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Peer Review File

TDP43 aggregation at ER-exit sites impairs ER-to-Golgi transport

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Editorial note: This manuscript has been previously reviewed at another journal that is not operating a transparent peer review scheme. This document only contains reviewer comments and rebuttal letters for versions considered at *Nature Communications*.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript was transferred to Nature Communications with my previous reviews. During the revision, the authors have clearly addressed most of my concerns. It would be ideal if additional assays for COPII function were included, but the reviewer agree that getting these new results published without much delay would benefit multiple fields.

Reviewer #2 (Remarks to the Author):

My concerns have been fully addressed. The work waslargely strengthened. I support publication of this work in Nature Communications.

Reviewer #4 (Remarks to the Author):

The authors have conducted additional experiments, edited the text, and made several other changes that have improved the manuscript.

As written and presented, however, the authors have not sufficiently addressed the concern about stressors and protein overexpression. Without specifically measuring the frequency of TDP43/SEC16A aggregation in relation to TDP43/SEC16A expression levels, the authors cannot make any strong arguments against this conclusion.

Many readers may be tempted to dismiss the findings as artifacts of overexpression or artificial stressors. Careful wording of the text and additional discussion on this point -- if not an additional experiment measuring TDP43/SEC16A aggregation as a function of TDP43 expression levels -- are required.

Reviewer #5 (Remarks to the Author):

TPD-43 plays a role in the propagation of neurodegenerative disease. In the manuscript of Wu et al. this propagation is connected with ER-to-Golgi transport by pinpointing aggregations of proteins in stress granules and at ER exit sites. The manuscript focuses on the aggregations using a Hsp104 mutant (yeast) which is known to reverse the toxicity of TDP-43. The findings of Wu et al. are relevant for the scientific community as thy reveal the co-aggregation of SEC16 and COPII with TDP43, impairing the ER-to-Golgi transport. The study uses a combination of imaging experiments and a mass spectrometry setup to elucidate in detail the above mentioned aggregation connections that down the line are of interest for better understanding neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis (ALS). The work of Wu et al. offers the scientific community the opportunity to use the presented results for further needed studies to tackle TDP-43 connections of aggregation and pharmaceutical intervention.

Major points and concerns:

Figure S1depicts the affinity-based aggregation purification using a Hsp104 flag-tagged mutant. The authors did state-of-the-art mass spectrometry using TMT-labeling in a context of flag-immunoprecipitations to present 337 enriched proteins. Figure S1F highlights the enrichment of these protein aggregates in a Hsp104 context. Figure S1E falls short of a being a good presentation of these proteins since it only shows enrichment but not the bead control side is shown. S1F is usually depicted as a complete volcano plot also for bead binders and contaminations. Overall, the Hsp104 data should be part of the main publication and not a part of the supplement since it reflects the technical comprehensiveness of this study. Using a HEK293T cell system for these immunoprecipitiations has experimental benefits and drawbacks when addressing ALS questions. The mass spectrometry raw data should be after publication of the manuscript be made available to the scientific community on the PRIDE proteomics identification database. This will make future studies comparable with other cell lines.
Nucleoporin proteins, such as NUP88 and NUP214 play two important roles in the cellular

context. One as nuclear pore complexes and secondly their secondary functional requirements during mitosis. Addressing these relevant details in the discussion part of the manuscript is important since it would address the overall importance of these proteins in aggregates and not in functional locations. This may lead to further investigation of the topic by the scientific community. This discussion should be embedded resonating other citable publications.
Figure 6 provides results of TDP43_ERES retain secretory cargo. The model depicted in Figure 6H is one of the key takeaways from this manuscript. I believe the readers would benefit of a better depiction of this model, using a more streamlined presentation of the endoplasmatic reticulum, the involved proteins, and the Golgi apparatus.

Minor points and concerns:

Hsp104 is a protein aggregation model system from yeast and in a context of HEK293T, it does not reflect the human conditions of neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis (ALS). HEK293T cells are a viable cell system for large scale experiments, especially since mass spectrometry based immunoprecipitation (IP) readouts require a certain amount of starting material when gone through gradients and IPs. Since using other human cell lines for detecting IP partners could have not given the reader the full readout that approach in this manuscript has its drawbacks at the same time is a valid approach the used to address this issue, this has to be more detailed in their discussion part and put it into context.

Originality, significance, validity:

The publication shows a original approach and is in coherence with previous publications while showing its own significance in the importance of proteotoxic stress impairing the ER-to-Golgi transport. The supplemental material is extensive and detailed, in order to support the authors findings and theories.

Statistics:

The statistics used are in coherence with the experimental setups and reflect up-to-date statistics. As mentioned above, adding the mass spectrometric raw files to the PRIDE database will make them accessible for future comparative studies.

Conclusions:

As above mentioned the conclusions should add a compact short discussion on the nucleoporin proteins and the Hsp104 yeast model protein.

References:

The references are comprehensive and no excessive self-referencing was detected.

Clarity and context:

The abstract, results, and discussion is coherently written and easy to scientifically comprehend.

Author response:

Reviewer #1 (Remarks to the Author)

This manuscript was transferred to Nature Communications with my previous reviews. During the revision, the authors have clearly addressed most of my concerns. It would be ideal if additional assays for COPII function were included, but the reviewer agree that getting these new results published without much delay would benefit multiple fields.

We appreciate the reviewer's positive comment and indeed plan to further investigate the effects of COPII in the future.

Reviewer #2 (Remarks to the Author)

My concerns have been fully addressed. The work was largely strengthened. I support publication of this work in Nature Communications.

We appreciate the reviewer's positive comment.

Reviewer #4 (Remarks to the Author):

The authors have conducted additional experiments, edited the text, and made several other changes that have improved the manuscript.

As written and presented, however, the authors have not sufficiently addressed the concern about stressors and protein overexpression. Without specifically measuring the frequency of TDP43/SEC16A aggregation in relation to TDP43/SEC16A expression levels, the authors cannot make any strong arguments against this conclusion.

Many readers may be tempted to dismiss the findings as artifacts of overexpression or artificial stressors. Careful wording of the text and additional discussion on this point -- if not an additional experiment measuring TDP43/SEC16A aggregation as a function of TDP43 expression levels -- are required.

We appreciate the reviewer for recognizing the improvement on our manuscript and the suggestion to clarify the relationship between TDP43/SEC16A expression levels and their co-aggregation.

In response, we have performed Pearson's correlation analyses between TDP43/SEC16A co-aggregation and TDP43 or SEC16A expression levels. The analysis shows that there is a weak but significant positive correlation between co-aggregation and these levels (r=0.24 for TDP43, and r=0.29 for SEC16A, with p<0.05, please see Fig. S2c, d in the further revised manuscript). However, we note that this weak correlation may not be so unexpected since when the expression levels were too low, co-aggregates also became difficult to visualize. In addition, this analysis also shows that the heterogeneity of co-aggregation cannot be explained simply by the degree of overexpression: If, for example, cells are categorized by TDP43 expression levels into below-median and above-median, ~10% of cells with below-median expression levels formed greater than average level of TDP43/SEC16A coaggs, whereas ~38% of cells with above-median expression had less than average coaggs (Fig. S2c). Similar observations can be made for SEC16A (Fig. S2d).

We speculate that, in addition to expression levels, co-aggregation heterogeneity could also result from heterogeneity in stress tolerance among cells, such as G3BP1 expression level and hence SG formation (Fig. S4d-j), or stochasticity in the nucleation of protein aggregation (Wang et al., 2022, Consortium, 2020). We would like to reiterate that the observation of co-aggregation was also made for endogenous expression of these proteins in both RPE-1 cells (Fig. S3d, e) and in ALS neurons (Fig. 7). Furthermore, overexpression of TDP43 is linked to ALS as we discussed in the previous revision. We have added the new analysis and additional discussion, similar as the above, in the revised manuscript (Page 8 Lines 4-10 and Page 19 Lines 13-20).

Reviewer #5 (Remarks to the Author):

TPD-43 plays a role in the propagation of neurodegenerative disease. In the manuscript of Wu et al. this propagation is connected with ER-to-Golgi transport by pinpointing aggregations of proteins in stress granules and at ER exit sites. The manuscript focuses on the aggregations using a Hsp104 mutant (yeast) which is known to reverse the toxicity of TDP-43. The findings of Wu et al. are relevant for the scientific community as thy reveal the co-aggregation of SEC16 and COPII with TDP43, impairing the ER-to-Golgi transport. The study uses a combination of imaging experiments and a mass spectrometry setup to elucidate in detail the above mentioned aggregation connections that down the line are of interest for better understanding neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis (ALS). The work of Wu et al. offers the scientific community the

opportunity to use the presented results for further needed studies to tackle TDP-43 connections of aggregation and pharmaceutical intervention.

We thank the reviewer for the positive overall comment on our study.

Major points and concerns:

Figure S1depicts the affinity-based aggregation purification using a Hsp104 flag-tagged mutant. The authors did state-of-the-art mass spectrometry using TMT-labeling in a context of flag-immunoprecipitations to present 337 enriched proteins. Figure S1F highlights the enrichment of these protein aggregates in a Hsp104 context. Figure S1E falls short of a being a good presentation of these proteins since it only shows enrichment but not the bead control side is shown. S1F is usually depicted as a complete volcano plot also for bead binders and contaminations. Overall, the Hsp104 data should be part of the main publication and not a part of the supplement since it reflects the technical comprehensiveness of this study. Using a HEK293T cell system for these immunoprecipitiations has experimental benefits and drawbacks when addressing ALS questions. The mass spectrometry raw data should be after publication of the manuscript be made available to the scientific community on the PRIDE proteomics identification database. This will make future studies comparable with other cell lines.

We appreciate these suggestions for our MS experiment and presentation. In Figure S1f we showed "Enrichment" as the vertical axis instead of "-log p-value" as in a volcano plot because our MS experiment was performed only once so p-values could not be calculated. This is also why we did not place MS data in main figures. Nonetheless, we validated the candidates from MS results by careful microscopy imaging (Figure 1g-i and S2a, b).

We agree with the reviewer that, to specifically address ALS, it would be better to perform IP-MS on more relevant systems, such as hiPSC-induced motor neurons (iMNs) or ALS patient samples. However, we started our research from a basic cell biology perspective aiming to identify proteins that are generally aggregation-prone under stressful conditions. We therefore chose HEK293T cell line for the comprehensiveness of its proteome and convenience of handling (Page 6 Lines 5-6). The identification of ALS-associated TDP43 and SEC16A in aggregates unexpectedly led to the follow-up study as presented. As suggested by Reviewer 4 (Major Concern 1), we were careful not to overstate on the disease relevance of our cell biology results beyond what we could validate in ALS patient derived samples or iMNs (Figure 7 and S10). In the revised manuscript, we have explained the advantage and limitations of using HEK293T in Discussion (Page 16 Lines 13-16) as suggested by the reviewer.

Our MS collaborators have deposited the MS raw data to the publicly accessible Japan ProteOme STandard Repository (jPOSTrepo) under identifier 9443 with the link https://repository.jpostdb.org/entry/JPST002060 (Page 30 Lines 26-28).

Nucleoporin proteins, such as NUP88 and NUP214 play two important roles in the cellular context. One as nuclear pore complexes and secondly their secondary functional requirements during mitosis. Addressing these relevant details in the discussion part of the manuscript is important since it would address the overall importance of these proteins in aggregates and not in functional locations. This may lead to further investigation of the topic by the scientific community. This discussion should be embedded resonating other citable publications.

We thank the reviewer for reminding us of the disassembly of nuclear pore complexes during mitosis. We have added a discussion about the possibility that nucleoporins were incorporated into cytoplasmic aggregates during mitosis (Kutay et al., 2021), and a recent report about stress-induced cytoplasmic Nup foci in *C. elegans* (Thomas et al., 2023) (Page 17 Lines 22-27).

Figure 6 provides results of TDP43_ERES retain secretory cargo. The model depicted in Figure 6H is one of the key takeaways from this manuscript. I believe the readers would benefit of a better depiction of this model, using a more streamlined presentation of the endoplasmatic reticulum, the involved proteins, and the Golgi apparatus.

We appreciate this suggestion and have provided a broader cellular context of our proposed model, as well as streamlined presentation of ER and Golgi (Figure 6h).

Minor points and concerns:

Hsp104 is a protein aggregation model system from yeast and in a context of HEK293T, it does not reflect the human conditions of neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis (ALS). HEK293T cells are a viable cell system for large scale experiments, especially since mass spectrometry based immunoprecipitation (IP) readouts require a certain amount of starting material when gone through gradients and IPs. Since using other human cell lines for detecting IP partners could have not given the reader the full readout that approach in this manuscript has its drawbacks at the same time is a valid approach the used to address this issue, this has to be more detailed in their discussion part and put it into context.

$Hsp104^{DWB}$ was employed as a *bio-orthogonal* probe that binds misfolded proteins in general, including those in human cells, and therefore enriched in aggregates. This specific mutant does not disassemble aggregates (DeSantis et al., 2012)

(mentioned in Page 5 Lines 6-12 of the revised manuscript). The fact that it is not involved in neurodegeneration or present in human cells at all (*i.e.* being bio-orthogonal) is an advantage of using it as a reporter.

As explained to Major Concern 1, we started this study from a cell biology perspective, not ALS-specific, so HEK293T was an ideal choice for us then, but we have validated the ALS relevance of our findings by using microscopy imaging of relevant samples (Figure 7 and S10). As suggested by the reviewer, now we have explained the advantage and limitations of using HEK293T in Discussion (Page 16 Lines 13-16).

Originality, significance, validity:

The publication shows a original approach and is in coherence with previous publications while showing its own significance in the importance of proteotoxic stress impairing the ER-to-Golgi transport. The supplemental material is extensive and detailed, in order to support the authors findings and theories.

Statistics:

The statistics used are in coherence with the experimental setups and reflect up-to-date statistics. As mentioned above, adding the mass spectrometric raw files to the PRIDE database will make them accessible for future comparative studies.

Conclusions:

As above mentioned the conclusions should add a compact short discussion on the nucleoporin proteins and the Hsp104 yeast model protein.

As mentioned above, we have included discussions about nucleoporins (Page 17 Lines 22-27) and yeast $Hsp104^{DWB}$ (Page 5 Lines 6-12). We thank the reviewer for these suggestions.

References:

The references are comprehensive and no excessive self-referencing was detected.

Clarity and context:

The abstract, results, and discussion is coherently written and easy to scientifically comprehend.

References

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REVIEWERS' COMMENTS

Reviewer #4 (Remarks to the Author):

The authors have largely addressed my concerns, and should be congratulated on a comprehensive study.

Since their original submission, additional evidence has arisen regarding the validity and relevance of TDP43 overexpression to disease pathogenesis (PMID: 38853250). Based in part on this, the authors may wish to temper their conclusions regarding TDP43 overexpression and its relationship to disease.

Reviewer #5 (Remarks to the Author):

Wu et al. have fully addressed my concerns, concerning figures, discussion, making high-throughput data accessible to the scientific community. I fully support the publication of this manuscript in Nature Communications.

Author response:

Reviewer #4 (Remarks to the Author):

The authors have largely addressed my concerns, and should be congratulated on a comprehensive study.

Since their original submission, additional evidence has arisen regarding the validity and relevance of TDP43 overexpression to disease pathogenesis (PMID: 38853250). Based in part on this, the authors may wish to temper their conclusions regarding TDP43 overexpression and its relationship to disease.

We have cited this recent finding of how TDP43 overexpression modulates RNA splicing on Page 20 Lines 11-13 and made sure that there is no overstatement about TDP43 overexpression and its relationship to disease in the manuscript. We thank the reviewer for continuously helping us improve the manuscript.

Reviewer #5 (Remarks to the Author):

Wu et al. have fully addressed my concerns, concerning figures, discussion, making high-throughput data accessible to the scientific community. I fully support the publication of this manuscript in Nature Communications.

We appreciate the support of this reviewer.