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Loss of TMEM106B potentiates lysosomal and FTD-like pathology in progranulin deficient mice

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1st Editorial Decision 1st Apr 2020

Dear Christian,

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 and novel, and all support the publication of your study. Referee 1 raises a number of points, however, upon cross-commenting, all referees agree that it will be sufficient to address these points in the manuscript text.

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 to the best of your abilities. 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.

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

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

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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.

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

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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>.

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

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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 #1:

Frontotemporal Dementia (FTD) is a common form of neurodegenerative disease in individuals under the age of 60. About 15% of familia FTD cases are cause by dominant mutations in the Progranulin (GRN) gene, which lead to haploinsufficiency in a protein thought to be involved in the endolysosomal pathway. Recent studies have also shown that GRN mutation carriers with SNPs in TMEM106b, a key regulator of lysosome function, have an increased risk for FTD, suggesting that TMEM106b may functionally interact with progranulin to regulate disease onset. Interestingly, two recent studies showed that loss of TMEM106B ameliorates lysosomal phenotypes in Grn-/- neurons, and overexpression of TMEM106B in neurons exacerbates lysosomal defects in Grn-/- neurons during aging. Together, these results support that reducing TMEM106B can be protective of neurodegeneration in PGRN deficiency.
In this manuscript, Werner et al. generated Grn-/-;Tmem106b-/- double knock out mice and showed that loss of progranulin and TMEM106b led to a more severe phenotype than Grn-/- and Tmem106b-/- single mutants with earlier onset of motor impairment, astrogliosis, and microgliosis. Transcriptomic analyses showed that these phenotypes are likely caused by dysregulation of genes involving abnormal microglial activation and impaired autophagy. In addition, the authors

provided evidence that loss of progranulin and TMEM106b also resulted in more severe defects in proteostasis, with abnormal increases in lysosomal enzymes, autophagy protein p62, protein ubiquitination, and aggregation of insoluble TDP-43.
Overall, the results in this manuscript are quite intriguiing and, if supported by additional evidence, may have major impacts in clarifying the contribution of TMEM106b in the pathogenesis of FTD caused by GRN mutations. That said, there are several areas in this study that need to be addressed to improve clarity. The specific comments and suggestions are detailed below.
Major Points:
1. The transcriptomic analysis using NanoString Neuropathology nCounter provides important information. However, there are several major issues with the results and their interpretations. First, although the volcano plots provide cutoff for p value (0.05), the authors do not indicate what is the cutoff for fold change. Reading from the supplementary table for Tmem106b-/- data, many DEGs have log2 fold change of +/-0.3-0.4. These would have been considered borderline or flat out insignificant in other more stringent criteria. While the authors never provide a similar supplementary table for Grn-/- mouse brain, the same is true for this mutant. This would be consistent with previously published results using more genome-wide transcriptomic analysis in Grn-/- mice (Lui et al., Cell 2016). Second, based on the number of differentially expressed genes, it is obvious that Grn-/-;Tmem106b-/- brain showed much more transcriptomic changes (173 DEGs). Judging from the Venn diagram, however, only 11 genes are shared among the 3 mutant mouse brain. What are these genes? Will these 11 genes remain using more stringent fold-change criteria? What Gene Ontology or functional group(s) they belong to? Are they consistently up- or down-regulated in all 3 mutant brain? Third, as the authors indicated, NanoString Neuropathology panel only provides very limited genes related to neuroinflammation. In fact, NanoString also provides the mouse Neuroinflammation panel using the same nCounter platform. Since the results in Grn-/-;Tmem106b-/- mutants show severe glial pathology, repeating the analysis using the Neuroinflammation panel makes more sense and should provide more mechanistic insights regarding the glial pathology that is most relevant to this study. Finally, for all transcriptomic work validation is the key. To convince the readers, it will be critical for the authors to validate several key genes that are top of their "gene set" list in Figure 2E.
2. As a follow-up to Point #1, the western blot results for TREM2 in Figure 3A are rquite problematic. First, these results are very different from data published by the same lab (e.g Gotzl et al., EMBO Mol Med 2019) in that there are too many bands and all look very faint. Despite this problem, quantification show statistically significant increase in single mutants and double mutants. It will be important for the authors to repeat these experiments and show consistency with their own published data.
3. The results in Figure 2C-F show images supporting increases in GFAP+ astrocytes and IBA1+ microglia. First of all, these results were never quantified to support regional specificity of the glial pathology. Based on these images, one is under the impression that Grn-/-;Tmem106b-/- mouse brain is loaded with reactive microglia and astrocytes everywhere. More importantly, these results are in direct contrast to those in the co-submitted results from two other group, both showed more prominent glial pathology in the spinal cord, but less in cerebellum or cortex. Given these conflicting results, it will be important to perform a general survey of glial pathology using IHC DAB staining for GFAP and IBA1, and provide a table or graphic representation of the microglia or astocyte density.
4. The results in Figure 3F show prominent increase in lipofuscin which colocalizes with IBA1 and CD68. In addition, Figure 4 shows biochemical evidence supporting defects in autophagy (marked increase in p62) and lysosomal enzymes, cathepsins B and D. While these results are interesting, it is unclear which cell type(s) contain the most abundant p62 or cathepsins?
5. In Figure 4B, the authors stated that Grn-/-;Tmem106b-/- mice "show robustly elevated p62/SQSTM1 levels in RIPA and urea lysates compared to single knockout and WT mice," however, the post hoc analysis shows that there is no statistical difference between the double knock out

mice and the single knock out mice. The only difference is between the WT mice and each of the three knock out lines.
6. The second top gene set in Figure 2 is "autophagy". However, the authors never provided information regarding which genes are dysregulated in this category. It will be important to provide additional experiments beyond western blots for p62 and CatD to better support the authors' central claims of impaired autophagy. For example, what do the levels of LC3-II, ATG12, ATG7, etc, look like across mouse lines?
7. The results in Figure 5 are quite confusing. First, it is unclear which cell type(s) have the TDP-43 protein aggregates in 5A and 5B. In fact, unlike the TDP-43 staining in wild type, Grn-/- and Tmem106b-/- cells, many cells in Grn-/-;Tmem106b-/- do not have or show significant reduction in TDP-43. Essentially all TDP-43 aggregates, including those highlighted in boxed area, are not associated with any DAPI staining. Why? These results are very different from those seen in FTLD-GRN cases. How do one interpret these results. The authors claimed that Grn-/-;Tmem106b-/- mice show reduced holoprotein TDP-43 in the RIPA fraction, but their graph does not indicate any statistical significance. Additionally, the authors should indicate whether the increase in pTDP-43 in the Tmem106b-/- mice is significantly elevated compared to WT or Grn-/- mice. Further, what might this increase mean in the context of their findings? Similarly, are we to assume that the apparent increase in the C-terminal fragment of TDP-43 in the Grn-/- mice is not significantly elevated compared to WT? Finally, the western blots for TDP-43 CTF is quite problematic. For one, the size of TDP-43 CTF is close to 36-38 KDa. This is very different from those reported in FTLD-TDP cases. In this blot, the full length TDP-43 appears to be equal for all four genotypes. How do one interpret this results compared to those from NP40 extraction.
8. Throughout the manuscript, the authors used the word "massive" or "massively" to describe motor deficits, transcriptomic changes, astrogliosis, lysosomal defects, TDP-43 proteinopathy, etc. In the Abstract alone, the word "massive" was used twice in back-to-back sentences. This is quite unusual for scientif literature. Is this really necessary?
9. In Discussion, the authors provided several scenarios on how TMEM106b might regulate lysosomal function and thereby affects the role of progranulin in lysosomes. None appears to be convincing. A major problem is rooted in the lack of effective assays to characterize the function of TMEM106b. The fact that different Tmem106b mutant lines appear to show different phenotypes make it more difficult to understand the discrepancy from different groups.

Minor Concerns:
• Fig. 4F: The authors claim that the increase in CatD and CatB expression is "correlated" with increased activity levels. They do not make a case for correlation here; they instead demonstrate that the increased expression coincides with increased activity (at least with CatD). For CatB, the authors should comment on the discrepancy between the supposed increase in CatBp in the double knock out compared to WT mice and the lack of functional increase compared to WT. The only increase in CatB activity is compared to the Tmem106b-/-, where we did not see any significant reduction in expression relative to the double knock out.
• Fig. 5A/Sup.1: The authors should include more images with increased magnification, as it is difficult to see even the arrows that indicate the cytoplasmic TDP-43 inclusions. Additionally, these magnified images should be included for every mouse line in order to prevent any biasing across the lines. The authors could also quantify the number of inclusions found across mouse lines, which could be more helpful than the relative metric of quantified western blot expression.

Referee #2:

This is a review of the manuscript by Werner et al entitled "Loss of TMEM106b Potentiates Lysosomal and FTD-like pathology in progranulin deficient mice." It has been firmly established that polymorphisms in TMEM106B dramatically alter the risk of dementia in humans with concurrent GRN mutations. Both genes encode lysosomal proteins, suggesting the possibility of pathogenic convergence. However, direct, compelling evidence of such convergence from cellular or mouse studies has been lacking.

Here, the authors provide compelling evidence that this is indeed the case through a series of experiments in mice lacking GRN, TMEM106B, or both genes. They conclusively show that dual knockdown of GRN and TMEM106B dramatically worsens pathologic and behavioral phenotypes as well as inflammatory signatures in mice compared to single knockout mice.

These findings are incredibly important for the field, providing the first direct evidence that it is likely that loss of TMEM106B that potentiates GRN insufficiency. These data will open up new lines of research to investigate how such loss mechanistically worsens GRN-related lysosomal dysfunction. I recommend that the manuscript be accepted after minor revisions. I have recommended a number of ways to further improve the manuscript below, which I believe could be accomplished using data that is likely on hand, or simply requires textual revision.

Major comments
• One of the longstanding critiques in our field is that only GRN KO mice have significant phenotypes (e.g. GRN het mice are very similar, if not identical, to WT mice). Though not absolutely necessary for publication here, it would be very helpful to the field to include any phenotypic data generated from GRN het/TMEM106B KO or GRN het/TMEM106B het mice. Does full or partial TMEM106B loss "bring out" any relevant pathologic, behavioral, or transcriptional phenotypes of GRN het mice?
• Figure 1C: It appears that nuclear loss of TDP-43 may also be occurring in the double KO mice. Can TDP-43 nuclear levels be quantified from existing images?

Minor comments
• Change colors of Figure 1 (bright blue and green are visually unappealing, and white text on light blue is of insufficient contrast.
• Use of header "Impaired autophagy in Grn -/-/Tmem106b -/- mice" is not supported by the accompanying data. The problem could lie in a number of different points within the autophagosome/lysosome axis, and without detailed cellular studies the precise molecular impairment cannot be assigned to "impaired autophagy". For example, there could be increased autophagy present (e.g. high p62), but a failure of autophagosome/lysosome fusion, or a failure of lyososome degradation of engulfed autolysosome material. I would recommend using more conservative and precise language in the conclusions here, and perhaps adding potential mechanism and future directions regarding where the precise blockade in function is to the discussion section.

• Introduction and Abstract: identify need and relevance
- Their conclusion that the defect is in protein turnover specifically due to lysosomal dysfunction, as opposed to more general lysosome function (eg lipid metabolism), is not necessarily supported by the data. Note that lysosomes do more than just degrade things (signaling, transport).
- ALS is not a major clinical manifestation of GRN mutations, in contrast to the suggestion within

the introduction.
- figure 3A did not scan properly
- figure 5C did not scan properly

• Discussion:

- The authors note very striking changes in microglial gene expression. It would be nice if they could comment in the discussion section regarding potential autonomous vs non cell autonomous mechanisms of microglial dysregulation in the setting of dual GRN/TMEM106B loss. Is all of this due to autonomous microglial dysfunction, reaction to sick neurons (e.g. due to the transport impairments), or both? Are there potential cell autonomous mechanisms linking lysosome dysfunction with microglia dysfunction?

Referee #3:

The current submission by Georg Werner et al., explores the potential role of TMEM106B progranulin induced Frontotemporal lobar degeneration (FTLD-GRN) using progranulin deficient mice models.
The authors generated a new TMEM106B-/-; GRN-/- double knock-out mice model and using single, double knock-out mice models show that loss-of-function of both TMEM106B, GRN leads to motor deficits, dysregulated expression of microglial, autophagy genes together with impaired autophagy and accelerated TDP-43 aggregation. They therefore conclude that the TMEM106B risk alleles may modulate FTLD-GRN via a loss-of-function mechanism. The results are interesting and are an important contribution to the field of FTD caused by GRN mutations.
Comments:
- Was the paralysis accompanied by loss of motor neurons in the ventral horn of the spinal cord?
- The significant hits from the differential gene expression data, as presented in figure 2 should also be included in more detail as supplementary information.
- TMEM106B plays a crucial role is regulating lysosomal size, morphology and their acidification (pH). Given this fact, experiments and data relating to such measurements would be interesting to add.
- The accumulation of ubiquinated proteins and pTDP-43 is remarkable, as it is the hallmark of FTLD/ALS, which was missing in the GRN -/- mice. This aspect could be discussed more, especially the relation between lysosomal dysfunction, TDP-43 accumulation and PGRN functioning.

Cross-comments from referee 2:

The majority of comments from reviewer 1 can be addressed in textual revisions or rebuttals. Regarding the transcriptomic changes and setting of cutoff values, I do agree with their questions related to why the log fold change settings were relatively modest. One possibility would be for the authors to use noise modeling to empirically set a LFC threshold, rather than an arbitrary cutoff.

Point by point response to the comments of reviewer 1:

1. The transcriptomic analysis using NanoString Neuropathology nCounter provides important information. However, there are several major issues with the results and their interpretations. First, although the volcano plots provide cutoff for p value (0.05), the authors do not indicate what is the cutoff for fold change. Reading from the supplementary table for Tmem106b-/- data, many DEGs have log2 fold change of +/-0.3-0.4. These would have been considered borderline or flat out insignificant in other more stringent criteria. While the authors never provide a similar supplementary table for Grn-/- mouse brain, the same is true for this mutant. This would be consistent with previously published results using more genome-wide transcriptomic analysis in Grn-/- mice (Lui et al., Cell 2016). Second, based on the number of differentially expressed genes, it is obvious that Grn-/-;Tmem106b-/- brain showed much more transcriptomic changes (173 DEGs). Judging from the Venn diagram, however, only 11 genes are shared among the 3 mutant mouse brain. What are these genes? Will these 11 genes remain using more stringent fold-change criteria? What Gene Ontology or functional group(s) they belong to? Are they consistently up- or down-regulated in all 3 mutant brain? Third, as the authors indicated, NanoString Neuropathology panel only provides very limited genes related to neuroinflammation. In fact, NanoString also provides the mouse Neuroinflammation panel using the same nCounter platform. Since the results in Grn-/-;Tmem106b-/- mutants show severe glial pathology, repeating the analysis using the Neuroinflammation panel makes more sense and should provide more mechanistic insights regarding the glial pathology that is most relevant to this study. Finally, for all transcriptomic work validation is the key. To convince the readers, it will be critical for the authors to validate several key genes that are top of their "gene set" list in Figure 2E.

We carefully reanalyzed our transcriptomic data from the NanoString nCounter Neuropathology panel. We provide full data transparency, for all genotypes, we uploaded raw and processed data in Gene Expression Omnibus (GEO) database and we provide excel tables for all data shown in the manuscript. In addition, we set a cutoff for the fold change by 20% up- or down-regulation and the number of significantly changed genes are adjusted, accordingly.
We totally agree with the reviewer that the Venn diagram showing the overlap between the genotypes did not provide much information. Instead, we now show the overlapping genes (significant for all indicated genotypes and at least for one genotype a 20% change compared to WT) in a bar graphs, indicating the log2 fold-change for all applicable genotypes, including standard error and the gene names (new Fig 2D).
Our data are indeed consistent with Lui et al., Cell 2016 and effects on C1qa, C1qb, C1qc, Cd68, and Trem2 are accelerated in the double knockout mice. We discuss this finding and refer to Lui et al., Cell 2016.
As the reviewer suggested, we now also used the NanoString nCounter mouse Neuroinflammation panel to further verify the glial pathology (new Fig 3 A-E). For this analysis, we used an additional mouse cohort and increased the number of mice to 4-5. The findings achieved with the Neuropathology panel were confirmed and additional information about immune response, inflammation and astrocytes was obtained. We agree with the reviewer that validation is the key step in gene expression analysis. We confirmed the dysregulation of key genes at protein level in most affected pathways, like microgliosis, astrogliosis, autophagy, lysosomal enzymes, and myelination/oligodendrocytes (Fig 4A (GFAP, IBA1), Fig 5A (CD68, TREM2, ApoE), Fig 6A (cathepsins), Fig 7 B (P62, ubiquitin, LC3), Fig EV2 (MOG, MAG)).

2. As a follow-up to Point #1, the western blot results for TREM2 in Figure 3A are rquite problematic. First, these results are very different from data published by the same lab (e.g Gotzl et al., EMBO Mol Med 2019) in that there are too many bands and all look very faint. Despite this problem, quantification show statistically significant increase in single mutants and double mutants. It will be important for the authors to repeat these experiments and show consistency with their own published data.

The GRN KO mice investigated by Götzl et al. were much older (9 months) and thus showed increased expression of DAM associated proteins, whereas in the current study we investigated these mice at an age of 4.5 months month, where microglial activation is by far not as strong. Thus, these data are in line with our previous findings. This has now been pointed out in the text. Furthermore, in Götzl et al. we acutely isolated microglia, which allows a much more sensitive detection of proteins expressed exclusively in microglia. Unfortunately, due to the pandemics we did not have enough mice available for microglia isolation.

3. The results in Figure 2C-F show images supporting increases in GFAP+ astrocytes and IBA1+ microglia. First of all, these results were never quantified to support regional specificity of the glial pathology. Based on these images, one is under the impression that Grn-/-;Tmem106b-/- mouse brain is loaded with reactive microglia and astrocytes everywhere. More importantly, these results are in direct contrast to those in the co-submitted results from two other group, both showed more prominent glial pathology in the spinal cord, but less in cerebellum or cortex. Given these conflicting results, it will be important to perform a general survey of glial pathology using IHC DAB staining for GFAP and IBA1, and provide a table or graphic representation of the microglia or astocyte density.

We divided former Fig 3 into Fig 4 and Fig 5, and added *in vivo* TSPO PET scans to provide detailed information of brain regions with high microglial activity (new Fig 4E). Additionally, we added an extended view figures (Figure EV3 and EV4) to provide information about the micro- and astrogliosis throughout the entire brain as well as the spinal cord. In line with the co-submitted manuscripts, strong micro- and astrogliosis occurs in the spinal cord whereas gliosis in cortex is milder.

4. The results in Figure 3F show prominent increase in lipofuscin which colocalizes with IBA1 and CD68. In addition, Figure 4 shows biochemical evidence supporting defects in autophagy (marked increase in p62) and lysosomal enzymes, cathepsins B and D. While these results are interesting, it is unclear which cell type(s) contain the most abundant p62 or cathepsins?

We focused on the cell type distribution of p62 aggregates and found mostly neuronal and few microglia depositions (new Fig 7E).

5. In Figure 4B, the authors stated that Grn-/-;Tmem106b-/- mice "show robustly elevated p62/SQSTM1 levels in RIPA and urea lysates compared to single knockout and WT mice," however, the post hoc analysis shows that there is no statistical difference between the double knock out mice and the single knock out mice. The only difference is between the WT mice and each of the three knock out lines.
The former Fig 4B (now new Fig 7C) shows a significant increase of p62 in the double knockout compared to WT and single knockout mice in the RIPA and urea fraction. We kindly ask the reviewer to re-check Fig. 7B and C.

6. The second top gene set in Figure 2 is "autophagy". However, the authors never provided information regarding which genes are dysregulated in this category. It will be important to provide additional experiments beyond western blots for p62 and CatD to better support the authors' central claims of impaired autophagy. For example, what do the levels of LC3-II, ATG12, ATG7, etc, look like across mouse lines?

We now provide complete transparency about the NanoSting evaluation and GO pathways associated to genes. The elevation of the autophagy pathway mainly refers to the upregulation of lysosomal proteins and sugar cleaving enzymes (Gusb, Hexb, Naglu, Man2b1, Galc). Genes associated with initiation of autophagy are either not present in the panel or not upregulated. We also added LC3 to the biochemical analysis (new Fig 7B and C) and found a significant increase of LC3II is detected in double knockout mice.

7. The results in Figure 5 are quite confusing. First, it is unclear which cell type(s) have the TDP-43 protein aggregates in 5A and 5B. In fact, unlike the TDP-43 staining in wild type, Grn-/- and Tmem106b-/- cells, many cells in Grn-/-;Tmem106b-/- do not have or show significant reduction in TDP-43.
Essentially all TDP-43 aggregates, including those highlighted in boxed area, are not associated with any DAPI staining. Why? These results are very different from those seen in FTLD-GRN cases. How do one interpret these results.
The authors claimed that Grn-/-;Tmem106b-/- mice show reduced holoprotein TDP-43 in the RIPA fraction, but their graph does not indicate any statistical significance.
Additionally, the authors should indicate whether the increase in pTDP-43 in the Tmem106b-/- mice is significantly elevated compared to WT or Grn-/- mice.
Further, what might this increase mean in the context of their findings?
Similarly, are we to assume that the apparent increase in the C-terminal fragment of TDP-43 in the Grn-/- mice is not significantly elevated compared to WT?
Finally, the western blots for TDP-43 CTF is quite problematic. For one, the size of TDP-43 CTF is close to 36-38 KDa. This is very different from those reported in FTLD-TDP cases. In this blot, the full length TDP-43 appears to be equal for all four genotypes. How do one interpret this results compared to those from NP40 extraction.

We want to point out that some reduction of nuclear TDP-43 is seen in Grn-/-&Tmem106b-/- mice and most aggregates are therefore cytoplasmic and not nuclear (new Fig 8 EV6). This is similar to GRN-associated FTLD patients who also show neocortical neuronal and microglial cytoplasmic inclusions with a granular appearance in addition to a substantial number of lenticular intranuclear inclusions in several brain areas (Richard A. Armstrong and Nigel J. Cairns Histol Histopathol 2011; John C van Swieten and Peter Heutink Lancet Neurol 2008; Ian R. A. Mackenzie Acta Neuropathol 2007).
The reviewer is correct, we do not see reduced levels of TDP-43 in the RIPA fraction and we corrected this passage in the text. p-TDP-43 in the double knockout mice is significantly increased compared to WT and Tmem106b knockout mice (Fig 8). Only significant changes are indicated, this is now noted in the figure legend. The phosphorylation of TDP-43 might indicate aberrant accumulation of either holoprotein or C-terminal fragments. Using multiple comparison ANOVA with Tukey's post-hoc test, C-terminal fragments of TDP-43 in the Grn-/- mice are not significantly elevated compared to WT. In humans and in mice TDP-CTFs with a molecular weight between 25 kDa and 35-38 kDa are frequently reported (M. Igaz et al. American Journal of Pathology 2008; Fuyuki Kametani et al. Scientific Reports 2016).

8. Throughout the manuscript, the authors used the word "massive" or "massively" to describe motor deficits, transcriptomic changes, astrogliosis, lysosomal defects, TDP-43 proteinopathy, etc. In the Abstract alone, the word "massive" was used twice in back-to-back sentences. This is quite unusual for scientif literature. Is this really necessary?

We corrected the manuscript accordingly.

9. In Discussion, the authors provided several scenarios on how TMEM106b might regulate lysosomal function and thereby affects the role of progranulin in lysosomes. None appears to be convincing. A major problem is rooted in the lack of effective assays to characterize the function of TMEM106b. The fact that different Tmem106b mutant lines appear to show different phenotypes make it more difficult to understand the discrepancy from different groups.

We revised the discussion based on the reviewer's recommendation. Based on the recent publications (X. Zhou et al. Brain 2020, T. Feng et al. Brain 2020) of two groups, which previously have not seen a phenotype in the Tmem106b ko mice, finally, a stronger phenotype is getting

increasingly clear. The problem of the discrepant phenotypes of Tmem106b mice might be associated with an incomplete knockout model. The important role of Tmem106b in neuronal transport of endo-/lysosomes is further supported by the strong phenotype of Grn/Tmem106b double knockout mice.

Response to minor concerns of reviewer #1

• Fig. 4F: The authors claim that the increase in CatD and CatB expression is "correlated" with increased activity levels. They do not make a case for correlation here; they instead demonstrate that the increased expression coincides with increased activity (at least with CatD).
For CatB, the authors should comment on the discrepancy between the supposed increase in CatBp in the double knock out compared to WT mice and the lack of functional increase compared to WT. The only increase in CatB activity is compared to the Tmem106b-/-, where we did not see any significant reduction in expression relative to the double knock out.

Former Fig 4F / new Fig 6C: We agree with the reviewer that we did not address a correlation between cathepsin expression and activity. We addressed this and accordingly changed the text. The reviewer is correct, the significant change of activity between double knockout and single Tmem106b knockout is not reflected by altered protein levels and might be owed to the small mouse numbers.

• Fig. 5A/Sup.1: The authors should include more images with increased magnification, as it is difficult to see even the arrows that indicate the cytoplasmic TDP-43 inclusions.
Additionally, these magnified images should be included for every mouse line in order to prevent any biasing across the lines.
The authors could also quantify the number of inclusions found across mouse lines, which could be more helpful than the relative metric of quantified western blot expression.

We included TDP-43 images with increased magnification for all mouse lines (new Fig 8 and EV6). Due to the small number of available mice during the pandemic, we were not able to quantify images.

Point by point response to the comments of reviewer 2:

• One of the longstanding critiques in our field is that only GRN KO mice have significant phenotypes (e.g. GRN het mice are very similar, if not identical, to WT mice). Though not absolutely necessary for publication here, it would be very helpful to the field to include any phenotypic data generated from GRN het/TMEM106B KO or GRN het/TMEM106B het mice. Does full or partial TMEM106B loss "bring out" any relevant pathologic, behavioral, or transcriptional phenotypes of GRN het mice?

We analyzed Grn Het/Tmem106b Ko mice for the hind-leg clasping reflex and rotarod performance. Notably, these mice had not to be sacrificed at any age because of animal welfare practice and did not show any hind limb clasping. However, rotarod performance was significantly better compared to double knockout mice, but still significantly impaired compared to WT mice (see new Fig. 1 C-D). Thus, we may see a gene dose effect and could probably detect for the first time an obvious phenotype in heterozygous Grn KO mice.

• Figure 1C: It appears that nuclear loss of TDP-43 may also be occurring in the double KO mice. Can TDP-43 nuclear levels be quantified from existing images?

As requested, we included TDP-43 images with increased magnification for all mouse lines for a better visualization (new Fig. 8). Unfortunately, due to small numbers of mice available during the pandemic, which caused breading ban, we were not able to quantify images.

Response to minor concerns of reviewer #2

• Change colors of Figure 1 (bright blue and green are visually unappealing, and white text on light blue is of insufficient contrast.

We changed the colors in Fig 1 accordingly but kept the color code of “green” for wild type exons and “blue” for changes within introns.

• Use of header "Impaired autophagy in Grn -/-/Tmem106b -/- mice" is not supported by the accompanying data. The problem could lie in a number of different points within the autophagosome/lysosome axis, and without detailed cellular studies the precise molecular impairment cannot be assigned to "impaired autophagy". For example, there could be increased autophagy present (e.g. high p62), but a failure of autophagosome/lysosome fusion, or a failure of lyososome degradation of engulfed autolysosome material. I would recommend using more conservative and precise language in the conclusions here, and perhaps adding potential mechanism and future directions regarding where the precise blockade in function is to the discussion section.

We changed the heading “Enhanced lysosomal and autophagic dysfunction in Grn–/–/Tmem106b–/– mice” and used a more precise language for describing our findings. In accordance, we revised the autophagy chapter in the discussion.

• Introduction and Abstract: identify need and relevance

In the synopsis, we again point out that the interplay between TMEM106B and GRN remained elusive and that we shed some light on the mechanism with this study.

- Their conclusion that the defect is in protein turnover specifically due to lysosomal dysfunction, as opposed to more general lysosome function (eg lipid metabolism), is not necessarily supported by the data. Note that lysosomes do more than just degrade things (signaling, transport).

- ALS is not a major clinical manifestation of GRN mutations, in contrast to the suggestion within the introduction.

We totally agree that the lysosome has many more functions than simple protein degradation and probably these functions are dependent on each other. However, we have most evidence of impaired protein degradation, which is indicated by accumulation of ubiquitinylated and p62-positive aggregates, by autophagosome accumulation and by the mRNA expression profile. We agree that ALS is not a major clinical manifestation of GRN mutations and corrected that sentence accordingly.

• Discussion:

- The authors note very striking changes in microglial gene expression. It would be nice if they could comment in the discussion section regarding potential autonomous vs non cell autonomous mechanisms of microglial dysregulation in the setting of dual GRN/TMEM106B loss. Is all of this due to autonomous microglial dysfunction, reaction to sick neurons (e.g. due to the transport impairments), or both? Are there potential cell autonomous mechanisms linking lysosome dysfunction with microglia dysfunction?

We revised our discussion by adding a paragraph on cell autonomous vs. non-cell autonomous function being involved in the accelerated phenotype of the double knockout mice

Point by point response to the comments of reviewer 3:

- Was the paralysis accompanied by loss of motor neurons in the ventral horn of the spinal cord?

To address this point, we added stainings of the spinal cord and confirmed a strong micro- and astroglios in the young double knockout mice (new Fig. EV3). However, there was no obvious effect on the number of motor neurons. Unfortunately, for a detailed analysis the mouse number was too small and due to the pandemics we could not breed additional animals.

- The significant hits from the differential gene expression data, as presented in figure 2 should also be included in more detail as supplementary information.

We carefully reanalyzed our transcriptomic data from the NanoString nCounter Neuropathology panel. We provide full data transparency, for all genotypes, we uploaded raw and processed data in Gene Expression Omnibus (GEO) database and we provide excel tables for all data shown in the manuscript. Now, we set a cutoff for the fold change by 20% up- or down-regulation and the number of significantly changed genes are adjusted, accordingly. We exchanged the Venn diagram showing the overlap between the genotypes into a bar graph diagram, thereby we show the overlapping genes (significant for all indicated genotypes and at least for one genotype a 20% change compared to WT), indicating the log2 fold-change, including standard error and the gene names (new Fig 2D). To further address the inflammatory phenotype we also performed NanoString nCounter Neuroinflammation panel on a complete new mouse cohort (new Fig 3 A-E).

- TMEM106B plays a crucial role is regulating lysosomal size, morphology and their acidification (pH). Given this fact, experiments and data relating to such measurements would be interesting to add.

We believe that this would be beyond the topic of our manuscript, which already increased dramatically by the addition of a significant amount of new data and new figures.

- The accumulation of ubiquinated proteins and pTDP-43 is remarkable, as it is the hallmark of FTLD/ALS, which was missing in the GRN -/- mice. This aspect could be discussed more, especially the relation between lysosomal dysfunction, TDP-43 accumulation and PGRN functioning.

We agree with the reviewer and we included a detailed discussion on protein and TDP-43 accumulation in the Grn-/-/Tmem106b-/- mice in respect to a potential lysosomal dysfunction.

1st Revision - Editorial Decision 4th Aug 2020

Dear Christian,

Thank you for the submission of your revised manuscript. We have now heard back from referee 1, who fully supports its publication. Only a few minor issues need to be corrected before we can proceed with the official acceptance:

- Given that you uploaded a movie that is called movie EV1, the EV figures need to be renumbered from figures EV1 to EV5. Please also correct all callouts in the manuscript file. Also, a callout to figure 7F is missing and needs to be added. The movie needs to be uploaded as a zipped file together with its legend.

- All authors of the manuscript need to be mentioned in the author contributions, please correct.

- Please upload the final figures in a different format, eg jpg, tiff, psd files.

- The file "animals used in this study" needs to be uploaded as a regular file, or this information could be part of the methods, where it seems to fit best. If you do not want to add it to the main manuscript, it could be moved to an Appendix file. If you prefer to have all supplementary information together in one file, all extra data could be moved to the Appendix file (extra figures and the info about mouse strains).

- The manuscript has several source data files per figure. You need to upload one single source data file per figure, and all source data need to be clearly labeled with the figure panel they refer to. The current files are confusing. I suggest that you zip all source data files that belong to one figure and upload the zipped file. Please make sure that all source data are clearly labeled so that the reader can identify to which panel they belong. The excel source data files also need a heading (eg in the first tab) that explains what these data are.

- Please add all accession numbers to the Data Availability Section. You need to make sure that the data are publicly accessible on the day of online publication.

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

The synopsis file you sent is fine, but for our website we also need a short summary (1-2 sentences) of your findings and their significance and 3-4 bullet points highlighting key results.

In the abstract, please explain what GRN is, and what kind of gene or protein TMEM106B is. When you describe the findings of your study in the abstract, please use the present tense, this must be corrected.

I look forward to seeing a final version of your manuscript as soon as possible.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor

Referee #1:

The revised manuscript by Werner and colleagues has been extensively revised to address my previous critiques. I applaud the authors for adding the NanoString Neuroinflammation panel, which provides additional insights to the existing data from the Neuropathology panel. In addition, the authors also updated many figure panels to improve the presentation and clarity of their data. Overall, this is a much improved study, and along with the other two papers from Dr. Rademakers and Dr. Hu, will have a significant impact in advancing our understanding on Tmem106B as a disease modifier for progranulin deficiency.

I support the publication of this study in EMBO Reports and urge the authors to make their NanoString data available to the readers on Omnibus (page 15).

The authors made the requested changes.

2nd Revision - Editorial Decision 10th Aug 2020

Dr. Christian Haass
Ludwig-Maximilians-University (LMU)
DZNE, Munich Cluster for Systems Neurology (SyNergy)
Feodor-Lynen-Strasse 17
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Germany

Dear Christian,

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,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

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Corresponding Author Name: Anja Capell and Christian Haass
Journal Submitted to: EMBO reports
Manuscript Number: EMBOR-2020-50241V2

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?	Biochemical experiments showed only very little variations between individual mice of same genotype, therefore a common number of 3 animals per genotype were used.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Numbers of animals used in study were limited, sample size was calculated accordingly to previous experience for biochemical analysis and NanoString technology taking biological variance into account. For immune histological data animal number allowed only to make semi-quantitative estimates of the observed trends.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	For rotarod experiments, mice identification numbers and experimental values were documented without knowledge of the genotype. Tested groups contained equal gender distribution
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	Animals of different genotypes were housed togehter, after behavioural testing animal identifcations were conected to genotype groups.
5. For every figure, are statistical tests justified as appropriate?	Yes, all figures include a statement to the statistical tests. Raw data and statistical analysis with display of the exact P-vaues and number of replication are provided in the appendix.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We observed little variations between individual mice of same genotype, and saw equal distribution in an independent cohort.
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	The groups compared have a similar n numbers. For most of the experiments the variance or the percentage of the variance were similar between the compared groups.

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).	See paragraphs "Western blotting and antibodies " and "Immunofluorescence and image acquisition" of the Materials and Methods section for detailed discription and citation of the used.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used for this study.

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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.	Animal experiments were performed in accordance to local animal handling laws. Housing conditions included standard pellet food and water provided ad libitum, 12-hour light-dark cycle at temperature of 22 °C with cage replacement once per week and regular health monitoring. The Grn knockout mice were provided by the group of Masuki Nishihara (Kayasuga et al, 2007).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments with live mice were approved according to the "Tierschutzgesetz (TierSchG)" (animal protection law) by the government of Upper Bavaria.
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.	All animal experiments were performed in accordance to local animal handling laws.

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
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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
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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