EMBO reports

O-GlcNAcylation of TDP-43 suppresses proteinopathies and promotes TDP-43's mRNA splicing activity

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1st Editorial Decision 25th Sep 2020

Dear Dr. Du,

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, all referees acknowledge that the findings are interesting. However, they also all point out that significant revisions will be required. I think all points raised are reasonable and should be addressed. If you prefer, or if you disagree, we can discuss the revisions also over the phone or via video chat. Just let me know.

I would thus 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. 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 should 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.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

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.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

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.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at

<https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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 when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #2:

In this work, Zhao et al. have investigated the effects of O-GlcNAcylation on TDP-43 functional properties, especially splicing of the STMN2 gene. The importance of this modification was then tested in a yeast system to investigate its effects on TDP-43 toxicity and in a Drosophila system, showing that mutation of the site can rescue motor defects and can extend lifespan. Overall, the observation is interesting and the effects of O-GlcNAcylation on TDP-43 may be novel and of interest to the field. However, several experimental clarifications are in order.

1) In Figure 1B it is not clear what is the band migrating at 35kDa when in Figure 1A only one band seems to correspond to the O-GlcNAcylated TDP-43. Could it be a degradation fragment that is enriched by the O-GlcNAcylation specific antibody or is it an artefactual band?. Because its intensity is as high as the TDP-43 signal this should be clarified.

2) In general, it would be important to quantify how much TDP-43 is normally affected by this modification. From the Western blots in Figure 1, the signal from the non-modified TDP-43 seems much higher than that of the modified protein. Have the authors quantified the extent of this modification in any way?. Ideally, they should have tried to make an antibody specific against the GlcNAcylated epitopes (identified in Figure 3) and screened cells using this more specific tools.
3) The results in the yeast system do not really address the importance of TDP-43 O-GlcNAcylation but rather the importance of OGT expression in suppressing TDP-43 toxicity. No evidence is provided, in fact, that OGT overexpression is acting only through the modification of TDP-43. In fact, it may well act on some other yeast cellular factor that would then be responsible for repressing TDP-43 toxicity. This possibility should be addressed at least by showing that yeast cells are not affected in any way by OGT expression in the absence of TDP-43.
4) The asterisk in Figure 2B is not explained anywhere. Please clarify. In addition, the authors should note that it is not ideal practice to crop the western blots in close correspondence to signal bands. Therefore, a better picture should be shown for such an experiment that shows the entire blotted surface.
5) Figure 2C is lacking an IHC for cells simply treated with Glucosamine. It is also rather strange that in the only cell shown in the figure the signal for pTDP-43 is all in the nucleus when it is well known that pTDP-43 localizes to the cytoplasm. Are the authors sure that this is a representative image of the experiment?.
6) Figure 2E should be quantified and also Figure 3E and 3F from independent repetitions.
7) The results in Figure 4 are interesting but they do not really link TDP-43 GlcNAcylation with rescue of larval locomotion. At the very least, the authors should show that the WT TDP-43 is GlcNAcylated in Drosophila and in human cells and that this is impaired by the T199A, T233A, and 2TA mutants). Has this been investigated?
8) This problem is also reflected in the splicing assays where the mutants have an effect on a variety of substrates but they may be acting independently of GlcNAcylation on the RNA binding activity of TDP-43. In this case, the authors should have performed some of these splicing experiments also using the conditions explored in the first part of the study. For example, does the splicing of the CFTR exon 9 reporter change according to expectations in conditions where TDP-43 GlcNAcylation should be increased?. Such a result, coupled with the mutant experiments in Figure 5 would make a much more compelling argument.
9) Statistical significance is lacking from Fig.5H

Referee #3:

This manuscript describes the impact of the post-translational modification O-GlcNAcylation on TDP-43 function. Importantly, this PTM has not been previously reported for TDP-43. Using a combination of in vitro and in vivo approaches, the authors identify that TDP-43 can be directly modified by the enzymes that add and remove O-GlcNAc (OGT and OGA, respectively) and identify the targeted residues. Moreover, using Drosophila, the authors demonstrate that these O-GlcNAc targeted sites can influence fly lifespan and locomotion. Lastly, the impact of these PTM on TDP-43 function in the splicing of a number of genes is demonstrated.
Overall, the story is highly interesting and while there are limitations and overstatements in some of the individual experiments (most of which can be addressed, see below), the collective dataset seems to support that O-GlcNAcylation of TDP-43 is relevant to TDP-43 function in splicing. However, quantification of key experiments is absolutely required to solidify the work.
Lastly, a major issue is that this manuscript does not cover whether O-GlcNAcylation is relevant to ALS/FTD pathogenesis. For example, is there any evidence that this modification or any of the

enzymes implicated in the process are linked to ALS/FTD?

Other points to be addressed:
• Page 4, first sentence of last paragraph, typo "N-acetylgulcosamine"
• Page 4, last sentence, typo "hyerphosphorylation"
• Legend of Fig 3E: "TPD-43" typo
• Page 5, the following sentence is out of place: "Regardless of normal feeding, fasted or refed conditions, the O-GlcNAcylation of TDP-43 was detected in mice with different modified levels (Fig EV1A and B). Thus, we conclude that TDP-43 is O-GlcNAcylated by OGT in vivo." The rationale of this experiment is only introduced on page 8. Also, what is the significance of having looked in the primordial pallium? (vs. an ALS-relevant CNS region?)
• Page 6, what do the authors' mean "...we test if OGT could improve TDP-43 proteinopathy by defend TDP-43 hyperphosphorylation." ? The language is not clear here. Note, the word "defend" is actually used a few times... Do the authors mean "prevent"?
• Page 8, last sentence: "Although we noticed that the expression level of the 2TA mutant was slightly lower than the other TDP-43 proteins, higher expression level in T199A mutant did not gave rise to the shortest lifespans of Drosophila, indicating that the different phenotypes may not result from the divergence of TDP-43 expression (Fig 4D)." The word "slightly" should be removed as the difference in levels is obvious.
• What is the rationale for using different O-GlcNAc antibodies (RL2 vs CTD110.6)?
• Throughout the manuscript, claims regarding protein/O-GlcNAc levels are not sufficiently supported. Quantification is needed throughout. Example, Fig 1G and 1H: authors claim "substantial increase" and "significantly decreased" but there is no quantification provided. Similar comment for Fig EV2A, 2F, and 4F.
• Input lanes should be included for IPs in Figure 2A, 2B and 3F. Authors might consider combining Fig 2A with Fig 1C.
• It is broadly held that phosphorylated TDP-43 is cytoplasmic. However, in Fig 2C, ethacrynic acid results in pTDP-43 signal that is predominantly nuclear. Can the authors comment?
• The O-glcNAc blots in Fig 3E and EV3A are not very convincing. Can better blots be provided?
• The in vivo experiments (Fig 4) are highly interesting. The levels of the enzymes regulating the O-GlcNAcylation should be evaluated since a change in the level of these could also explain the data. This is especially relevant since the authors also indicate that TDP-43 can bind OGT transcripts. Also, the authors should also include data demonstrated that TDP-43 is O-GlcNAcylated in flies.
• In a variety of splicing assays, the authors have employed the ALS-linked mutation Q331K as a control. (Indeed, I found these experiments very well designed and nicely controlled). However, I wonder: is Q331K O-GlcNAcylated?
• Is the protein level of OGT impacted by the TDP-43 mutations? OGA?

Referee #4:

Zhao and co-authors report on the effect of O-GlcNAc modification of the RNA binding protein TDP-43 and the role of glycosylation of this protein on its proteotoxicity and regulation of its activities on splicing. Briefly, the authors show that TDP-43 is O-GlcNAc modified, they may the sites of O-GlcNAc modification, show that these sites are important for proteotoxicity in yeast and fly models, and finally show that these sites are also important for the splicing functions played by TDP-43. Overall, this is an extensive body of work using a range of different methods and models. The work builds on a range of previous observations regarding the protective effects of increasing

O-GlcNAc in a range of proteotoxicity models including neurodegenerative disease models including the tauopathies.

This is an original body of work that is conceptually appealing. The work is consistent with data on other aggregation prone proteins involved in neurodegenerative diseases including tau and alpha-synuclein. In those cases the sites have been specifically shown to decrease the aggregation propensity of those proteins. OGA inhibitors have advanced into human clinical trials. There is accordingly high potential significance to the work as it may stimulate developing inhibitors for ALS, which is a major unmet medical need. This manuscript reports on preclinical support that could stimulate the pursuit of targeting ALS using this approach. The manuscript could be publishable but there are a number of questions that come up and need to be considered prior to publication.

The general approaches are sound and the data is largely clear when considered in composite. Some exceptions are noted below.

The main point is that I am not certain that the authors have truly shown that O-GlcNAc on TDP-43 and OGT catalytic activity is essential for blocking gain of toxicity and loss of function. In particular the double mutant may not show toxicity because it fails to get phosphorylated at these two positions. In addition these two sites of modification are directly in the RRM2 region and so these mutations may affect RNA binding in an unknown manner by loss of the hydroxyl group. The presence of modifications in a folded domain is also unusual for O-GlcNAc.

To the point of the catalytically active OGT being needed. The data can in many points be rationalized in terms of OGT having some relevant non-catalytic role. The use of the OGT mutant only extends to Figure 2. Further use of this mutant would help strengthen the paper a great deal. An example is in Figure 2 Panel E where these appears to be a small difference, if any, between the WT and Mut OGT. Some replication would help. But also using the mutant in downstream studies would be more convincing. Also, nowhere in the fly studies is data shown suggesting catalytic activity is needed - recognizing this is difficult some consideration of this and discussion is needed in the manuscript.

One issue is also the extent to which TDP-43 is glycosylated in these studies. The general model implies that when OGT is expressed the modification directly antagonizes toxicity. If this is the case one would expect the stoichiometry of modification to be quite high. I would recommend the authors consider and discuss this point and perhaps try to address this if possible.

Another question is the use of ethynacrinic acid. Why this compound - no clear rationale is offered.

One further concern is that for many of the immunoblot experiments only one example is shown and the claims depend on the results. There are several examples like this. The authors should identify observations that are central and provide clear replication and some statistical analysis using true independent replicates.

Page 7. The double mutant should be purified from mammalian cells and assessed for O-GlcNAc levels. This in vitro modification system is less clear.

There is also a lack of use of various antagonists of OGT or the OGA that could be used to support the observations using different methods. Though not essential for publication the authors could use such methods in some studies to strengthen the work.

Figure 1: Why are there two bands for TDP-43 in Panel B?
Figure 1: Less important but increase in O-GlcNAc on TDP-43 by glucosamine.
Figure 2: Panel A and E. Are these differences significant. They do not seem so to me. Some validation would be needed.
Figure 3: Panel F. The catalytically dead OGT is needed.
Figure 4: Panel F. I see no significant differences. Some repeat and statistics are needed. But largely I am not sure if this is useful for the manuscript and not sure it adds anything.
Figure 5: Indication of controls for protein expression levels should be included.
Figure EV3 and text: MS mapping of T199. How was this done. This is not a tryptic peptide. Explanation is needed.
Figure EV1: I do not see significant differences here between normal, fed, and fasted.

The choice of references seems good. Page 4: Arnold 1996 reference to bovine tau. Replace with Lefebvre 2003 that relates to human tau.

The manuscript is fairly well written. But the authors do not discuss the effect sizes in many cases for immunoblots and in many cases these differences are central to the claims of the manuscript. In addition, the scope of the work and the wide range of models and methods used necessitates careful expert review and also makes reading quite difficult.

This is an interesting manuscript on an important topic that represents a major undertaking. Some elements are very convincing including the yeast and fly data. The manuscript therefore has real potential and is appealing as it addresses a potential disease modifying mechanism for ALS. However, there are some technical weaknesses in terms of showing the catalytically active OGT is needed and replication and quantification of key experiments. The authors need to focus on the key elements and provide a clear reply to the concerns. However, the work would certainly be of high interest and I would support a major revision of this manuscript. I believe that the work could be an excellent contribution to the journal and prove to be of wide interest to a range of readers.

Referee #2

In this work, Zhao et al. have investigated the effects of O-GlcNAcylation on TDP-43 functional properties, especially splicing of the STMN2 gene. The importance of this modification was then tested in a yeast system to investigate its effects on TDP-43 toxicity and in a Drosophila system, showing that mutation of the site can rescue motor defects and can extend lifespan. Overall, the observation is interesting and the effects of O-GlcNAcylation on TDP-43 may be novel and of interest to the field. However, several experimental clarifications are in order.

We thank the reviewer's positive comments and believe that the following suggested revisions have strengthened the manuscript as a result of their inclusion.

1) In Figure 1B it is not clear what is the band migrating at 35kDa when in Figure 1A only one band seems to correspond to the O-GlcNAcylated TDP-43. Could it be a degradation fragment that is enriched by the O-GlcNAcylation specific antibody or is it an artefactual band? Because its intensity is as high as the TDP-43 signal this should be clarified.

We tried to re-examine the 35-kDa band using RL2 antibody. However, the commercial antibody with same lot number was used up, so a new Lot. of the RL2 antibody has been purchased and used here (Abcam, cat: ab2739, Lot: GR3306462-2). We reperformed the experiment twice. Unfortunately, we could not observe any band migrating at 35-kDa this time. We speculate that the previous 35-kDa band was an unspecific band that might originate from the previous Lot. Antibody. Another possibility is that the cells used in original Fig 1B were not very healthy, so that the truncated 35-kDa fragment was present. We consistently noticed that cells treated with hydrogen peroxide or arsenite tend to produce the truncated fragment of TDP-43. To avoid misleading, a new representative blot without the truncated form is shown in the revised manuscript (new Fig 1B).

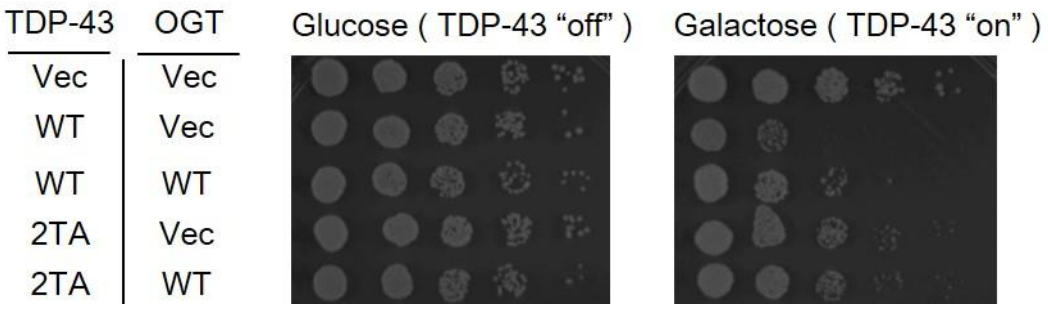
2) In general, it would be important to quantify how much TDP-43 is normally affected by this modification. From the Western blots in Figure 1, the signal from the non-modified TDP-43 seems much higher than that of the modified protein. Have the authors quantified the extent of this modification in any way? Ideally, they should have tried to make an antibody specific against the GlcNAcylated epitopes (identified in Figure 3) and screened cells using this more specific tools.

We agree with the reviewer's suggestion. It is definitely important to quantify the extent of O-GlcNAcylation of TDP-43 *in vivo*. Actually, we have tried to make a specific antibody recognizing the O-GlcNAcylated T199, but it is unsuccessful (data not shown). We have surveyed the literature and communicated with some experts in glycobiology, and we were alerted that it is extremely difficult to generate a site-specific O-GlcNAcylated antibody based on the current technology. Alternatively, quantitative mass spectrometry might be an ideal way to determine the extent of modification. Unfortunately, it still lacks any mature approach to do this due to the chemically unstable linkage of O-GlcNAcylated moiety. To solve this problem, we chose a semi-quantitative assay to determine the extent of TDP-43 O-GlcNAcylation. Basically, cells were treated with Thiamet-G (TMG), an effective inhibitor of OGA (Ki = 21 nM), for different times, and the O-GlcNAcylation levels

of global proteins in cells were compared. We noticed that the O-GlcNAcylation levels are not further increased over the time from 12 to 48 hours, suggesting that this modification may reach a stable state at this condition (Fig EV1A). We supposed the O-GlcNAcylation extent of TDP-43 at this situation (treated with 48 hours) as 100%, and the relative O-GlcNAcylation levels of TDP-43 in untreated cells was quantified with the signal intensity in treated cells. We found that around 20% TDP-43 was modified by O-GlcNAcylation (new Figure EV1B and C). We hope that this experiment could answer the reviewer's question.

3) The results in the yeast system do not really address the importance of TDP-43 O-GlcNAcylation but rather the importance of OGT expression in suppressing TDP-43 toxicity. No evidence is provided, in fact, that OGT overexpression is acting only through the modification of TDP-43. In fact, it may well act on some other yeast cellular factor that would then be responsible for repressing TDP-43 toxicity. This possibility should be addressed at least by showing that yeast cells are not affected in any way by OGT expression in the absence of TDP-43.

We understand that the reviewer proposed that OGT may modify other factors instead of TDP-43 to indirectly reflect the repression of TDP-43 toxicity in yeast. Actually, in new Fig 2A, left panel (TDP-43 "off"), we have shown that in the absence of TDP-43, constitutive expression of OGT alone does not affect yeast cell growth (row 3). To further address that expression of OGT rescues the growth defect caused by TDP-43 toxicity is mainly due to the O-GlcNAcylation of TDP-43, OGT was co-expressed with the O-GlcNAcylation deficitive 2TA mutant. We observed that overexpression of OGT and 2TA mutant did not further promote yeast cellular growth, indicating that the major impact of OGT on TDP-43 toxicity (rebuttal Figure 1).



Rebuttal Figure 1. Yeast cells that express galactose-inducible GFP-tagged either WT or 2TA hTDP-43 constructs in combination with co-expressing mCherry-tagged WT OGT were spotted onto plates containing glucose or galactose. Cell growth was assessed after 2 days.

4) The asterisk in Figure 2B is not explained anywhere. Please clarify. In addition, the authors should note that it is not ideal practice to crop the western blots in close correspondence to signal bands. Therefore, a better picture should be shown for such an experiment that shows the entire blotted surface.

The asterisks in original Fig 2B indicated the heavy chains of IgG. We have added the information in the new Fig 3 legend. We have also shown the entire blots containing the heavy chains and added ''Input" Western blots in the new Fig 3C.

5) Figure 2C is lacking an IHC for cells simply treated with Glucosamine. It is also rather strange that in the only cell shown in the figure the signal for pTDP-43 is all in the nucleus when it is well known that pTDP-43 localizes to the cytoplasm. Are the authors sure that this is a representative image of the experiment?.

We thank the reviewer's constructive suggestion. We have re-performed the experiments by treating cells with either glucosamine (GlcNAc), ethacrynic acid (EA), or a combination. We noticed that the phosphorylated TDP-43 was found in cytoplasmic in most literatures. However, some studies also showed that the phosphorylated TDP-43 is located in both nucleus and cytosol (Chen *et al*, 2019; Cohen *et al*, 2015; Nonaka *et al*, 2009). In our previous experiment, we repeatedly observed phosphorylated TDP-43 signals are predominantly nuclear by biological repeats. We speculated that different experiment protocols might be the reason of divergent results. To do this, we treated cells with 60 μM ethacrynic acid for 24 h (rather than 20 μM ethacrynic acid for 12 h as before), which allows TDP-43 to translocate into cytosol and be phosphorylated in this condition. The cell population of phosphorylated TDP-43 and the foci numbers of phosphorylated TDP-43 per cell were quantified as well. The data are shown in the revised Fig 3D and E.

Chen HJ, Topp SD, Hui HS, Zacco E, Katarya M, McLoughlin C, King A, Smith BN, Troakes C, Pastore A *et al* (2019) RRM adjacent TARDBP mutations disrupt RNA binding and enhance TDP-43 proteinopathy. ***Brain* 142**: 3753-3770

Cohen TJ, Hwang AW, Restrepo CR, Yuan CX, Trojanowski JQ, Lee VM (2015) An acetylation switch controls TDP-43 function and aggregation propensity. ***Nat Commun* 6**: 5845

Nonaka T, Arai T, Buratti E, Baralle FE, Akiyama H, Hasegawa M (2009) Phosphorylated and ubiquitinated TDP-43 pathological inclusions in ALS and FTLD-U are recapitulated in SH-SY5Y cells. ***FEBS Lett* 583**: 394-400

6) Figure 2E should be quantified and also Figure 3E and 3F from independent repetitions.

As the reviewer suggested, these panels are quantified. New data are shown in revised Fig 3G, 4F, and 4H, respectively.

7) The results in Figure 4 are interesting but they do not really link TDP-43 GlcNAcylation with rescue of larval locomotion. At the very least, the authors should show that the WT TDP-43 is GlcNAcylated in Drosophila and in human cells and that this is impaired by the T199A, T233A, and 2TA mutants). Has this been investigated?

We agree with this reviewer's opinion, since we did not perform a rescue experiment by expression of either WT or GlcNAc-defective mutants of TDP-43 in *Drosophila*. The difficulty of this assay is that we need to knockout two alleles of fly *TBPH* (the human homolog of *TARDBP)* with expression of various constructs of TDP-43 or generate both alleles mutated *TBPH* fly, which needs to spend much time and many efforts. To overcome this issue, we have overexpressed various TDP-43 constructs in *Drosophila* neurons, and the O-GlcNAcylation levels of TDP-43 were examined (new Fig 5D). We consistently observed decreased O-GlcNAc levels of TDP-43 mutants compared to the

WT. In addition, the O-GlcNAcylation levels of various TDP-43 proteins in human cells are shown in new Fig 4G. We think these results can, at least partially, link O-GlcNAcylation of TDP-43 with rescue of larval locomotion.

8) This problem is also reflected in the splicing assays where the mutants have an effect on a variety of substrates but they may be acting independently of GlcNAcylation on the RNA binding activity of TDP-43. In this case, the authors should have performed some of these splicing experiments also using the conditions explored in the first part of the study. For example, does the splicing of the CFTR exon 9 reporter change according to expectations in conditions where TDP-43 GlcNAcylation should be increased? Such a result, coupled with the mutant experiments in Figure 5 would make a much more compelling argument.

We thank the reviewer's constructive suggestions. To answer whether TDP-43 mutations affect its RNA binding capacity, RNA-EMSA is performed using total cell lysate from GFP-TDP-43 transfected 293T cells incubated with a biotin-labelled RNA probe. The results indicate that O-GlcNAcylation defective mutants of TDP-43 did not affect their RNA binding abilities, which rule out the possibility that loss of splicing ability in the mutants is due to changes of RNA binding ability in their own (Fig EV4).

As the reviewer suggested, the CFTR splicing assays were performed with either knockdown or overexpression of WT or catalytic-inactive OGT, in order to directly link the impact of OGT on RNA splicing with O-GlcNAcylation of TDP-43 but not other substrates. We observed that knockdown of OGT itself was sufficient to attenuate TDP-43 dependent CFCR exon 9 splicing, and double knockdown of OGT and TDP-43 displayed a comparable splicing signal with that by knockdown of TDP-43 alone (Fig 6B). Furthermore, we found that only overexpression of WT OGT, but not the catalytic inactive mutant of OGT, promoted CFTR exon 9 spliced, implying a potential role of O-GlcNAcylation (Fig 6C, lane 1-3). We also observed that, in the absence of TDP-43, overexpression of OGT failed to promote CFCR splicing (Fig 6C, lane 4 vs. 5). Introduction of WT TDP-43 but not O-GlcNAc-deficitive 2TA mutant into siTDP-43 cells was able to rescue CFCR splicing ability, which indicates that the effect of OGT on CFCR splicing mainly relies on TDP-43, but not other substrates (Fig 6C, lane 7-12).

Based on these data, we conclude that O-GlcNAcylation of TDP-43 is critical for its RNA splicing activity.

9) Statistical significance is lacking from Fig.5H

We have performed at least three biological repeats and statistical analyses of relative truncated *STMN2* signals and relative mRNA expression levels of *STMN2* shown in new Fig 6I and J, respectively.

Referee #3:

This manuscript describes the impact of the post-translational modification O-GlcNAcylation on TDP-43 function. Importantly, this PTM has not been previously reported for TDP-43. Using a combination of in vitro and in vivo approaches, the authors

identify that TDP-43 can be directly modified by the enzymes that add and remove O-GlcNAc (OGT and OGA, respectively) and identify the targeted residues. Moreover, using Drosophila, the authors demonstrate that these O-GlcNAc targeted sites can influence fly lifespan and locomotion. Lastly, the impact of these PTM on TDP-43 function in the splicing of a number of genes is demonstrated.
Overall, the story is highly interesting and while there are limitations and overstatements in some of the individual experiments (most of which can be addressed, see below), the collective dataset seems to support that O-GlcNAcylation of TDP-43 is relevant to TDP-43 function in splicing. However, quantification of key experiments is absolutely required to solidify the work.

We are grateful to the reviewer's positive comments. Meanwhile, we performed more experiments and quantification analyses to support our conclusions. We believe that the following suggested revisions have strengthened the manuscript.

Lastly, a major issue is that this manuscript does not cover whether O-GlcNAcylation is relevant to ALS/FTD pathogenesis. For example, is there any evidence that this modification or any of the enzymes implicated in the process are linked to ALS/FTD?

This is a good question. Since it is the first time that our study shows that TDP-43 can be O-GlcNAcylated, there is no more evidence to link its O-GlcNAcylation with ALS/FTLD pathogenesis so far. However, we had been thinking to examine whether ALS/FTLD pathogenesis is directly relevant to O-GlcNAcylation of TDP-43. Unfortunately, it is really difficult to obtain ALS/FTLD postmortem brain samples in China, especially after worldwide outbreak of COVID-19. Actually, several reports have suggested that elevated O-GlcNAc levels in spinal motor neurons protect age-dependent oxidative stress and improve cell survival, and decreased O-GlcNAC levels are found in the spinal cords of ALS model animals (Hsieh *et al*, 2019; Ludemann *et al*, 2005; Shan *et al*, 2012). In addition, neuron-specific depletion of OGT leads to impaired mobility or progressive neurodegeneration in mice (O'Donnell *et al*, 2004). These data obviously indicate that O-GlcNAcylation in motor neurons or spinal cords is closely related with ALS/FTLD pathogenesis. We have cited these studies in the main text (Page 14, the first paragraph).

Hsieh YL, Su FY, Tsai LK, Huang CC, Ko YL, Su LW, Chen KY, Shih HM, Hu CM, Lee WH (2019) NPGPx-Mediated Adaptation to Oxidative Stress Protects Motor Neurons from Degeneration in Aging by Directly Modulating O-GlcNAcase. ***Cell Rep* 29**: 2134-2143 e2137

Ludemann N, Clement A, Hans VH, Leschik J, Behl C, Brandt R (2005) O-glycosylation of the tail domain of neurofilament protein M in human neurons and in spinal cord tissue of a rat model of amyotrophic lateral sclerosis (ALS). ***J Biol Chem* 280**: 31648-31658

Shan X, Vocadlo DJ, Krieger C (2012) Reduced protein O-glycosylation in the nervous system of the mutant SOD1 transgenic mouse model of amyotrophic lateral sclerosis. ***Neurosci Lett* 516**: 296-301

O'Donnell N, Zachara NE, Hart GW, Marth JD (2004) Ogt-dependent X-chromosome-linked protein glycosylation is a requisite modification in somatic cell function and embryo viability. ***Mol Cell Biol* 24**: 1680-1690

Other points to be addressed:

• Page 4, first sentence of last paragraph, typo "N-acetylgulcosamine"

Thanks. It has been changed.

• Page 4, last sentence, typo "hyerphosphorylation"

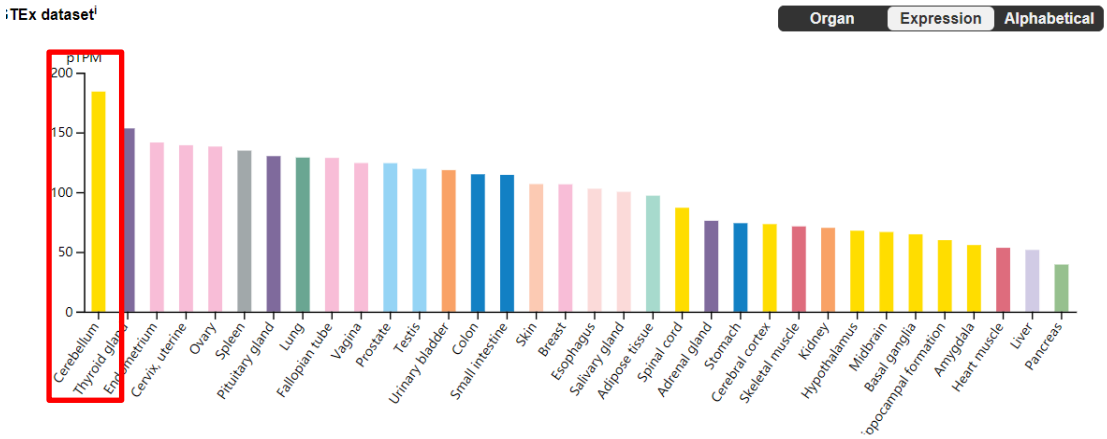
Thanks. It has been changed.

• Legend of Fig 3E: "TPD-43" typo

Thanks. The typo in Fig 1E has been changed.

• Page 5, the following sentence is out of place: "Regardless of normal feeding, fasted or refed conditions, the O-GlcNAcylation of TDP-43 was detected in mice with different modified levels (Fig EV1A and B). Thus, we conclude that TDP-43 is O-GlcNAcylated by OGT in vivo." The rationale of this experiment is only introduced on page 8. Also, what is the significance of having looked in the primordial pallium? (vs. an ALS-relevant CNS region?)

The purpose of the experiment shown in the original Fig EV1 is to confirm that O-GlcNAcylated TDP-43 can also be detected at animal levels. The reason that we chose primordial pallium is that TDP-43 expression is higher in brain based on the database of Genotype-Tissue Expression (GTEx), and the primordial pallium is relatively easy to be obtained (Rebuttal Figure 2). Due to a page limitation (Reports type), we apologize that this was not mentioned previously in the main text. In fact, we have also examine O-GlcNAcylated levels of TDP-43 in cerebral cortex regions of normal mice or type-I diabetes mice, and a similar conclusion can be drawn (data not shown). Since these results are less important compared to other evidence presented, we decided to remove this data in the revised manuscript.



Rebuttal Figure 2. Expression levels of TDP-43 in various tissues based on GTEx. The red frame highlights the highest level of TDP-43 in cerebellium.

• Page 6, what do the authors' mean "...we test if OGT could improve TDP-43 proteinopathy by defend TDP-43 hyperphosphorylation." ? The language is not clear here. Note, the word "defend" is actually used a few times... Do the authors mean "prevent"?

Yes, we have changed “defend” to “prevent”, and the sentence is modified as “*Next, we*

test if OGT could relieve TDP-43 proteinopathy by preventing TDP-43 hyperphosphorylation".

• Page 8, last sentence: "Although we noticed that the expression level of the 2TA mutant was slightly lower than the other TDP-43 proteins, higher expression level in T199A mutant did not gave rise to the shortest lifespans of Drosophila, indicating that the different phenotypes may not result from the divergence of TDP-43 expression (Fig 4D)." The word "slightly" should be removed as the difference in levels is obvious.

Thanks, the word “slightly” has been removed.

• What is the rationale for using different O-GlcNAc antibodies (RL.2 vs CTD110.6)?

Both O-GlcNAc antibodies have been widely used in examining the O-GlcNAc levels of proteins in previous studies. To validate the signals we detected here represent a real O-GlcNAcylated TDP-43, both RL2 and CTD110.6 antibodies are initially utilized. Afterwards, the CTD110.6 antibody was mainly utilized in following experiments. Of note, we noticed that the RL2 antibody is better than the CTD110.6 antibody in immunoprecipitation assays, but less than the latter in immunoblotting assays.

• Throughout the manuscript, claims regarding protein/O-GlcNAc levels are not sufficiently supported. Quantification is needed throughout. Example, Fig 1G and 1H: authors claim "substantial increase" and "significantly decreased" but there is no quantification provided. Similar comment for Fig EV2A, 2F, and 4F.

The quantification analysis regarding original Fig 1G, 1H, and 2F were performed, and the data are added in the revised manuscript shown as the new 1H, 1J, 3I. As for original Fig EV2A (new Fig 2A), we think that it is unusual to perform statistical analysis for a yeast spot assay. In original Fig 4F (new Fig 5F), we were trying to show that flies fed with high sugar diets have extended lifespan than that fed with regular sugar diets is not due to the changes of TDP-43 expression. Actually, we observed that protein levels of TDP-43 in each pair are equivalent. Therefore, the quantification data are unnecessary.

• Input lanes should be included for IPs in Figure 2A, 2B and 3F. Authors might consider combining Fig 2A with Fig 1C.

As the reviewer suggested, Input blots regarding original Fig 2A, 2B and 3F are included in the revised manuscript (new Fig 3A, C and G). Moreover, quantification assays for original Fig 2A and 3F were shown as well (new Fig 3B and 4H).

• It is broadly held that phosphorylated TDP-43 is cytoplasmic. However, in Fig 2C, ethacrynic acid results in pTDP-43 signal that is predominantly nuclear. Can the authors comment?

It is true that phosphorylated TDP-43 was found in cytoplasmic shown in most of papers. However, we also noticed that some studies showed that phosphorylated TDP-43 is localized, at least partially, in nucleus (Chen *et al.*, 2019; Cohen *et al.*, 2015; Nonaka *et al.*, 2009). In our experiment, we repeatedly observed phosphorylated TDP-43 signals are predominantly nuclear by independent biological repeats. To figure out the reason, we decided to treat cells with higher concentration of ethacrynic acid (increased from 20 μM

to 60 μM) and longer time (extended from 12 hrs to 24 hrs). Now we can see that the majority of pTDP-43 signals are shown in cytoplasmic. The new representative images and quantification data are shown in new Fig 2D and 2E.

Chen HJ, Topp SD, Hui HS, Zacco E, Katarya M, McLoughlin C, King A, Smith BN, Troakes C, Pastore A *et al* (2019) RRM adjacent TARDBP mutations disrupt RNA binding and enhance TDP-43 proteinopathy. ***Brain* 142**: 3753-3770

Cohen TJ, Hwang AW, Restrepo CR, Yuan CX, Trojanowski JQ, Lee VM (2015) An acetylation switch controls TDP-43 function and aggregation propensity. ***Nat Commun* 6**: 5845

Nonaka T, Arai T, Buratti E, Baralle FE, Akiyama H, Hasegawa M (2009) Phosphorylated and ubiquitinated TDP-43 pathological inclusions in ALS and FTLD-U are recapitulated in SH-SY5Y cells. ***FEBS Lett* 583**: 394-400

• The O-glcNAc blots in Fig 3E and EV3A are not very convincing. Can better blots be provided?

We thank the reviewer's constructive criticism. A Western blots in new Fig 4E (original Fig 3E) have been added, and the quantification data are also shown as new Fig 4F. Since we have provided sufficient data to support the specificity of O-GlcNAcylation on TDP-43, the original results in Fig EV3A are removed.

• The in vivo experiments (Fig 4) are highly interesting. The levels of the enzymes regulating the O-GlcNAcylation should be evaluated since a change in the level of these could also explain the data. This is especially relevant since the authors also indicate that TDP-43 can bind OGT transcripts. Also, the authors should also include data demonstrated that TDP-43 is O-GlcNAcylated in flies.

As shown in new Fig 5D, both O-GlcNAc levels of TDP-43 and protein levels of OGT were examined using fly heads. As expected, the O-GlcNAc levels of TDP-43 mutants are decreased compared to the WT (new Fig 5D), which is consistent with the results in human cells (new Fig 4G). In addition, we found that OGT levels are not affected by overexpression of either WT or various mutant TDP-43, which is not too surprising considering 1) the exon-7 inclusion form is still the main population of mRNA encoding OGT in cells even overexpressing TDP-43 (new Fig EV5B); 2) the OGT isoform lacking exon 7 may be unstable so that it can not be translated into protein. It will be interesting to explore the possibility in the future.

• In a variety of splicing assays, the authors have employed the ALS-linked mutation Q331K as a control. (Indeed, I found these experiments very well designed and nicely controlled). However, I wonder: is Q331K O-GlcNAcylated?

To examine if Q331K mutant is O-GlcNAcylated, GFP-TDP-43 variants were expressed in HEK 293T cells, and the O-GlcNAc levels of TDP-43 were examined. We found that both WT and Q331K, but not 2TA mutant of TDP-43 showed obvious O-GlcNAcylation signals, which indicates that Q331K mutant-caused ALS symptom probably does not rely on the O-GlcNAc level of TDP-43 (new Fig EV3)..

• Is the protein level of OGT impacted by the TDP-43 mutations? OGA?

We have examined the protein levels of OGT and OGA using both mammalian cells and *Drosophila* organism. In cells, OGT and OGA levels are not affected by TDP-43 mutations (new Fig EV5B). In *Drosophila*, Western blots against OGT shows that OGT levels are not affected (new Fig 5D). However, the commercial antibody against OGA does not recognize the *Drosophila* OGA protein, given that their amino acid sequences are divergent (data not shown).

Referee #4:

Zhao and co-authors report on the effect of O-GlcNAc modification of the RNA binding protein TDP-43 and the role of glycosylation of this protein on its proteotoxicity and regulation of its activities on splicing. Briefly, the authors show that TDP-43 is O-GlcNAc modified, they may the sites of O-GlcNAc modification, show that these sites are important for proteotoxicity in yeast and fly models, and finally show that these sites are also important for the splicing functions played by TDP-43. Overall, this is an extensive body of work using a range of different methods and models. The work builds on a range of previous observations regarding the protective effects of increasing O-GlcNAc in a range of proteotoxicity models including neurodegenerative disease models including the tauopathies.

This is an original body of work that is conceptually appealing. The work is consistent with data on other aggregation prone proteins involved in neurodegenerative diseases including tau and alpha-synuclein. In those cases the sites have been specifically shown to decrease the aggregation propensity of those proteins. OGA inhibitors have advanced into human clinical trials. There is accordingly high potential significance to the work as it may stimulate developing inhibitors for ALS, which is a major unmet medical need. This manuscript reports on preclinical support that could stimulate the pursuit of targeting ALS using this approach. The manuscript could be publishable but there are a number of questions that come up and need to be considered prior to publication.

We really appreciate this reviewer for such positive comments. We really hope that our study can facilitate the preclinical research using this approach to targeting ALS patients. As the reviewer suggested, we have added the following suggested revisions that should significantly strengthen the manuscript.

The general approaches are sound and the data is largely clear when considered in composite. Some exceptions are noted below.

The main point is that I am not certain that the authors have truly shown that O-GlcNAc on TDP-43 and OGT catalytic activity is essential for blocking gain of toxicity and loss of function. In particular the double mutant may not show toxicity because it fails to get phosphorylated at these two positions. In addition these two sites of modification are directly in the RRM2 region and so these mutations may affect RNA binding in an unknown manner by loss of the hydroxyl group. The presence of modifications in a folded domain is also unusual for O-GlcNAc.

We thank the reviewer for constructive criticisms. Currently, there is no any reports or evidence to suggest that T199 and T233 sites can be phosphorylated. What we can prove

here is that these two sites is able to be O-GlcNAcylated by mass spectrometry and by several biochemical assays (Fig EV2 and 4E-H).

It is a concern that the T199A and T233A mutations may affect RNA binding ability, thereby exhibiting less cellular toxicity. To rule out this possibility, RNA-EMSA assays were performed using total cell lysate from GFP-TDP-43 transfected HEK 293T cells incubated with a biotin-labelled RNA probe as described previously (Chen *et al.*, 2019). As shown in the new Fig EV4, various TDP-43 proteins exhibit comparable RNA binding ability when incubated with a biotin-labelled RNA probe, which suggests that these mutations show loss of cellular toxicity is not due to altering RNA binding ability.

In fact, many studies have shown that O-GlcNAc sites could be in a folded domain. Here are a few examples: 1) the chromatin-associated fumarase (FH) can be O-GlcNAcylated at S75 site, which is located in a folded domain (Wang *et al*, 2017); 2) the TATA-box binding protein (TBP) can be O-GlcNAcylated at T114 site, which is required for inhibition of the interaction of TBP and BTAF1 (Hardiville *et al*, 2020); 3) the histone deacetylase 4 (HDAC4) can be O-GlcNAcylated at S642 site, which is present at a HDAC4 domain (Kronlage *et al*, 2019). Together, we think that the presence of O-GlcNAcylation in a folded domain is not rare.

Chen HJ, Topp SD, Hui HS, Zacco E, Katarya M, McLoughlin C, King A, Smith BN, Troakes C, Pastore A *et al* (2019) RRM adjacent TARDBP mutations disrupt RNA binding and enhance TDP-43 proteinopathy. ***Brain*** **142**: 3753-3770

Hardiville S, Banerjee PS, Selen Alpergin ES, Smith DM, Han G, Ma J, Talbot CC, Jr., Hu P, Wolfgang MJ, Hart GW (2020) TATA-Box Binding Protein O-GlcNAcylation at T114 Regulates Formation of the B-TFIID Complex and Is Critical for Metabolic Gene Regulation. ***Mol Cell*** **77**: 1143-1152 e1147

Kronlage M, Dewenter M, Grosso J, Fleming T, Oehl U, Lehmann LH, Falcao-Pires I, Leite-Moreira AF, Volk N, Grone HJ *et al* (2019) O-GlcNAcylation of Histone Deacetylase 4 Protects the Diabetic Heart From Failure. ***Circulation*** **140**: 580-594

Wang T, Yu Q, Li J, Hu B, Zhao Q, Ma C, Huang W, Zhuo L, Fang H, Liao L *et al* (2017) O-GlcNAcylation of fumarase maintains tumour growth under glucose deficiency. ***Nat Cell Biol*** **19**: 833-843

To the point of the catalytically active OGT being needed. The data can in many points be rationalized in terms of OGT having some relevant non-catalytic role. The use of the OGT mutant only extends to Figure 2. Further use of this mutant would help strengthen the paper a great deal. An example is in Figure 2 Panel E where these appears to be a small difference, if any, between the WT and Mut OGT. Some replication would help. But also using the mutant in downstream studies would be more convincing. Also, nowhere in the fly studies is data shown suggesting catalytic activity is needed - recognizing this is difficult some consideration of this and discussion is needed in the manuscript.

As the reviewer suggested, quantification analysis regarding original Fig 2E were performed and the data are shown in new Fig 3G. The reason that we did not include catalytic inactive mutant of OGT in the following experiments is that we have identified several OGT-catalyzed O-GlcNAc sites of TDP-43, which is sufficient to support that

O-GlcNAcylation on TDP-43 is required for regulation of TDP-43 functions. Thus, we only utilized the WT and O-GlcNAc defective mutants of TDP-43 to perform the following experiments.

It is worthy to mention that we attempted to obtain a fly strain with co-expression of TDP-43 and catalytic inactive OGT. However, only the WT UAS-HA-OGT/+, but not the catalytic inactive UAS-HA H498N OGT/+ was available at the Bloomington Stock Center. Thus, we could not get that fly strain at that time.

Since we cannot totally rule out that the non-catalytic role of OGT participates in regulating TDP-43 functions, some comments are present in the second paragraph, Page 9 of the main text.

One issue is also the extent to which TDP-43 is glycosylated in these studies. The general model implies that when OGT is expressed the modification directly antagonizes toxicity. If this is the case one would expect the stoichiometry of modification to be quite high. I would recommend the authors consider and discuss this point and perhaps try to address this if possible.

This is a good suggestion. We have been considered that a quantitative mass spectrometry assay is the best way to determine the stoichiometry of TDP-43 O-GlcNAcylation. However, this is a very tough experiment to be achieved upon current technology. Therefore, we alternatively took advantage of semi-quantitative assay to do this. Basically, we treated cells with 10 μM TMG for different times, an effective inhibitor of OGA (Ki = 21 nM), for different times, and the O-GlcNAcylation levels of global proteins in cells were compared. We noticed that the O-GlcNAcylation levels are not further increased over the time from 12 to 48 hours, suggesting that this modification may reach a stable state at this condition (Fig EV1A). We supposed the O-GlcNAcylation extent of TDP-43 at this situation (treated with 48 hours) as 100%, and the relative O-GlcNAcylation levels of TDP-43 in untreated cells was quantified with the signal intensity in treated cells. We found that around 20% TDP-43 was modified by O-GlcNAcylation (new Figure EV1B and C). Upon UDP-GlcNAc supplement or OGT overexpression, we believe that the extent of O-GlcNAcylation levels of TDP-43 would be significantly increased, which may obviously influence its function. We hope that this experiment could sufficiently answer the reviewer's question.

Another question is the use of ethynacrinic acid. Why this compound - no clear rationale is offered.

It has been reported that ethacrynic acid can induce the phosphorylation level of TDP-43 and insolubility, and C-terminal fragments (Iguchi *et al*, 2012). Therefore, we used this compound to induce TDP-43 proteinopathy. This reference is added in the revised manuscript.

Iguchi Y, Katsuno M, Takagi S, Ishigaki S, Niwa J, Hasegawa M, Tanaka F, Sobue G (2012) Oxidative stress induced by glutathione depletion reproduces pathological modifications of TDP-43 linked to TDP-43 proteinopathies. ***Neurobiol Dis*** **45**: 862-870

One further concern is that for many of the immunoblot experiments only one example is shown and the claims depend on the results. There are several examples like this. The authors should identify observations that are central and provide clear replication and some statistical analysis using true independent replicates.

As the reviewers suggested, we have performed at least three biological replicates (some of these experiments have been biologically repeated before revision) in many immunoblot experiments and the quantification data are shown in the revised manuscript, which includes new Fig 1H, 1J, 3B, 3G, 3I, 4F, 4H, 6I, and 6J.

Page 7. The double mutant should be purified from mammalian cells and assessed for O-GlcNAc levels. This in vitro modification system is less clear.

We speculate that this reviewer thought that the *in vitro* data shown in original Fig 3E is not sufficient to support that 2TA mutant blocks O-GlcNAc modification of TDP-43 *in vivo*. Actually, the result shown in the original Fig 3F was performed in mammalian system and it suggested that O-GlcNAc level of 2TA mutant is significantly reduced compared to that of WT (new Fig 4G). Moreover, the quantification analysis from three biological repeats are shown in new Fig 4H.

There is also a lack of use of various antagonists of OGT or the OGA that could be used to support the observations using different methods. Though not essential for publication the authors could use such methods in some studies to strengthen the work.

As suggested, the inhibitors of OGT or OGA were searched from the literatures, and two OGA inhibitor (PUGNAc and TMG) and one OGT inhibitor (OSMI-1) are selected in this assay. As expected, we observed that inhibition of OGT reduces TDP-43 O-GlcNAc level, whereas inhibition of OGA increases TDP-43 O-GlcNAc levels (new Fig 1C).

Figure 1: Why are there two bands for TDP-43 in Panel B?

Thanks for pointing it out. We initially thought the lower band represents the 35-kDa truncated fragment of TDP-43. However, when we utilized a new Lot of RL2 antibody to perform the same co-immunoprecipitation experiment, we could not see the lower band. We propose that this may be due to different Lot of the RL2 antibody. Another possibility is that the cells used in original Fig. 1B are not healthy, so that the truncated 35-kDa fragment is present. We consistently noticed that cells treated with hydrogen peroxide or arsenite tend to produce the truncated fragment of TDP-43. To avoid misleading the readers, we replaced the original Fig 1B with a new blot without the truncated form by using the recently-ordered RL2 antibody (Abcam, cat: ab2739, Lot: GR3306462-2).

Figure 1: Less important but increase in O-GlcNAc on TDP-43 by glucosamine.

We apologize that we wrote a wrong compound name of “glucosamine”. We actually used acetylglucosamine (GlcNAc) in our experiment. The errors have been changed in the revised manuscript.

Figure 2: Panel A and E. Are these differences significant. They do not seem so to me. Some validation would be needed.

Both original Fig 2A and 2E are biologically repeated and the quantification data are shown in the new Fig 3B and 3G. In addition, “input” blots were supplemented in new Fig 3A as well.

Figure 3: Panel F. The catalytically dead OGT is needed.

We do not quite understand this question. In original Fig 3F (new Fig 4G), we examined O-GlcNAc levels of different TDP-43 constructs, and showed mutating the identified O-GlcNAcylation residues decreased the O-GlcNAc levels of TDP-43.

Figure 4: Panel F. I see no significant differences. Some repeat and statistics are needed. But largely I am not sure if this is useful for the manuscript and not sure it adds anything.

In Fig 4F, we wanted to show that flies fed with high sugar diets have extended lifespan than that fed with regular sugar diets is not due to the expression changes of TDP-43. Indeed, expression levels of TDP-43 in each pair of diets are equivalent. Therefore, we don’t think that quantification data is needed here.

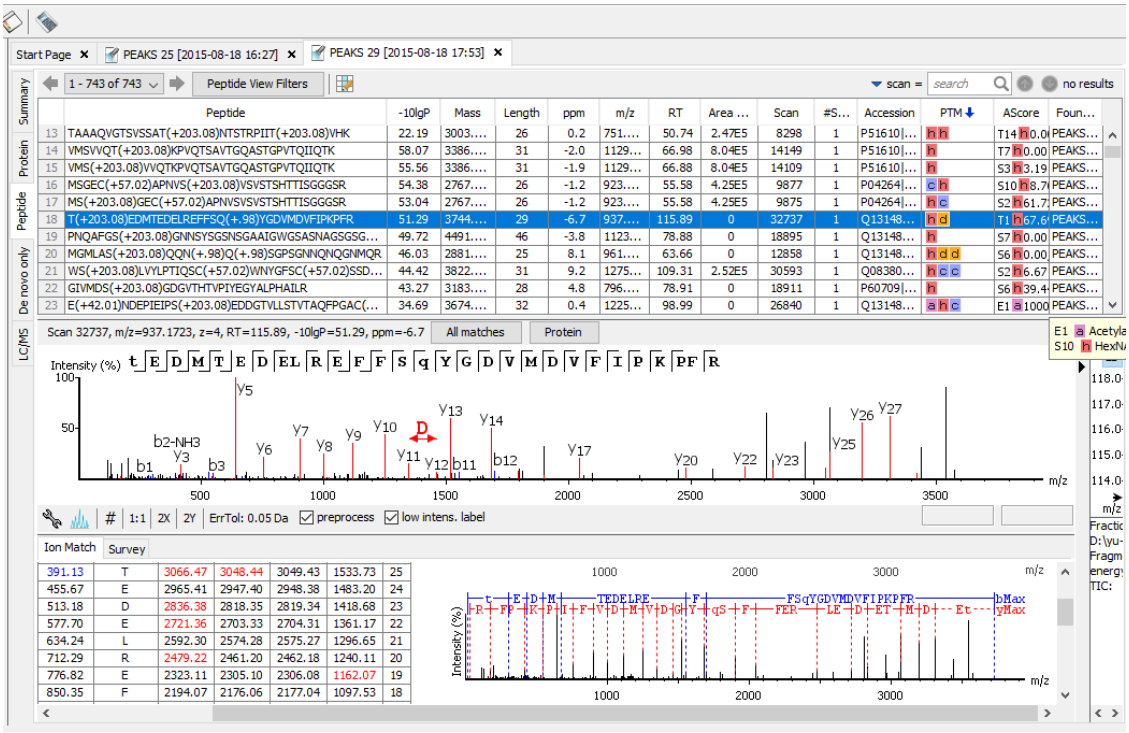
Figure 5: Indication of controls for protein expression levels should be included.

In original Fig 5 (new Fig 6), we have labelled with “WB” in each panel to indicate the protein levels. We hope that we understand this question correctly.

Figure EV3 and text: MS mapping of T199. How was this done. This is not a tryptic peptide. Explanation is needed.

We thank the reviewer for his/her constructive criticism. Actually, the immunoprecipitated TDP-43 protein was subjected to in-gel trypsin digestion, and the digested peptides were injected into Orbitrap Fusion mass spectrometer, and detected with combination of HCD and ETD modes. Based on the mass spectrum patterns, the predicted y-ions and b-ions are identified, and the peptide sequences can be precisely confirmed (rebuttal Figure 3). In addition, the *O*-GlcNAc diagnostic oxonium ions at m/z 204.04 was present (new Fig EV2A, top panel). Therefore, we believe the identified peptide is O-GlcNAC on T199. We have added more details into the *Methods* section.

As for why this tryptic peptide lost the N-terminal Cysteine residue, we don’t know. It is very likely that the commercial trypsin (Promega, V5071) used here nonspecifically cut at the C-terminus of Cysteine from a typical trypsin-digested peptide.



Rebuttal Figure 3. Results show that the peptide of T199 is identified using PEAKS studio software (Bioinformatics Solutions Inc.). PTM: “H” represents O-linked HexNAcylation; “D” represents deamidated; “C” represents carbamidomethylation.

Figure EV1: I do not see significant differences here between normal, fed, and fasted.

The difference between normal, fed and fasted samples is indeed not significant. The data shown here is to explain that TDP-43 in the primordial pallium, one of most abundant expression regions in mice organisms, can be O-GlcNAcylated. The main point is not to compare the difference between these differently treated samples. Since both reviewer 3 and 4 thought the results are less important or lack of rationale, we decided to remove them from the revised manuscript, which does not affect our main conclusion.

The choice of references seems good. Page 4: Arnold 1996 reference to bovine tau. Replace with Lefebvre 2003 that relates to human tau.

The cited reference of Arnold 1996 is the paper that shows O-GlcNAcylation of Tau was identified in human brains, which is suitable to be cited here (Arnold *et al*, 1996). The reference of Lefebvre 2003 is to examine the balance of phosphorylation and O-GlcNAcylation of Tau. We thought this reference is better to cite in the following sentence: *“Increasing O-GlcNAc levels can block phosphorylation of tau and attenuate the formation of tau aggregates, common pathological features of tau-associated neurodegenerative disorders”* (Lefebvre et al, 2003). Both references are cited in the revised manuscript.

Arnold CS, Johnson GV, Cole RN, Dong DL, Lee M, Hart GW (1996) The microtubule-associated protein tau is extensively modified with O-linked N-acetylglucosamine. ***J Biol Chem* 271**: 28741-28744

Lefebvre T, Ferreira S, Dupont-Wallois L, Bussiere T, Dupire MJ, Delacourte A, Michalski JC, Caillet-Boudin ML (2003) Evidence of a balance between phosphorylation and O-GlcNAc glycosylation of Tau proteins--a role in nuclear localization. ***Biochim Biophys Acta*** **1619**: 167-176

The manuscript is fairly well written. But the authors do not discuss the effect sizes in many cases for immunoblots and in many cases these differences are central to the claims of the manuscript. In addition, the scope of the work and the wide range of models and methods used necessitates careful expert review and also makes reading quite difficult.

W have performed at least three biological replicates in many immunoblot experiments and the quantification data are shown in the revised manuscript, which includes Fig 1H, 1J, 3B, 3G, 3I, 4F, 4H, 6I, and 6J. We hope that these quantification data are sufficient to support our main conclusions. As for the models, we tried to focus on the mammalian cells and *Drosophila* system, which are commonly used in studying neurodegenerative diseases. For the yeast model, we thought it is a better system to examine the cellular toxicity and aggregation property of TDP-43 in eukaryotic cells, which has been developed by Dr. Araon Gitler from Stanford University. We believe that these model systems would together consolidate our studies from different aspects.

This is an interesting manuscript on an important topic that represents a major undertaking. Some elements are very convincing including the yeast and fly data. The manuscript therefore has real potential and is appealing as it addresses a potential disease modifying mechanism for ALS. However, there are some technical weaknesses in terms of showing the catalytically active OGT is needed and replication and quantification of key experiments. The authors need to focus on the key elements and provide a clear reply to the concerns. However, the work would certainly be of high interest and I would support a major revision of this manuscript. I believe that the work could be an excellent contribution to the journal and prove to be of wide interest to a range of readers.

We really appreciate the reviewer's very positive comments.

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Dear Dr. Du,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees. They still have a few more minor suggestions that I would like you to address and incorporate before we can proceed with the official acceptance of your manuscript.

A few other editorial changes are also required:

- Hong-Yu Hu and Zhiyuan Luo are missing from the author contributions, please add.

- Please correct the callout for Table EV1.

- Please upload all EV figures as individual figure files. And please upload the EV table as EV table file. The legends for the EV figures need to be removed from the figure files and need to be added to the main manuscript file after the main figure legends.

- Please upload all movies as independent ZIP files with their legends.

- Please fill in the dual research statement in the author checklist, this is missing.

- The source data (SD) for Fig 3E and 3F are labeled incorrectly, please correct. The box around the first row of bands in SD Fig 5H needs to be a little bigger to include the bands that can be seen in the figure panel.

- The "Conclusion" subtitle needs to be removed and replaced with "Discussion", or the text needs to be rearranged as you see fit.

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

- The synopsis image you sent is good. We also need a short (1-2 sentences) summary of your findings and their significance, and 2-3 bullet points highlighting key results for our website. Please send us this information along with the final manuscript.

I would like to suggest a few changes to the title and abstract. Please let me know whether you agree with the following and whether the text faithfully represents the data:

O-GlcNAcylation of TDP-43 suppresses proteinopathies and promotes TDP-43's mRNA splicing activity

Pathological TDP-43 aggregation is characteristic of several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD-TDP), however, how TDP-43 aggregation and function is regulated remains poorly understood. Here, we show that O-GlcNAc transferase OGT-mediated O-GlcNAcylation of TDP-43 suppresses ALS-associated proteinopathies and promotes TDP-43's splicing function. Biochemical and cell-based assays indicate that OGT's catalytic activity suppresses TDP-43 aggregation and hyperphosphorylation, whereas abolishment of TDP-43 O-GlcNAcylation impairs its RNA splicing activity. We further show that TDP-43 mutations in the O-GlcNAcylation sites improve locomotion defects of larvae and

adult flies and extend adult lifespans, following TDP-43 overexpression in Drosophila motorneurons. We finally demonstrate that O-GlcNAcylation of TDP-43 promotes proper splicing of many mRNAs, including STMN2, which is required for normal axonal outgrowth and regeneration. Our findings suggest that O-GlcNAcylation might be a target for the treatment of TDP-43-linked pathogenesis.

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

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #2:

The authors have carefully revised the manuscript and addressed most comments made by this reviewer. In general, they should be commended for having done a good job and it is understandable that it may not have been possible to address all possible issues in the first work on this issue.

Referee #3:

General comment:
The additional experiments and controls (notably the IP input lanes, Fig 1C-D and EV5) have considerably improved this manuscript. The only remaining question is whether the OGT enzyme has other functions unrelated to O-GlcNAc, as suggested by the decreased locomotor abilities of adult flies overexpressing OGT. However, the authors have acknowledged this possibility and have used appropriate mutants to link the observations to its known catalytic activity. Overall, the authors have adequately addressed all of the comments.

Specific comments:
Page 5: "Of note, overexpression of OGT in the absence of TDP-43 would not affect cell toxicity (Fig 2A, left panel, row 2 vs 1)". This sentence does not correspond with the figure since there is no condition in which OGT is overexpressed in the absence of TDP-43. Can the authors please clarify?
Figure 2B and 2D: Please indicate whether it is WT or Q331K TDP-43 that is expressed here.
Figure 3A: The input and IP do not correspond. The increase of O-GlcNAc should be seen with addition of the GlcNAc. (looks like the blot is flipped)
Figure 3G: The authors might want to reconsider the statistical analyses presented in this figure to address whether there is a difference between lanes 2 and 3, as well as lanes 5 and 6. (The effectiveness of the H498N mutation does not look as strong in the presence of EA).
Page 10, inserted paragraph: Please replace CFCR by CFTR. (5 times)
The authors state "Based on previous CLIP-Seq dataset, we found that that TDP-43 directly binds the intron region between exon 6 and exon 7 of OGT transcript (Fig EV5A)." As there are other TDP-43 binding peaks (one of which is higher than the one between exons 6 and 7), it is unclear why the authors have focused in on this one. Can the authors expand on their rationale?

Referee #4:

I have carefully reviewed the revised manuscript provided by the authors as well as their set of replies. I believe that the authors have made good efforts, including a series of additional experiments, to improve the manuscript. While not perfect, many of the main issues have been fairly addressed in this revised manuscript. The findings support the core hypothesis. I find the manuscript is a significant body of work that, while having some limitations as do all studies, strongly advances a single key message that meets the specified criteria for publication of broad interest, importance to a specific field, strong evidence, and novelty. I believe the manuscript can be accepted with some minor revisions to the text that can be managed by the editorial office.

Minor text revisions:
1) The authors have made a fair effort to determine the levels of O-GlcNAc modification. But this is not an accurate method to determine the extent of modification of TDP-43. The steady state level of O-GlcNAc on this protein may be well below the level observed with inhibitor treatment. The authors assume stoichiometry is 100% with inhibitor treatment but this is most unlikely. The level will depend on many factors including the rate of biosynthesis and degradation and removal by OGA. An inhibitor will not complete block removal but only affects the rate at which it is removed by the enzyme by changing the apparent affinity of the substrate for the enzyme. Based on this data I think it is important for the authors to fairly note that alternative indirect mechanisms may be operative that protect cells against TDP-43 toxicity.
2) Figure 3A. O-GlcNAc blot panel reversed? Please examine and revise if needed.
3) Arnold in JBC discusses only Bovine Tau. Please correct.
4) The limitations and caveats of the experiments to the conclusions need to also be fairly and clearly noted in the conclusions section. All studies have limitations. No conclusions from one study are entirely reliable. Replication of the literature is essential. Therefore, such discussion regarding limitations does not detract from the work but does fairly direct readers to consider alternatives and potential misinterpretations that may only later become apparent.
5) Language editing is needed.

I convey my congratulations to the authors, and in particular to the more junior members of the team for completion of a major body of research. I expect the work will stimulate new thinking and pursuit of new research within the community.

Editor:

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The author contributions have been added.

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We have changed Table EV1 as Supplementary Table S1.

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All these have been done appropriately as suggested.

- Please upload all movies as independent ZIP files with their legends.
Done as suggested.

- Please fill in the dual research statement in the author checklist, this is missing.
This has been added.

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They have been corrected.

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Thanks. It has been replaced.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript.
Thanks for these editing. We have addressed all comments appropriately.

- The synopsis image you sent is good. We also need a short (1-2 sentences) summary of your findings and their significance, and 2-3 bullet points highlighting key results for our website. Please send us this information along with the final manuscript.
Thanks. We have added these information with the final manuscript on the cover page.

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The title and abstract have been edited appropriately.

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

Best regards,
Esther

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Senior Editor
EMBO reports

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The authors have carefully revised the manuscript and addressed most comments made by this reviewer. In general, they should be commended for having done a good job and it is understandable that it may not have been possible to address all possible issues in the first work on this issue.
We are grateful to the reviewer’s positive comments and understanding.

Referee #3:

General comment:
The additional experiments and controls (notably the IP input lanes, Fig 1C-D and EV5)

have considerably improved this manuscript. The only remaining question is whether the OGT enzyme has other functions unrelated to O-GlcNAc, as suggested by the decreased locomotor abilities of adult flies overexpressing OGT. However, the authors have acknowledged this possibility and have used appropriate mutants to link the observations to its known catalytic activity. Overall, the authors have adequately addressed all of the comments.

We appreciate this reviewer for positive comments.

Specific comments:

Page 5: "Of note, overexpression of OGT in the absence of TDP-43 would not affect cell toxicity (Fig 2A, left panel, row 2 vs 1)". This sentence does not correspond with the figure since there is no condition in which OGT is overexpressed in the absence of TDP-43. Can the authors please clarify?

We are sorry we made a mistake. It should be “row 3 vs 1”. In Fig 2A, the TDP-43’s expression was induced by galactose. In the left panel, the yeast was cultured on medium only with glucose, which didn’t induce TDP-43 expression. So TDP-43 in the left panel was absent, and OGT is constitutively overexpressed without TDP-43 in the row 3. We have clarified this information in the main text.

Figure 2B and 2D: Please indicate whether it is WT or Q331K TDP-43 that is expressed here.

Thanks. It is WT TDP-43 and is indicated in the figures.

Figure 3A: The input and IP do not correspond. The increase of O-GlcNAc should be seen with addition of the GlcNAc. (looks like the blot is flipped)

We apologized this error. The blot of input has been flipped.

Figure 3G: The authors might want to reconsider the statistical analyses presented in this figure to address whether there is a difference between lanes 2 and 3, as well as lanes 5 and 6. (The effectiveness of the H498N mutation does not look as strong in the presence of EA).

As the reviewer suggested, we added statistical analyses between lanes 2 and 3, lanes 5 and 6. As shown in Fig 3G, the difference between expression of WT OGT and catalytic inactive OGT is significant without adding EA ($P < 0.01$), but no statistical significance when treated with EA ($P = 0.08$). The results suggest that OGT may only play a minor role in suppression of TDP-43 aggregation under stress conditions. We have added this comment in the main text.

Page 10, inserted paragraph: Please replace CFCR by CFTR. (5 times)

Thanks. They have been corrected.

The authors state "Based on previous CLIP-Seq dataset, we found that that TDP-43 directly binds the intron region between exon 6 and exon 7 of OGT transcript (Fig EV5A)." As there are other TDP-43 binding peaks (one of which is higher than the one between

exons 6 and 7), it is unclear why the authors have focused in on this one. Can the authors expand on their rationale?

This is a good question. Actually, there are two stronger binding peaks shown at the intron regions between exon 3 and 4, as well as exon 6 and 7 (Fig. EV5A). However, only silenced exon 7 was confirmed according to a previous study (Tollervey *et al*, 2011). Therefore, we only pointed out the peaks between exon 6 and 7. To avoid such misleading, we have revised our sentence as "*Based on previous CLIP-Seq dataset, we found that that TDP-43 displayed a strong binding at the intron regions between exon 3 and 4 or exon 6 and 7 of OGT transcript and but was only proven to silence exon 7 expression*" in the main text.

Tollervey JR, Curk T, Rogelj B, Briese M, Cereda M, Kayikci M, Konig J, Hortobagyi T, Nishimura AL, Zupunski V *et al* (2011) Characterizing the RNA targets and position-dependent splicing regulation by TDP-43. ***Nat Neurosci* 14**: 452-458

Referee #4:

I have carefully reviewed the revised manuscript provided by the authors as well as their set of replies. I believe that the authors have made good efforts, including a series of additional experiments, to improve the manuscript. While not perfect, many of the main issues have been fairly addressed in this revised manuscript. The findings support the core hypothesis. I find the manuscript is a significant body of work that, while having some limitations as do all studies, strongly advances a single key message that meets the specified criteria for publication of broad interest, importance to a specific field, strong evidence, and novelty. I believe the manuscript can be accepted with some minor revisions to the text that can be managed by the editorial office.

We thank the reviewer's positive comments.

Minor text revisions:

1).The authors have made a fair effort to determine the levels of O-GlcNAc modification. But this is not an accurate method to determine the extent of modification of TDP-43. The steady state level of O-GlcNAc on this protein may be well below the level observed with inhibitor treatment. The authors assume stoichiometry is 100% with inhibitor treatment but this is most unlikely. The level will depend on many factors including the rate of biosynthesis and degradation and removal by OGA. An inhibitor will not complete block removal but only affects the rate at which it is removed by the enzyme by changing the apparent affinity of the substrate for the enzyme. Based on this data I think it is important for the authors to fairly note that alternative indirect mechanisms may be operative that protect cells against TDP-43 toxicity.

We totally agree with that the steady state level of O-GlcNAc on the protein may be much below the level observed with inhibitor treatment. Nevertheless, we certainly can draw a conclusion that TDP-43 is O-GlcNAcylated by OGT in vivo. As suggested by the reviewer, we pointed out alternative indirect mechanisms may be operative that protect cells against TDP-43 toxicity (page 6).

2) Figure 3A. O-GlcNAc blot panel reversed? Please examine and revise if needed.
Apologize for this error. We have flipped the blot over.

3) Arnold in JBC discusses only Bovine Tau. Please correct.
Thanks. We have corrected it.

4) The limitations and caveats of the experiments to the conclusions need to also be fairly and clearly noted in the conclusions section. All studies have limitations. No conclusions from one study are entirely reliable. Replication of the literature is essential. Therefore, such discussion regarding limitations does not detract from the work but does fairly direct readers to consider alternatives and potential misinterpretations that may only later become apparent.
We are grateful for reviewer's constructive suggestion and the limitations and caveats have been added in the discussion part (page 13-14).

5) Language editing is needed.
We thank for reviewer's suggestion. This manuscript was proofread by Professor Lin Guo, a colleague with 30+ years' experience in the US.

I convey my congratulations to the authors, and in particular to the more junior members of the team for completion of a major body of research. I expect the work will stimulate new thinking and pursuit of new research within the community.
We are really grateful for this reviewer's encouragement. We believe that O-GlcNAcylation of TDP-43 and other proteins would be extensively investigated by the community, which may shed lights on the clinical interference of ALS disease, as well as neurodegenerative diseases.

2nd Revision - Editorial Decision 9th Mar 2021

Dr. Hai-Ning Du
Wuhan University
College of Life Sciences
299 Bayi Road
Wuhan 430072
China

Dear Dr. Du,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Best regards,
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Esther Schnapp, PhD
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Corresponding Author Name: Jiwu Wang, Hai-Ning Du
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Manuscript Number: EMBOR-2020-51649V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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?	At least three biological replicates were performed in all of these experiments. For yeast cell imaging, >50 cells for each biological experiments were counted. For fly experiments, the sample sizes are labelled in the legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	None. The criteria were pre-established.
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.	All the samples were chosen randomly.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes, to the best of our knowledge.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Methods section "Data analysis" unpaired student's t-test was used for comparision of two groups. In Fig. 5E, Kaplan-Meier survival analysis was used.

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http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	Yes, standard deviation (SD) is shown as indicated.
Is the variance similar between the groups that are being statistically compared?	Yes.

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).	All the antibodies are listed in methods sections.
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 source of cell lines are listed appropriately and tested 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.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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 also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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 c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
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).	NA
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).	NA
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 in a public repository or included in supplementary information.	NA

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.	NA