiology*. doi: 10.1111/nan.12732.

Yu, H. *et al.* (2021) 'HSP70 chaperones RNA-free TDP-43 into anisotropic intranuclear liquid spherical shells', *Science*. American Association for the Advancement of Science, 371(6529). doi: 10.1126/SCIENCE.ABB4309.

1st Revision - Editorial Decision **27th Oct 2021**

Dear Magda,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from referees 2 and 3.

As you will see, referee 3 is still not fully convinced that the manuscript provides a clear advance over the already published paper, but referee 2 sees its merits for the field and does support its publication, and we have therefore decided that we can offer to publish it. However, a strict requirement for its publication is that we must receive all final and correct manuscript files latest on the 1st of November and preferably earlier.

As referee 3 suggests, please add the strongest points, i.e. the use of a different system to confirm the spreading of TDP-43, as well as the opportunities this system may bring, to the main manuscript text.

A few other editorial changes will also be required:

- Your manuscript has 5 main figures but is layed out as a full article. Please either add one main figure, or combine the results and discussion sections to publish your paper as a short report. The total character count for a short report should not exceed 29.000 (including spaces and figure legends, but excluding references and materials and methods).

- A Data Availability Section must be added at the end of the methods section. If you have not deposited any data into public databases, a sentence to explain this should be added to this section.

- Please add up to 5 keywords to the ms file.

- Carlo Scialo needs to be added to the author contributions.

- The reference format needs to be corrected to the EMBO reports (Harvard) style.

- The abstract needs to be correct to present tense throughout.

- The grant #s for the 2 Alzheimer Forschung Schweiz awards need to be added to the funding info.

- Fig 3C is called out after 4A. Fig EV3 panel callouts are missing. Fig EV4C_E callouts are missing. Fig EV5A-F callouts are missing. Appendix Fig S4 callout is missing. Appendix Table callouts miss the 'S'. Please correct.

- There is a callout to "Supplementary Table 3" which needs removing/correcting.

- A legend/title needs to be added to your Dataset. It can be added to the first tab in the excel file.

- A table of content with page numbers is missing from the Appendix file. The table names need correcting to 'Appendix Table S#'.

- The figure legends should follow-on after the Reference list.

- Please clarify the duplication in Fig 5A and EV4D.

I attach to this email a related ms file with comments by our data editors. Please address all comments in the final ms file.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

Please make sure that the synopsis image has the correct size. A delay in submitting any of the requested information will prevent the publication of your study by EMBO reports.

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Best regards,
Esther

Esther Schnapp, PhD

Senior Editor
EMBO reports

Referee #2:

The manuscript greatly improved during revision. Results from the state-of-the-art imaging techniques in cellular TDP-43 models and patient tissue should be very interesting to the field.

Referee #3:

The revised manuscript by Polymenidou and co-workers provides additional explanations to highlight the potential of their innovative seeding approach that will certainly be key to address critical questions in the future, as the authors advance in their rebuttal letter.
As discussed previously, a similar work came out early this year and therefore the manuscript by De Rossi et al. represents a confirmatory study. Nevertheless, they use a different WT-based protein system that is more reliable in cellular models, albeit prior results are more physiologically relevant in the sense that they were conducted on in vivo systems. Still, Polymenidou and coworkers also proved the spreading of TDP-43 and advanced new imaging data of high quality. Perhaps the most relevant aspect in terms of novelty to consider this manuscript beyond a confirmatory study that uses a new seeding paradigm system would be the attempt to advance an underlying mechanism for the seeding capacity of TDP-43 (with a focus on phosphorylation as a key PTM).

Distinct phosphorylation patterns as a key to distinguish between the two types of aggregates is very interesting, and in the original version of the manuscript the authors relied on the short C-terminal, eleven-residue stretch spanning residues 403-414. Since these are the very last residues of this 414-protein, the interpretation of them as being reliable indicators of distinct underlying mechanisms needs further assessment. These terminal residues may be too floppy within the aggregate cores and the differences may not be necessarily a distinguishing feature of disease subtype, as elaborated below.

Bearing in mind the time constraints, as well as the 2019 paper in which Polymenidou and co-workers informed characterization of phosphorylated FTLD-TDP pathological aggregates, it was proposed a mass spectrometry (MS)-based analysis of proteolyzed fragments to robustly characterize the distinguishing features of this post-translational modification in the two types of aggregates. This would strengthen this novel message to go well beyond the confirmatory nature of the study. Particularly, the methodology was shown by Kametani et al in 2016 to straightforwardly characterize brain-derived TDP-43 aggregates from two distinct patients. This aspect is relevant because it is now well established by cryo-EM and by different solid-state NMR studies that fibrillar aggregates of TDP-43 may contain residues from the entire ca. 150-residue long C-terminal domain (CTD), some of them are well-fixed in the fibril cores and other very dynamic, depending on the particular aggregate. In fact in cross-polarization-based solid-state NMR experiments on TDP-43 fibrils the stretch 401-414 remains invisible, pointing to structural heterogeneity due to enhanced dynamics. Similarly, another cyro-EM study of a different fibril conformation show this stretch loosely bound to the core.

In line with the above, many residues throughout the entire CTD are prone to PTMs such as phosphorylation, as found by MS analyses of proteolyzed fragments in Kametani et al. (2016), and therefore it was proposed that the revised manuscript should address this point to avoid drawing conclusions from the very terminal residues that may be too floppy and subject of strong conformational heterogeneity. It is unfortunate that the tight time constraints did not suffice to confirm that phosphorylation distinguish the two types of aggregates thoroughly by looking at those positions beyond the limited 11-residue C-terminal stretch. Certainly, this reviewer agree with the authors in that it was "impossible to acquire conclusive results in the available time for revision", and it is remarkable the amount of work they have accomplished, including extended data for the phosphorylation timeline of serines within the 403-414 C-terminal stretch.

In summary, in this very-well written manuscript the strongest points are the use of a different system to confirm the spreading of TDP-43, as well as the opportunities this system may bring (which are not shown in the manuscript but clearly stated in the rebuttal letter).
The weakest point is that by looking at phosphorylation in the extreme (i.e. in the very last 11 C-terminal residues) may not properly reflect reliable differences, as the termini could be too heterogeneous if it is floppy within the aggregates (in the unique cryo-EM structure available these residues do not establish strong interactions to be immobilized in the fibrils). Characterization of more "inner" residues would have provided the key as they would be protected against PTMs, and thus reliably reflecting different patterns from distinct underlying mechanisms.

Below are the required answers to the four editorial questions that appear in the Referee Instructions section.

1. Does this manuscript report a single key finding? If YES, please describe it in one sentence.

YES. The manuscript describes different seeding properties of FTLD-TDP type A and type aggregates, supporting the current state of knowledge.

2. Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (NO)?

NO. The manuscript describe a confirmatory finding that has already been documented using other methods (TDP-43 ΔNLS instead of TDP-43 WT) or in other organisms (e.g. in mice).

3. Is it of general interest to the molecular biology community? If YES, please say why, in a single sentence. If NO, please state which more specialized community you feel it is aimed at (or none), in a single word or phrase.

IN PART. The in vitro and in cellulo spreading of TDP-43 has been proposed earlier as the authors noted (Nonaka et al., 2013; Shimonaka et al., 2016; Freiler et al., 2015), and more recently in vivo, for instance in Porta et al., (2018) or in Asakawa et al., (2021).

Although the field is of very high relevance due to the involvement of TDP-43 proteinopathies in fatal diseases, most recent publications of this nature seem to appear in specialized yet high-impact journals within the neuropathology/neurobiology community, e.g.:

Ding et al., (2021) "Spreading of TDP-43 pathology via pyramidal tract induces ALS-like phenotypes in TDP-43 transgenic mice". Acta Neuropathologica Communications (PMID: 33461623).

Porta et al., (2021) "Distinct brain-derived TDP-43 strains from FTLD-TDP sybtypes induce diverse morphological TDP-43 aggregates and spreading patterns in vitro and in vivo". Neuropathology and Applied Neurobiology (PMID: 33461623).

4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longer-format article (NO)?

IN PART. The major finding reported corroborates previous results in a very thorough manner. However, the authors' approach to propose a possible underlying mechanism (here, with the focus on phosphorylation) is limited to the C-terminal eleven-residue stretch and could not be robustly documented due to the limited time constraints.

Referee #2:

The manuscript greatly improved during revision. Results from the state-of-the-art imaging techniques in cellular TDP-43 models and patient tissue should be very interesting to the field.

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

Referee #3:

The revised manuscript by Polymenidou and co-workers provides additional explanations to highlight the potential of their innovative seeding approach that will certainly be key to address critical questions in the future, as the authors advance in their rebuttal letter.

We thank the referee for acknowledging the value of our work for future studies.

As discussed previously, a similar work came out early this year and therefore the manuscript by De Rossi et al. represents a confirmatory study. Nevertheless, they use a different WT-based protein system that is more reliable in cellular models, albeit prior results are more physiologically relevant in the sense that they were conducted on in vivo systems. Still, Polymenidou and coworkers also proved the spreading of TDP-43 and advanced new imaging data of high quality. Perhaps the most relevant aspect in terms of novelty to consider this manuscript beyond a confirmatory study that uses a new seeding paradigm system would be the attempt to advance an underlying mechanism for the seeding capacity of TDP-43 (with a focus on phosphorylation as a key PTM).

We thank the referee for recognizing the contribution of our study beyond the previously published work. We followed the referee's recommendation and added text in our discussion to further emphasize the strongest points and opportunities that our study brings.

Distinct phosphorylation patterns as a key to distinguish between the two types of aggregates is very interesting, and in the original version of the manuscript the authors relied on the short C-terminal, eleven-residue stretch spanning residues 403-414. Since these are the very last residues of this 414-protein, the interpretation of them as being reliable indicators of distinct underlying mechanisms needs further assessment. These terminal residues may be too floppy within the aggregate cores and the differences may not be necessarily a distinguishing feature of disease subtype, as elaborated below. Bearing in mind the time constraints, as well as the 2019 paper in which Polymenidou and co-workers informed characterization of phosphorylated FTLD-TDP pathological aggregates, it was proposed a mass spectrometry (MS)-based analysis of proteolyzed fragments to robustly characterize the distinguishing features of this post-translational modification in the two types of aggregates. This would strengthen this novel message to go well beyond the confirmatory nature of the study. Particularly, the methodology was shown by Kametani et al in 2016 to straightforwardly characterize brain-derived TDP-43 aggregates from two distinct patients. This aspect is relevant because it is now well established by cryo-EM and by different solid-state NMR studies that fibrillar aggregates of TDP-43 may contain residues from the entire ca. 150-residue long C-terminal domain (CTD), some of them are well-fixed in the fibril cores and other very dynamic, depending on the particular aggregate. In fact in cross-polarization-based solid-state NMR experiments on TDP-43 fibrils the stretch 401-414 remains invisible, pointing to structural heterogeneity due to enhanced dynamics. Similarly, another cyro-EM study of a different fibril conformation show this stretch loosely bound to the core.
In line with the above, many residues throughout the entire CTD are prone to PTMs such as phosphorylation, as found by MS analyses of proteolyzed fragments in Kametani et al. (2016), and therefore it was proposed that the revised manuscript should address this point to avoid drawing conclusions from the very terminal residues that may be too floppy and subject of strong conformational heterogeneity. It is unfortunate that the tight time constraints did not suffice to confirm that phosphorylation distinguish the two types of aggregates thoroughly by looking at those positions beyond the limited 11-residue C-terminal stretch. Certainly, this reviewer agree with the authors in that it was "impossible to acquire conclusive results in the available time for revision", and it is remarkable the amount of work they have accomplished, including extended data for the phosphorylation timeline of serines within the 403-414 C-terminal stretch.

We agree with the referee that the phospho-proteomic analysis would be particularly interesting in the context of our observations. However, as the referee also acknowledges the revision timeline did not allow us to perform these experiments in a conclusive manner. We have now initiated these studies and are planning to continue this work to uncover potential subtype-distinguishing phosphorylation events. This will be the focus of a future study.

In summary, in this very-well written manuscript the strongest points are the use of a different system to confirm the spreading of TDP-43, as well as the opportunities this system may bring (which are not shown in the manuscript but clearly stated in the rebuttal letter).

We thank the referee for this comment and suggestion. We have now added text in our discussion to highlight the key findings and potential future of our work.

The weakest point is that by looking at phosphorylation in the extreme (i.e. in the very last 11 C-terminal residues) may not properly reflect reliable differences, as the termini could be too heterogeneous if it is floppy within the aggregates (in the unique cryo-EM structure available these residues do not establish strong interactions to be immobilized in the fibrils). Characterization of more "inner" residues would have provided the key as they would be protected against PTMs, and thus reliably reflecting different patterns from distinct underlying mechanisms.

We agree with the referee and will perform the suggested experiments in an independent study.

Prof. Magdalini Polymenidou
University of Zurich
Department of Quantitative Biomedicine
Winterthurerstrasse 190
Zurich 8057
Switzerland

Dear Magda,

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Our study is exploratory and therefore, we could not to do a sample size calculation. Sample size calculation requires to know the effect of the studied treatment, and how it differs from control. When this study was started, no data were available on the seeding effect of pathological TDP-43.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not applicable, all data are included
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.	Patient used were selected on their physiopathiological characterization (i.e. non neurodgenerative control, FTLD-TDP-A or FTLD-TDP-C). Patients within one group were randomly selected.
For animal studies, include a statement about randomization even if no randomization was used.	not applicable
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.	Not applicable
4.b. For animal studies, include a statement about blinding even if no blinding was done	not applicable
5. For every figure, are statistical tests justified as appropriate?	The statistical department of the University of Zurich was consulted to assess the best way to analyze our data. Non parametric tests were used when the data did not reach the requirements. All statistical tests are indicated in the respective figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We use GraphPad Prism 9.2 for stastical analyses. This software control for all parameters necessary for completion of a statistic test.
Is there an estimate of variation within each group of data?	We showed the variability and distribution of our data in this paper.

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http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumc

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	This parameter was tested by GraphPad Prism 9.2 upon running statistical 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).	Yes, see appendix table 2
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	the HEK HA-TDP-43 used in this study as been generated in house (Laferriere et al, 2019). Our lab regularly check for mycoplasma contamination.

* 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.	not applicable
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	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.	All patient samples were received from the Queen Square Brain Bank (QSBB) for Neurological Disorders, at University College of London (UCL) and the Netherland Brain Bank (NBB) at the Netherlands Institute for Neuroscience, Amsterdam (Appendix table 1). All material has been collected from donors for or from whom a written informed consent for a brain autopsy and the use of the material and clinical information for research purposes had been obtained by the NBB or QSBB. Patient identity was anonymized by QSBB and NBB before shipment to the University of Zurich (UZH).
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'. Please confirm you have submitted this list.	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'. Please confirm you have followed these guidelines.	Not applicable

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures	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	We will be happy to share any dataset upon request, however, the amount of microscopy dataset used for this study makes it fastidious to generate a sharable repository for anyone to easily access and work on.
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-	Not applicable
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited	Not applicable

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Not applicable