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Neuron-specific translational control shift ensures proteostatic resilience during ER stress

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **19th Jan 2022**

Thank you for submitting your manuscript assessing the response to ER stress in PERK-deficient neurons to The EMBO Journal. We have now received reports from two experts in the field, which are included below for your information. In light of the referees' comments, we would now invite you to prepare and submit a revised version of the manuscript.

As you will see, the reviewers appreciate the findings, but also raise several major concerns that must be resolved before the study can be considered further for publication. In particular, both referees find that the tolerance of PERK-deficient neurons to ER stress should be further assessed experimentally (ref#1- point 1, ref#2- point 3) and the effect on ATF4 translation supported by additional, technically independent, experiments (ref #1-point 2, ref #2-point 1, 2). Please also carefully consider all other referee comments and revise the manuscript and figures as appropriate, as well as providing a detailed response to each comment.

Please note that it is our policy to allow only a single round of major revision. Acceptance depends on a positive outcome of a second round of review and therefore on the completeness of your responses included in the next, final version of the manuscript. It is thus important to clarify any questions and concerns at this stage and I encourage you to review the referees' comments and to contact me to discuss specific points or a preliminary revision plan in case there are any uncertainties regarding the revision.

Referee #1:

#EMBOJ-2021-110501

PERK is quite important for the regulation of mammalian protein synthesis and cell survival under ER stress, relating to degenerative neurologic disease. PERK deficiency in mice brings extensive tissue damage particularly to pancreas by the misfolding and the impairment of secretion of endocrine and exocrine secretory proteins. In contrast, the brain-specific PERK KO mice have not shown severe phenotype except for the behavioral abnormality, suggesting that neurons are tolerant to PERK deficiency. In this manuscript, Wolzak et al. identified the novel back-up system on transient protein synthesis reduction under ER stress using PERK-deficient neurons. The authors found two pathways; one is mediated by HRI-eIF2a and another is by ANG. Combination of two different pathways could complement PERK deficiency in neurons under proteostatic stress. Although these findings have been explored under limited condition (PERK-KO), the results are important for understanding the cell-type specific tolerance to ER stress. The data are mostly robust for supporting the main conclusions. The novel supplemental pathways could give new ideas to researchers for analyzing proteostatic stress response in neurons and translational research on neurodegenerative disease. However, at this stage, there are several concerns to be overcome before publishing this manuscript.

Major concerns:

1. One of the important claims in this manuscript is that PERK-deficient neurons fully retain the capacity to control translation during ER stress. Although the biochemical data supported this result, there is no data about the cell survival under ER stress. The authors should demonstrate that PERK-deficient neurons still retain the tolerance to ER stress (ref. PMID: 10882126). This result gives direct evidence and makes the authors' conclusion more rigid.

2. Most data were obtained by global protein synthesis and ATF4 translation, which were quantitatively measured by high content analysis of immunostaining. Although the typical images are presented as zoomed in photo, especially a single cell in most cases, these data are not good for the readers to understand the authors' claim. The authors should present the pictures containing several cells as far as possible, as shown in Fig.3GJ, Fig.4DF, and Fig.5C.

3. I was confused with the bar graphs representing "protein synthesis response" and "ATF4 response" in Fig.5BD, Fig.6BD, Fig.7OP, and Fig.S8B. The data should be shown like Fig.1EF and more explanation (how to calculate the response) should be needed.

4. Page 9, lines 17-19: examining the data shown in Fig.S8AB, this sentence (In accordance, ---) is not supported clearly enough.

5. Page 9, lines 19-22: as KD efficiencies were too low, the authors' conclusion is not supported.

6. Fig.2E upper panel, Fig.3L, Fig.6H: if "ns" by statistical analysis is correct, the authors' interpretations in the text would be uncertain. The authors should check these points.

Minor points:
1. Fig.5A-D: S52A should be changed to S51A.
2. Page 15: the paper by Tanaka et al. should be added in Ref.
3. "Moreno et al. (2013)" in references is duplicately typed.
4. MW size markers should be added in figures of gel electrophoresis.
5. Fig.S9I: there is no description about the result of TG treatment.

Referee #2:

The manuscript by Wolzak and colleagues shows that unlike astrocytes, PERK-deficient neurons, are able to reprogram proteostatic networks to thus respond to the ER stressor tunicamycin (TM). Briefly, TM increases phosphorylation of eIF2alpha, reduces general translation rates an increases translation of ATF4 by increasing oxidate stress, and activating the HRI and ANG pathways. Interestingly, these proteostatic rearrangement processes only takes place in PERk-deficient neurons (not astrocytes).
In my view, the findings are interesting, provocative and the implications may be important for neurodegeneration and other neurological disorders in which the ISR/UPR is activated. The authors provide compelling evidence of cell type specific reprograming in PERK-deficient neurons. While for the most part the data are solid, some claims are not fully supported by the data (see below). Below, I have a few suggestions/questions that I hope will strengthen the paper.
1) Specifically, the conclusion that "the control of ATF4 translation is uncoupled from the control of global protein synthesis during ER stress in PERK-deficient neurons is not fully supported by the data. Given that Fig. 5G shows the same up-regulation of ATF4 translation in WT and PERK-deficient neurons by TM treatment, and Fig. 1O shows the same down regulation of general translation in in WT and PERK-deficient neurons by TM treatment, I understand how ATF4 translation can be possibly uncoupled from the control of global protein synthesis during ER stress.

2) To measure changes in ATF4 in the nucleus assessed by immunofluorescence is not a direct way to measure ATF4 translation. I would suggest the authors to support their findings by using polysome profiling followed by qRT-PCR of ATF4 mRNA in the different fractions.

3) Are PERK-deficient cells more susceptible/resistant to TM-induced cell death? This is important since it would demonstrate whether the cell specific cell reprograming is able to confer proteostatic resilience or not

4) Does TM increase HRI levels or activity in PERK-deficient neurons?

5) Does inhibition of HRI prevents the increase in p-eIF2 induced by TM in PERK-deficient cells?

6) Can the authors speculate/discuss the potential mechanism(s) by which TM trigger oxidative stress only in PERK-deficient neurons?

7) The authors should better explain how the quantification and normalization to WT (controls) are performed. Replicates values of WT samples is always equal to 1, but there is clearly a distribution of values on the quantification graphs in each group... please explain

Response to Referee comments
EMBOJ-2021-110501
Wolzak et al., *"Neurons shift translational control to secure proteostatic resilience during ER stress"*

We thank the reviewers for the careful reading and positive evaluation of our manuscript. We have used their comments and suggestions to improve the manuscript as detailed point-to-point below.

Referee #1:

#EMBOJ-2021-110501

PERK is quite important for the regulation of mammalian protein synthesis and cell survival under ER stress, relating to degenerative neurologic disease. PERK deficiency in mice brings extensive tissue damage particularly to pancreas by the misfolding and the impairment of secretion of endocrine and exocrine secretory proteins. In contrast, the brain-specific PERK KO mice have not shown severe phenotype except for the behavioral abnormality, suggesting that neurons are tolerant to PERK deficiency. In this manuscript, Wolzak et al. identified the novel back-up system on transient protein synthesis reduction under ER stress using PERK-deficient neurons. The authors found two pathways; one is mediated by HRI-eIF2a and another is by ANG. Combination of two different pathways could complement PERK deficiency in neurons under proteostatic stress.
Although these findings have been explored under limited condition (PERK-KO), the results are important for understanding the cell-type specific tolerance to ER stress. The data are mostly robust for supporting the main conclusions. The novel supplemental pathways could give new ideas to researchers for analyzing proteostatic stress response in neurons and translational research on neurodegenerative disease. However, at this stage, there are several concerns to be overcome before publishing this manuscript.

We would like to thank Referee #1 for their positive assessment and valuable comments on the manuscript.

Major concerns:
1. One of the important claims in this manuscript is that PERK-deficient neurons fully retain the capacity to control translation during ER stress. Although the biochemical data supported this result, there is no data about the cell survival under ER stress. The authors should demonstrate that PERK-deficient neurons still retain the tolerance to ER stress (ref. PMID: 10882126). This result gives direct evidence and makes the authors' conclusion more rigid.

We thank the referee for this suggestion and agree that data regarding the survival of PERK-deficient neurons under ER stress would strengthen our mechanistic findings. Therefore, we quantitatively addressed the viability of PERK deficient neurons in three ways:

1. We quantified ATP content of the cultures using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) to address the metabolic activity of the neurons.
2. We quantified the number of nuclei by high-content automated microscopy to assess neuronal loss.
3. We quantified 5 parameters of neuronal morphology (neurite length, #segments/neuron, #extremities/neuron, #branch point/neuron and #roots/neuron) to also address more subtle effects on neuronal degeneration (Rosato et al., 2019).

The results of experiments 1 and 2 showed that ER stress does not affect the viability of WT or *Perk* KO neurons. Experiment 3 showed that 24 hrs ER stress only has a small effect on the morphology of neurons, but there are no differences observed between *Perk* KO and WT neurons. These experiments show that PERK-deficient neurons retain the tolerance to ER stress and support our conclusion that the cell type-specific reprograming identified in our study confers proteostatic resilience. The data are compiled in the new figure EV2 and described in the results on p5.

Ref: Rosato M, Stringer S, Gebuis T, Paliukhovich I, Li KW, Posthuma D, et al. Combined cellomics and proteomics analysis reveals shared neuronal morphology and molecular pathway phenotypes for multiple schizophrenia risk genes. Mol Psychiatry. 2021 Mar 1;26(3):784–99.

2. Most data were obtained by global protein synthesis and ATF4 translation, which were quantitatively measured by high content analysis of immunostaining. Although the typical images are presented as zoomed in photo, especially a single cell in most cases, these data are not good for the readers to understand the authors' claim. The authors should present the pictures containing several cells as far as possible, as shown in Fig.3GJ, Fig.4DF, and Fig.5C.

We thank the referee for pointing this out and agree that showing multiple cells facilitates the interpretation of the data by the reader. Following the reviewer's suggestion we have therefore adjusted all representative images obtained by high-content microscopy (Fig 1J, 1M, 3A, 3D, 4M, 6A, 6D, 6I, 6K, 6M, 7F, 7N, EV3G, EV4F, EV5A, EV5J, EV6G, S4G) to show fields of view with multiple adjacent cells.

3. I was confused with the bar graphs representing "protein synthesis response" and "ATF4 response" in Fig.5BD, Fig.6BD, Fig.7OP, and Fig.S8B. The data should be shown like Fig.1EF and more explanation (how to calculate the response) should be needed.

We thank the referee for binging it to our attention that the original graphs that we showed to quantify ISR signalling (especially those with ISR interventions) were confusing. We adjusted the quantification graphs in all figures and now show the data in the following ways:

1. Normalised **raw data**: These graphs show the data for all conditions used in the experiment (Y-axis: p-eIF2α/eIF2α, protein synthesis or nuclear ATF4 intensity).
2. The **response** to ER stress: In these graphs the effect of ER stress on p-eIF2α, protein synthesis or ATF4 is quantified by dividing the raw data of the condition + ER stress by the condition – ER stress of the same genotype/treatment. (Y-axis: p-eIF2α/eIF2α response, protein synthesis response or nuclear ATF4 intensity response (TM+/TM-)).
3. **Reduction** of the response to ER stress by an intervention: In these graphs the % reduction of the ISR response was calculated to more precisely determine the effect of interventions in WT versus PERK-deficient cells. (Y-axis: % reduction of response (intervention+/intervention-)).

Per experiment (N, biological replicate) the data were normalised to untreated WT cells or cells of the same genotype/treatment (defined in figure legends). All technical replicates (different wells or cells) were used for statistical analysis. NB: Since WB experiments only have one technical replicate per experiment, the condition to which the data were normalised has no error bar.

The information above was included to the Materials and Methods section (statistics paragraph) p31-32.

4. Page 9, lines 17-19: examining the data shown in Fig.S8AB, this sentence (In accordance, ---) is not supported clearly enough.

Indeed, we agree with the referee that only showing the % reduction of the ATF4 response by HRI-i (previously Fig. S8B, now Fig. EV5C) was insufficiently clear. To more clearly support our interpretation, we added new Fig. EV5B that shows that treatment with HRI-i had no effect on the ER stress-induced upregulation of ATF4 in WT neurons but did significantly reduce the upregulation of ATF4 in *Perk* KO neurons. Specifically, Fig. EV5C shows that inhibition of HRI in *Perk* KO neurons reduced the upregulation of ATF4 with 18%.

5. Page 9, lines 19-22: as KD efficiencies were too low, the authors' conclusion is not supported.

For the sake of brevity we did not provide all validation data for PKR and GCN2 KD, but we agree with the referee that these data are necessary to better support our conclusion. Therefore, in addition to mRNA expression data, we now also provide data that show the functional validation of KD. This demonstrates that the partial KD of PKR and GCN2 strongly reduces their function (new Fig. EV5E, EV5F, EV5H, EV5I, described in Results p10). Re-analysis of the data as suggested by referee #2 (see point 7 below) resulted in a small significant reduction of TM-induced ATF4 signal by GCN2 KD. Since this is observed in both WT and *Perk* KO neurons this does not underlie the specific adaptation in *Perk* KO cells, which is completely inhibited by HRI KD in *Perk* KO only.

6. Fig.2E upper panel, Fig.3L, Fig.6H: if "ns" by statistical analysis is correct, the authors' interpretations in the text would be uncertain. The authors should check these points.

We thank the referee for this comment. We reanalysed the data as suggested by referee #2 (point 7 below) and adjusted the graphs accordingly. All interpretations are now supported by the statistical analysis.

Minor points:
1. Fig.5A-D: S52A should be changed to S51A. The figure has been corrected.
2. Page 15: the paper by Tanaka et al. should be added in Ref. This has been corrected.
3. "Moreno et al. (2013)" in references is duplicately typed. This has been corrected.
4. MW size markers should be added in figures of gel electrophoresis. MW size have been added to all figures.
5. Fig.S9I: there is no description about the result of TG treatment. The description about the positive control in our stress granules experiment (the result of TG treatment) was indeed accidentally left out. The full description of this experiment is now included in the results on p13.

We thank the reviewer for indicating these mistakes and suggestions for improvement.

Referee #2:

The manuscript by Wolzak and colleagues shows that unlike astrocytes, PERK-deficient neurons, are able to reprogram proteostatic networks to thus respond to the ER stressor tunicamycin (TM). Briefly, TM increases phosphorylation of eIF2alpha, reduces general translation rates an increases translation of ATF4 by increasing oxidate stress, and activating the HRI and ANG pathways. Interestingly, these proteostatic rearrangement processes only takes place in PERk-deficient neurons (not astrocytes).
In my view, the findings are interesting, provocative and the implications may be important for neurodegeneration and other neurological disorders in which the ISR/UPR is activated. The authors provide compelling evidence of cell type specific reprograming in PERK-deficient neurons. While for the most part the data are solid, some claims are not fully supported by the data (see below). Below, I have a few suggestions/questions that I hope will strengthen the paper.

We would like to thank Referee #2 for their positive assessment and valuable comments on the manuscript.

1) Specifically, the conclusion that "the control of ATF4 translation is uncoupled from the control of global protein synthesis during ER stress in PERK-deficient neurons is not fully supported by the data. Given that Fig. 5G shows the same up-regulation of ATF4 translation in WT and PERK-deficient neurons by TM treatment, and Fig. 1O shows the same down regulation of general translation in in WT and PERK-deficient neurons by TM treatment, I understand how ATF4 translation can be possibly uncoupled from the control of global protein synthesis during ER stress.

The conclusion that "the control of ATF4 translation is uncoupled from the control of global protein synthesis during ER stress in PERK-deficient neurons" is based on the response of endogenous ATF4 analyzed in Figure 2A-F by nuclear accumulation and Fig. EV3J-K by WB. These data clearly indicate a strong reduction of the ER stress-induced ATF4 response in PERK-deficient neurons in sharp contrast with the intact global protein synthesis response (Fig. 1M-O and EV3C an EV3D).
The data in Fig. 5I and 5J that the referee refers to are derived from the translational ATF4 reporter, comprising of an EYFP reporter of which the translation is driven by the 5' UTR of ATF4 and therefore increased during ER stress. The EYFP signal accumulates in the nucleus because of an N-terminal NLS. We validated the reporter by demonstrating co-regulation with the endogenous nuclear ATF4 signal during ER stress (see figure for referees below). Please note that the signal is derived from the virally transduced reporter, this allows side-by-side comparison of control and TM treatment within one genotype, but direct comparison between WT and *Perk* KO cannot be made. In contrast to the endogenous signal, the reporter signal continues to increase during 24 hrs ER stress (see figure for referees below), which may be expected as the half-lives of the respective proteins are vastly different. The t1/2 of EGFP (that is very homologous to EYFP) is approximately 15 hrs (Danhier et al., 2015), whereas that of ATF4 is <60 mins (Ameri et al., 2004; Frank et al., 2010).

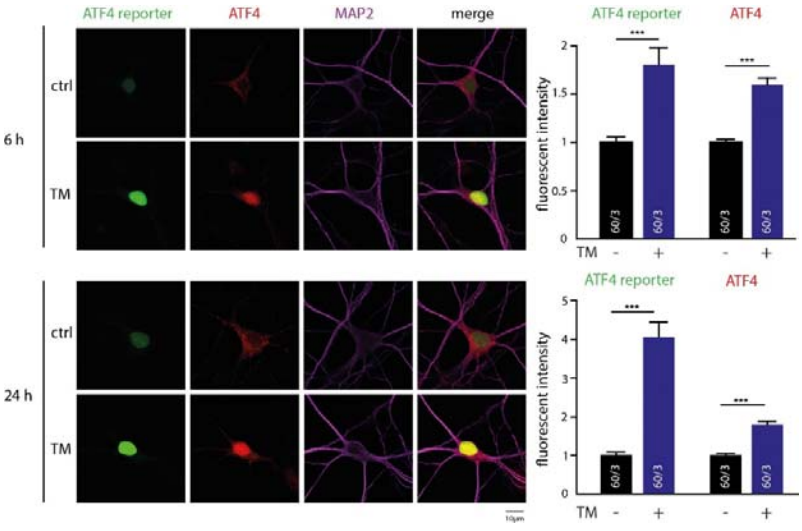

Figure for referees: Comparison of the 5'ATF4-uORF^{WT}-reporter and endogenous nuclear ATF4 accumulation.
WT neurons were transduced with the ATF4 reporter at DIV3 and treated with DMSO (Ctrl) or TM for 6 or 24 hrs as indicated and analysed by confocal microscopy at DIV14. Shown are representive images and quantification of nuclear EYFP direct fluorescence (ATF reporter) and endogenous ATF4 immunofluorescence (ATF4, Alexa Fluor 546). Data are presented as mean ± SEM from n=60 cells from N=3 experiments. Two-tailed Student's t test *** P<0.001.

The reporter is used to address the p-eIF2α-dependency of the residual ATF4 increase observed in Figures 2 and EV3. We agree with the referee that we have not sufficiently made this clear and have included a better explanation of the rationale of the experiment and the difference between the endogenous and reporter readouts for ATF4 on p10-11 in the results.

Ref:

- Ameri, K. et al. (2004) 'Anoxic induction of ATF-4 through HIF-1-independent pathways of protein stabilization in human cancer cells', Blood, 103(5), pp. 1876–1882. doi: 10.1182/blood-2003-06-1859.
- Danhier, P. et al. (2015) 'Combining Optical Reporter Proteins with Different Half-lives to Detect Temporal Evolution of Hypoxia and Reoxygenation in Tumors', Neoplasia (United States), 17(12), pp. 871–881. doi: 10.1016/j.neo.2015.11.007.
- Frank, C. L. et al. (2010) 'Control of activating transcription factor 4 (ATF4) persistence by multisite phosphorylation impacts cell cycle progression and neurogenesis', Journal of Biological Chemistry, 285(43), pp. 33324–33337. doi: 10.1074/jbc.M110.140699.

2) To measure changes in ATF4 in the nucleus assessed by immunofluorescence is not a direct way to measure ATF4 translation. I would suggest the authors to support their findings by using polysome profiling followed by qRT-PCR of ATF4 mRNA in the different fractions.

The mechanism of ATF4 induction by ER stress is well-established to involve an p-eIF2α- and 5'uORF-dependent selective translational upregulation, the newly synthesized transcription factor accumulates in the nucleus. Our primary neuronal cultures have very low RNA yields and are not well-suited to quantitatively perform large scale biochemical separations like polysomal profiling. Hence, we employed and extensively validated the nuclear ATF4 accumulation as proxy and are confident about the validity of the method as read-out for the ATF4 response:

1. We confirmed the reduced ATF4 response in *Perk* KO neurons by analyzing the protein levels on WB, demonstrating the nuclear increase is not due to subcellular relocalization of pre-existing proteins (Fig. EV3J and EV3K).
2. We demonstrated the p-eIF2α- and 5'ATF4-uORF-dependency of the observed ER stress-induced ATF4 responses using dominant negative eIF2α^{S51A} and 5'ATF4-uORF^{WT} and 5'ATF4-uORF^{MUT} reporters (Fig. 5G-5L).

However, we agree with the reviewer that nuclear accumulation of ATF4 upon ER stress is an indirect measure of ATF4 translation and therefore we specifically referred to the data as "ATF4 response" "ATF4 increase" rather than "ATF4 translation". We thank the reviewer for pointing out to us that we were not fully consistent in this and have rephrased the few instances where we still incorrectly use ATF4 translation when referring to our observations in Figures 2 and EV3 and throughout text.
In addition, we more explicitly indicated this as a limitation of the study with an outlook to future experiments in the discussion (p16).

3) Are PERK-deficient cells more susceptible/resistant to TM-induced cell death? This is important since it would demonstrate whether the cell specific cell reprograming is able to confer proteostatic resilience or not.

We thank the referee for this suggestion and provide new data that shows that PERK-deficient neurons are not more susceptible or resistant to TM-induced cell death as compared to WT neurons as described in the response to Referee #1 – point 1 (see above). The data are presented in the new Fig. EV2 and described in the results on p5 and support our conclusion that the cell specific reprogramming we identified in PERK-deficient neurons confers proteostatic resilience.

4) Does TM increase HRI levels or activity in PERK-deficient neurons?

We thank the reviewer for this interesting question. We performed WB analysis to show that neither PERK deletion nor ER stress affected the HRI protein levels. The data therefore suggest that the compensatory effect of HRI in PERK-deficient neurons is mediated by changes in the activity HRI rather than the levels.

The data are presented in the new Appendix Fig. S5 and discussed in the results on p10.

5) Does inhibition of HRI prevents the increase in p-eIF2 induced by TM in PERK-deficient cells?

We performed Western Blot analysis to show that TM-induced phosphorylation of eIF2α is indeed completely prevented by HRI KD in *Perk* KO, while it had no effect on WT neurons. Hence, the rescaled ER stress-induced phosphorylation of eIF2α is fully mediated by HRI in PERK-deficient neurons. The data are presented in the new Fig. 4K-4M and described in the results on p9.

We thank the reviewer for this comment as these data indeed strengthen our conclusion that HRI mediates the eIF2α-dependent part of the compensatory mechanism in PERK-deficient neurons.

6) Can the authors speculate/discuss the potential mechanism(s) by which TM trigger oxidative stress only in PERK-deficient neurons?

The information about cell type-specific PERK–mediated generation of ROS is currently very limited. In the revised manuscript we speculated more extensively on this topic in the discussion (p16-17).

7) The authors should better explain how the quantification and normalization to WT (controls) are performed. Replicates values of WT samples is always equal to 1, but there is clearly a distribution of values on the quantification graphs in each group... please explain

We agree with the reviewer that the quantification and normalization was not clear. A more detailed description of the quantifications has now been included in the Materials and Methods section under statistics (p31-32), see also response to referee #1 – point 3 above).

Following the reviewer's suggestion we have re-analysed all automated and confocal microscopy data, to incorporate the variation in the WT controls. The graphs and statistics have been adjusted accordingly in all figures and the analysis methods is described in the Materials and Methods section under statistics (p31-32).

Thank you for submitting your revised manuscript. Please also excuse the delay in communicating this decision to you which was due to delayed referee responses. We have now however received comments from referee #1, which you will find copied below. As you will see, the referee acknowledges that the manuscript has improved, but still raises a number of specific issues that must be resolved before we can consider the study further for publication. As mentioned in the previous decision letter, it is normally EMBO Journal's policy to allow only one round of major revision, such that it is now crucial that you address the remaining referee concerns fully in the this exceptional second round of revision. Please also remember to provide a detailed point-by-point response to the comments when you submit the revised manuscript.

Referee #1:

EMBOJ-2021-110501R
The revised manuscript has been improved. However, I note that several important concerns still remain. I feel there are so many mistakes in figures, which would confuse the readers. The authors should carefully check their manuscript and figures.

1. Cell survival: The viability of PERK deficient neurons was precisely examined and demonstrated. As a negative control, under the same condition, the data about the viability of PERK deficient astrocytes is required.
2. Fig.4 J: I do not still understand how to calculate "ATF4 response (%)". The authors should describe the calculating formula in legend as follows.
Ex. ATF4 response (%) = [ATF4 intensity (HRI KD+Ars) - ATF4 Intensity (HRI KD-Ars) / ATF4 intensity (WT+Ars) - ATF4 Intensity (WT-Ars)] x100
Calculating formula should be also described in legends of the following figures:
Fig.5 C,F (% reduction response); Fig.6 C (% reduction response); Fig.7 P,R (% reduction response); EV5 C (% reduction response).
3. Fig.4 I: Where are photos of nuclear ATF4 intensity?
4. Fig.4 N: Data from photos in (Perk KO+TM+HRI KD) do not support the results of O and P.
5. Fig.4 L,M: ATF4 response and Nuclear ATF4 response in Y axis, respectively, might be wrong. The authors should be checked.
6. Fig.4 L: Is statistic bar "ns" correct?
7. All microscopical figures: All scale bars (25 micrometer) are similar length in spite of the different cell size. The authors should indicate the real scale bars.
8. Fig.6 B: Why is WT+ISRIB defined as 1.0?
9. Fig.6 I: The photo (the bottom in the rightmost column) is quite hard to understand the result from J.
10. Page 12, line 4 from the bottom: (Fig. 6C-6F) should be corrected by (Fig. 6D-6F).

Response to Referee comments
EMBOJ-2021-110501
Wolzak et al., *"Neurons shift translational control to secure proteostatic resilience during ER stress"*

Referee #1:

EMBOJ-2021-110501R
The revised manuscript has been improved. However, I note that several important concerns still remain. I feel there are so many mistakes in figures, which would confuse the readers. The authors should carefully check their manuscript and figures.

We are happy that the reviewer considers the manuscript improved after revision and thank the reviewer for the time invested to provide feedback and pointing out a mistake in one of the figures (point 5) and a typo in the text (point 10). We have used the final minor comments of the referee to further improve the manuscript as detailed point-to point below.

1. Cell survival: The viability of PERK deficient neurons was precisely examined and demonstrated. As a negative control, under the same condition, the data about the viability of PERK deficient astrocytes is required.

We have determined the viability of PERK-deficient astrocytes using the same conditions and assay (CellTiter-Glo Luminescent Cell Viability Assay (Promega)) as used for neurons (Fig. EV2E). This showed that in contrast to neurons, PERK deficiency reduces the tolerance of astrocytes to ER stress. The observed small effect of PERK deficiency on the survival of astrocytes during ER stress is probably an underestimation due to the capacity of astrocytes to divide in culture. Neurons lack this capacity, and as we hypothesize in the discussion the inability of neurons to selfrenew may be an underlying reason for the presence of the neuron-specific translational control shift we identified in this study (Discussion p15-16).

2. Fig.4 J: I do not still understand how to calculate "ATF4 response (%)". The authors should describe the calculating formula in legend as follows.
Ex. ATF4 response (%) = [ATF4 intensity (HRI KD+Ars) - ATF4 Intensity (HRI KD-Ars) / ATF4 intensity (WT+Ars) - ATF4 Intensity (WT-Ars)] x100
Calculating formula should be also described in legends of the following figures:
Fig.5 C,F (% reduction response); Fig.6 C (% reduction response); Fig.7 P,R (% reduction response); EV5 C (% reduction response).

In Figure 5C, 5F, 6C, 6F, 7P, 7R and EV5C the % reduction of the ISR response by an intervention in WT and *Perk* KO neurons was calculated using the formulas:

$$\%\ \text{reduction of protein synthesis response} = \frac{[\text{Response+Intervention}]-[\text{Response}-\text{Intervention}]}{[\text{Response}-\text{Intervention}]} * 100$$

$$\%\ \text{reduction of ATF4 response} = \frac{[\text{Response+Intervention+}]-[\text{Response}-\text{Intervention}]}{[\text{Response}-\text{Intervention}]} * -100$$

In Figure 4J, EV5F and EV5I the remaining activity of HRI, PKR and GCN2 (i.e. increase in ATF4 intensity upon ISR activation) was determined after knockdown (KD) of the kinases by relating the increase in ATF4 intensity by KD neurons to WT neurons. We have further clarified this for the readers by replacing "% ATF4 response" by "Remaining ATF4 response (%)" in the graphs. The used formula is:

$$\text{Remaining ATF4 response (\%)} = \frac{\left(\frac{\text{ATF4 intensity KD+ISR activation}-\text{ ATF4 intensity KD Ctrl}}{\text{ATF4 intensity KD Ctrl}}\right)}{\left(\frac{\text{ATF4 intensity WT+ISR activation}-\text{ ATF4 intensity WT Ctrl}}{\text{ATF4 intensity WT Ctrl}}\right)} * 100$$

We added the general formulas to the Materials and Methods section under statistics (p31-32) and included the specific formulas in the individual figure legends.

3. Fig.4 I: Where are photos of nuclear ATF4 intensity?

We did not include the typical examples of the validation to preserve space. The representative images of Figure 4I are now provided in new Appendix Figure S5.

4. Fig.4 N: Data from photos in (Perk KO+TM+HRI KD) do not support the results of O and P.

We think that size differences in the shown neurons might have given a distorted view and agree with the referee that showing neurons of similar size is more clear. Therefore, we replaced the representative image of *Perk* KO + HRI KD + TM. We would like to stress that size differences do not affect our quantifications.

5. Fig.4 L,M: ATF4 response and Nuclear ATF4 response in Y axis, respectively, might be wrong. The authors should be checked.

We thank the referee for pointing this out and corrected this.

6. Fig.4 L: Is statistic bar "ns" correct?

Yes, this is correct, Tukey's post hoc test after two-way ANOVA shows that p=0.2485 (See Source Data).

7. All microscopical figures: All scale bars (25 micrometer) are similar length in spite of the different cell size. The authors should indicate the real scale bars.

All scale bars have been checked. We would like to point out that primary neuronal nuclei are smaller than astrocytic nuclei in culture and that the morphology of human and mouse cells differs. In addition, there are less pronounced inter-culture differences in cell/nuclear size. We alerted readers not familiar with these cultures to this by an explanation in the Materials and Methods section (p29).

8. Fig.6 B: Why is WT+ISRIB defined as 1.0?

As in all figures the WT control value is defined as 1.0. The ISR response of WT neurons (TM+/TM-) without ISRIB in this experiment is 0.734 (27% reduction of protein synthesis). As expected, ISRIB inhibits the response in WT cells to WT control value (0.9956), and therefore WT+ISRIB is approximately 1.0. This is the outcome of the experiment, not how we defined 1.0.

9. Fig.6 I: The photo (the bottom in the rightmost column) is quite hard to understand the result from J.

PERK-i treatment inhibits the clear TM-induced increase in nuclear ATF4 signal, which is the message of these data. A small increase in nuclear signal is still detected, but due to the lower signal/noise ratio not as clearly as the TM-induced increase in the absence of PERK-i. We replaced the typical example of *2b5*^{ho} + PERK-i + TM to more clearly visualize the nuclear ATF4 signal.

10. Page 12, line 4 from the bottom: (Fig. 6C-6F) should be corrected by (Fig. 6D-6F).

We thank the referee for pointing this out and corrected this.

2nd Revision - Editorial Decision **5th Jun 2022**

Thank you again for submitting your revised manuscript. We have received comments from referee # 1 (please see below), who now overall also supports publication. Please carefully consider the suggestion the referee makes regarding the axis labeling when compiling the final version of the manuscript and check the specific issues s/he lists for the figure legends. In addition, I would also ask you to please resolve a number of editorial issues that are listed in detail below. Please use the document that the data editors have added their comments to for any changes (see below).

Please feel free to contact me if you have further questions regarding the revision or any of the specific points. Thank you again for giving us the opportunity to consider your manuscript for The EMBO Journal.

Kind regards,
Stefanie

Stefanie Boehm
Editor
The EMBO Journal

Referee #1

Most problems have been resolved. However, personally, I feel the title of Y-axis (% reduction response) would be changed to more suitable title, because your calculation formula between "% reduction of protein synthesis response" and "% reduction of ATF4 response" is different in the point of +/-, making the readers confusing.
Finally, even just reding the final version of the manuscript, there are still some mistakes. The authors should carefully check the final version of the manuscript.
1. page 48, Fig.5 legend: "(Δ)Cre, green/red" to "(Δ)Cre, red".
2. page 49, Fig.6 legend: "Perk neurons" to "Perk-deficient neurons".

Herewith we submit the final revised version of our manuscript #EMBOJ-2021-110501 "*Neurons shift translational control to secure proteostatic resilience during ER stress"* by Wolzak et al.

We are very happy that both referees now support publication of the manuscript. In the final revised version we corrected the 2 textual mistakes pointed out by referee # 1 and we relabeled the axes of Figs. 5, 6, 7 and EV5 from "% reduction response" to "% change in response" and adjusted the text and figure legends accordingly. Furthermore, we resolved the editorial issues raised in point 1-4 regarding the disclosure and competing interests statement, reference format, data availability section and Appendix file. The editorial issues raised in point 5-7 were already communicated to us before and were already resolved in the first revision of the manuscript. Hence, we did not upload the "EMBOJ-2022-110501_figure_QC" document as this file did not contain the adjustments made in the second revision (R2) of the manuscript.

We would like to confirm that our mRNA sequencing dataset has been deposited to GEO and will be made public upon acceptance of the manuscript.

3rd Revision - Editorial Decision **9th Jun 2022**

Thank you for submitting the final revised version of your manuscript and addressing the remaining points. You will likely hear from me again soon regarding final textual edits of the transfer files, but for now I happy to inform you that we have formally accepted your study for publication in The EMBO Journal.

EMBO Press Author Checklist

Corresponding Author Name: W. Scheper	
Journal Submitted to: The EMBO Journal	
Manuscript Number: EMBOJ-2021-110501	

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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 χ2 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Data Availability Section

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods: Antibodies

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods: Table 1: Primers and probes used for qPCR

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

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Please detail **housing and husbandry conditions**.	Yes	Materials and Methods: Animals

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods: Statistics
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods: Statistics
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	Materials and Methods: Statistics
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods: Statistics

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends, Source data
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Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods: Animals
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