ALS-linked protein disulfide isomerase variants cause motor dysfunction

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1st Editorial Decision 21 July 2015

Thank you submitting your manuscript to The EMBO Journal. Your manuscript has now been reviewed by three referees and their comments are provided below.

As you can see, the referees find the analysis reporting on the effects of the ALS-linked PDI variants on motor function interesting. They raise a number of constructive comments that would further strengthen the findings reported. Given the referee comments, I would like to invite you to submit a revised version of the manuscript that addresses the concerns raised. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage.

Thank you for the opportunity to consider your work for publication. I look forward to seeing your revision.

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REFEREE REPORTS
Referee #1:

Woehlbier et al, investigate the effects of four new missense PDI variants in motor control and neuromuscular connectivity. Using a series of complementary methodologies the authors performed a very systematic study showing that the identified mutations in PDIA1 and ERp57 cause a
disturbance of the foldase function and structural and functional changes in the neuromuscular synapses thus inducing motor neuron pathology in zebrafish. In addition, they have demonstrated that loss of ERp57 in the nervous system trigger abnormal motor control without motor neuron loss, probably through the neuromuscular synapses alterations. This is an interesting functional study of PDI variants involved in the motor defects associated with a disruption of motor neuron connectivity.

Specific points to be address:

1. As the study identifies ER proteostasis imbalance as a risk factor for ALS, contributing to early events leading to the disease it would be interesting to see if the loss of one copy of ERp57 or expression of PDIA variants in the mice model of ALS (e.g. mutant SOD1 or mutant TDP-43 models) has an effect on the time of onset of ALS like symptoms.

2. It is not clear how old were the animals when the rotarod and hanging test were performed (Figures 5C and 5D). Do the motor defectd worsen during aging in the ERp57 conditional knockout animals?

3. As the loss of ERp57 in mice resulted in abnormal postsynaptic morphologies and a narrower band of acetylcholine receptor clusters along the length of the diaphragm, it would be of interest to see if ERp57 is involved in the synthesis and secretion of synaptic proteins.

Minor comments

1. The reference: Rutkevich LA, Williams DB (2011) Participation of lectin chaperones and thiol oxidoreductases in protein folding within the endoplasmic reticulum. Curr Opin Cell Biol23:157-166 is repeated twice in the references list and when referred to this publication in the section "Introduction" the year is omitted:

2. When authors claim that " Minor but significant differences were observed in the proteolytic pattern of the PDIA1 mutants compared with PDIA1wt (Fig 3E)", it is not clear what was the criteria for the significance claim without statistics.

3. In the supplementary table I it is shown that 27% and 25% of zebrafish show some morphological changes when PDIA1wt and ERp57wt mRNA are overexpressed. I am not that familiar with zebrafish but this percentage does not look not negligible. The authors should comment on this, as the current phrase: "the overall morphology of embryos expressing wild-type PDIA1 or ERp57 appeared largely unaffected at 24 hours post-fertilization" is not acceptable

4. Supplementary figure 3 is labeled as supplementary figure 4.

Referee #2:

The manuscript presents the functional characterization of four ALS-linked mutations recently identified in two major PDI genes, PDIA1 and PDIA3/ERp57. There is an impressive amount of models used to demonstrate the deleterious impact of the PDIA1 and PDIA3/ERp57 identified by Gonzalez-Perez earlier this year

Please see below comments and suggestions to improve the manuscript.

1)Figure 1A, it should be shown where is the V5 tag positionned (N or C term?)

2)Figure 1E-F, there is no information about how many animals were counted to present the histograms.

3)Figure 1 E-G, the impact of the different mutation is variable (in particular the D217N which looks like the wt and doesn't clearly emerge as deleterious)
4) Figure 2B, arrows should be added to show the neurites outgrowth because these are not clear on the figure. The difference between mock and ERp57wt is not convincing on this figure

5) Figure 3E, some explanation should be provided about the PK banding that reveals the WT and mutant proteins to be different

6) Figure 5I, why is there a band when the V5 antibody is used to detect the mock transfection?

7) Figure 4A, there is no mock transfection

8) Figure 5E, could be informative to show if the hetrozygous have a clearly intermediate phenotype

9) General comments: It could have been informative to: i) do a siRNA silencing in the cell model; ii) test if there are ALS relevant aggregates (ubiquitin, p62, TDP-43) in brain or spinal cord cross section of the ERP57 ko animals; propose an hypothesis regarding the death of the mice even as the number of neurons is not significantly altered (figure 5I)

Referee #3:

In this manuscript, the authors defined the involvement of PDIA1 and ERP57 mutations on ALS pathology. The authors performed a significant amount of genetic, biochemical, and in vivo studies to define the involvement of specific mutations in these PDIs on function and ALS pathology. All of the work in the manuscript is of high quality and the information is well presented in the manuscript.

I only have a few comments related to the work that I think should be addressed prior to publication. One global question is: what is the consequences of overexposing these PDI mutants on ER function? Does overexpression induce ER stress? Do you see alterations in ER protein folding/trafficking when you overexpress these mutants? The authors need to include some experiments with these overexpression studies to address the impact on ER function. ER stress could impact ALS pathology independent of PDI function and this needs to be addressed. Furthermore, it would be useful to provide more context on how alterations in PDI activity influence ALS phenotypes. It is mentioned in the intro that PDIs can localize with ALS-associated aggregates, but how does this happen? Are these proteins in the cytosol? If they are in the cytosol, how would alterations in ER-centric functions (e.g., association with calreticulin) influence disease progression? These points need to be addressed in the manuscript. In short, my main concern relates to how alterations in PDI activity influence ALS phenotypes which are often associated with the cytosol.

Apart from the above point, I only have a few other minor comments.

1. Are the axon lengths shorter for PDIA1 WT overexpression in Fig. 1E? It looks like overexpressing PDI WT or the mutant affect length in the same way.

2. Does the increased glycosylation of the ERP57(D217) mutant explain the increased association with calnexin/calreticulin for this mutant protein? In other words, is the interaction not a functional interaction between these proteins but instead represents a non-functional interaction where calnexin/calreticulin are involved in the folding of this protein.

3. It would be nice to include discussion on why overexposing PDI WT promotes neuronal morphology in Fig. 3-4. This positive result is referenced in the manuscript, but a proposed explanation would be appropriate.

4. In Fig 4E, it would be better to present the data as Kd.

1st Revision - authors’ response  13 November 2015

Response to Reviewers Referee #1:
Woehlbier et al., investigate the effects of four new missense PDI variants in motor control and neuromuscular connectivity. Using a series of complementary methodologies the authors performed a very systematic study showing that the identified mutations in PDIA1 and ERp57 cause a disturbance of the foldase function and structural and functional changes in the neuromuscular synapses thus inducing motor neuron pathology in zebrafish. In addition, they have demonstrated that loss of ERp57 in the nervous system trigger abnormal motor control without motor neuron loss, probably through the neuromuscular synapses alterations. This is an interesting functional study of PDI variants involved in the motor defects associated with a disruption of motor neuron connectivity.

Specific points to be address:
1. As the study identifies ER proteostasis imbalance as a risk factor for ALS, contributing to early events leading to the disease it would be interesting to see if the loss of one copy of ERp57 or expression of PDIA variants in the mice model of ALS (e.g. mutant SOD1 or mutant TDP-43 models) has an effect on the time of onset of ALS like symptoms.

Response:
We thank this reviewer for his/her constructive and positive feedback. We agree with reviewer 1 that a study investigating the loss of ERp57 or the expression of PDI variants in an ALS mouse models are of interest. However, we feel that this is a major undertaking and goes beyond the scope of the current already extensive study. Our laboratory has expertise in the use of classical ALS mouse models, and to develop a full serious study will take at least 2 years (mouse breedings, motor measurements and histological and biochemical analysis). We are indeed planning to perform these experiments but in the context of the mutations in PDIs. We are preparing a follow-up story for the next year and we already generated two transgenic mouse models, which carry either mutant form of human ERp57. We are currently analyzing these animals regarding their motor performance and we observed clear impairment in these two lines supporting the major conclusions of the current study (Figures shown to referees but removed from this review file). Furthermore, we have preliminary results showing that the ERp57^{D217N} transgenic mouse develop a similar NMJ and muscle phenotype (Figures shown to referees but removed from this review file) as we have observed for the ERp57 knockout animals. We are in the process of analyzing the ERp57^{Q481K} mouse in the same way. We plan to then (second stage of the project) cross these animals with mutant SOD1 and TDP43 mice to assess if the disease is accelerated. We decided not to cross the ERp57 KO mice with these ALS models because these animals are difficult to obtain because of the strong basal phenotype that they develop.

2. It is not clear how old were the animals when the rotarod and hanging test were performed (Figures 5C and 5D). Do the motor defect worsen during aging in the ERp57 conditional knockout animals?

Response:
We agree with the reviewer that this is a relevant question. The data of the rotarod shown refers to the average of 5 measurements (each measurement is the average of 3 replicates per day) performed in consecutive weeks. Measurements were initiated after a week of training in the rotarod. However animals are generally between 30 and 150 days of age. The data of the hanging test that is shown in Figure 5D refers to the average of the first 5 measurements performed in consecutive weeks independent of the age of the animal. Animal ages generally were between 30 and 150 days of age. We then stopped the measurement to avoid excessive manipulation of the mice. All these animals were obtained in a period of two years since the breeding is very inefficient and the rate of birth is not Mendellian as indicated in Table 1.

As requested by this reviewer, we now have included Appendix Fig S3A, which shows the progression of the phenotype over time. We do not observe an obvious worsening of the phenotype in rotarod performance in the time frame up to age 150 days. The hanging test was performed only as stated above.

3. As the loss of ERp57 in mice resulted in abnormal postsynaptic morphologies and a narrower band of acetylcholine receptor clusters along the length of the diaphragm, it would be of interest to see if ERp57 is involved in the synthesis and secretion of synaptic proteins.
Response:
We thank this reviewer for this idea. This question led us to explore a new avenue that gave us interesting results to propose a novel mechanism of action. We analyzed the expression of a panel of synapse-located proteins in our ERp57 deficient animals. Indeed we found that the expression of the synaptic protein SV2 is significantly decreased in the cortex of ERp57 heterozygous and knockout mice. More importantly, higher molecular weight species were detected in these experiments suggesting alterations in the folding of this protein leading to abnormal protein aggregation (new Fig 8). This phenomenon is in agreement with previous publications describing other clients of ERp57 (Solda et al. JBC 2006). Since this protein is a membrane glycoprotein synthesized through the secretory pathway, it is likely that it is a substrate of ERp57. Furthermore, we have recently observed that the loss of ERp57 in the brain also affects the levels of the Prion protein, another glycosylated synaptic protein (Torres et al, 2015 JBC). Our results are in agreement with the functional defects in the morphology of the NMJ. This is why here we propose a novel mechanism where alteration of ERp57 triggers neuronal dysfunction rather than toxic ER stress, which represent a novel mechanism of action that may be relevant to ALS. We have highlighted these issues in the new discussion. We thank this reviewer for this suggestion, which helped us to improve our manuscript.


Minor comments
1. The reference: Rutkevich LA, Williams DB (2011) Participation of lectin chaperones and thiol oxidoreductases in protein folding within the endoplasmic reticulum. Curr Opin Cell Biol23:157-166 is repeated twice in the references list and when referred to this publication in the section "Introduction" the year is omitted:

Response:
We thank reviewer 1 for his/her comment. We have removed one of the references.

2. When authors claim that "Minor but significant differences were observed in the proteolytic pattern of the PDIA1 mutants compared with PDIA1wt (Fig 3E)", it is not clear what was the criteria for the significance claim without statistics.

Response:
We agree with reviewer 1 and have therefore now repeated all these assays in 4 independent experiments and performed quantifications that are included in the main figures (Figure 3E). These results were consistent and thus we were able to quantify these changes showing statistically significant differences in the proteolytic pattern of mutant PDIA1.

3. In the supplementary table I it is shown that 27% and 25% of zebrafish show some morphological changes when PDIA1wt and ERp57wt mRNA are overexpressed. I am not that familiar with zebrafish but this percentage does not look not negligible. The authors should comment on this, as the current phrase: "the overall morphology of embryos expressing wild-type PDIA1 or ERp57 appeared largely unaffected at 24 hours post-fertilization" is not acceptable.

Response:
We agree that we did not refer to this point in the original submission. Over-expression of wild type forms of different genes related to ALS consistently induce global morphological phenotypes in a variable percentages (Kabashi et al., 2010; Ramesh et al., 2010; Kabashi et al., 2011), which are similar to the phenotypes we describe in our study after overexpression of PDIA1WT and ERp57WT. The origin of this global morphological effect is unclear. One possible explanation is that phenotypes arise from gain of protein function. Consistent with this idea, overexpression of PDIA1WT and ERp57WT leads to significant increase in neurons and neurites, a phenotype opposite to the overexpression of mutant forms that generate loss of protein function.
In summary, although having a percentage of embryos with global embryo morphological phenotypes is expected for the overexpression of PDIA1$^{WT}$ and ERp57$^{WT}$, our phrase to describe this effect was not sufficiently clear and could lead to confusion. Therefore, we have modified the text to the following: “While the majority of embryos expressing wild-type PDIA1 or ERp57 appeared normal in overall morphology at 24 hours post-fertilization (hpf), a large fraction of embryos expressing PDIA1$^{R300H}$, ERp57$^{D217N}$ and ERp57$^{Q481K}$ showed a striking shortening of the axis and tail curvature.” The relevant analysis here is to compare WT vs mutant PDI$s$. In addition, to avoid artefacts all morphological analysis were quantified in animals with overall healthy global phenotypes.


4. Supplementary figure 3 is labeled as supplementary figure 4.

Response:
We have fixed this.
We would like to thank again this reviewer for his/her constructive and positive feedback to improve the quality of the study. All of the comments were very useful for us.

Referee #2:
The manuscript presents the functional characterization of four ALS-linked mutations recently identified in two major PDI genes, PDIA1 and PDIA3/ERp57. There is an impressive amount of models used to demonstrate the deleterious impact of the PDIA1 and PDIA3/ERP57 identified by Gonzalez-Perez earlier this year. Please see below comments and suggestions to improve the manuscript.

1. Figure 1A, it should be shown where is the V5 tag positioned (N or C term?)
Response: We have added the V5 tag to Fig 1A. The V5 tag is positioned at the C-terminus, however before the ER-retention signal, as was published in Jessop et al. 2007 and 2009.

2. Figure 1E-F, there is no information about how many animals were counted to present the histograms.
Response: We thank reviewer 2 for his/her observation. We now have included the number of animals counted in the experiments to the figure for consistency.

3. Figure 1 E-G, the impact of the different mutation is variable (in particular the D217N which looks like the wt and doesn’t clearly emerge as deleterious)
Response: We agree with reviewer 2 that the results in the zebrafish are slightly variable between mutants. However, overall we observe a tendency of a deleterious effect of ERp57$^{D217N}$ e.g. in axon branching. The model represents an “acute test” to screen for in vivo phenotypes on a time window of 24h. This is why that possible effects in the long term that may trigger more evident neurodegeneration cannot be detected in this system. Precisely for this reason we have used several different experimental approaches to determine the effect of these mutations, since there might be variability in penetrance and severity of the effects of each mutation depending of the assay and model system used.
4. **Figure 2B, arrows should be added to show the neurites outgrowth because these are not clear on the figure. The difference between mock and ERp57wt is not convincing on this figure**

**Response:**
We have added arrows to Figure 2B to signal the neurite outgrowth.

5. **Figure 3E, some explanation should be provided about the PK banding that reveals the WT and mutant proteins to be different**

**Response:**
We agree with reviewer 1 and have therefore now repeated all these assays in 4 independent experiments and performed quantifications that are included in the main figures (Figure 3E). These results were consistent and thus we were able to quantify these changes showing statistically significant differences in the proteolytic pattern of mutant PDIA1.

We have now modified the text regarding this result to the following, to provide more insights about the identification of the nature of these bands using mass spectrometry: “In particular differences were observed in the ratio of the two major degradation products formed (band 1 and 2) (Fig 3E; bottom panel). Mass spectrometric analysis of these bands indicated that the mutations resulted in altered variation in cleavage around the x-linker region that lies between the b’ and a’ domains.”

6. **Figure 5I, why is there a band when the V5 antibody in used to detect the mock transfection?**

**Response:**
We believe there was a slight spillover of the next lane while loading the gel. We have 2 additional experiments showing the same result without this band. However, we chose to show the presented blot because in the other two experiments we also tested if the IP changes under treatment with tunicamycin, please see (Figures shown to referees but removed from this review file) in this letter as an example. Since tunicamycin had no effect we preferred not to show this result.

7. **Figure 4A, there is no mock transfection.**

**Response:**
The mock transfection in Figure 4A is the same as in Figure 3A since these samples are on the same blot, they were just split into two figures. We now added an explanation to the figure legend to indicate this issue, which is important because these two experiments are 100% comparable between them.

8. **Figure 5E, could be informative to show if the heterozygous have a clearly intermediate phenotype**

**Response:**
We thank this reviewer for noticing this omission that has been corrected. In the investigated timeframe there was no effect of the loss of one allele of ERp57 on the survival of the heterozygous animals, however these animals have evident motor dysfunction. We have now included these results in the new Fig 5E.

9. **General comments: It could have been informative to: i) do a siRNA silencing in the cell model; ii) test if there are ALS relevant aggregates (ubiquitin, p62, TDP-43) in brain or spinal cord cross section of the ERP57 KO animals; propose an hypothesis regarding the death of the mice even as the number of neurons is not significantly altered (figure 5I).**

**Response:**
We thank reviewer 2 for his/her insights. These are general questions that we addressed with specific experiments. We believe the new data provided here has now improved the quality of the study, helping defining the mechanism of action.

**Point (i):** As requested, we have performed the suggested knockdown of endogenous PDIA1 and ERp57 in NSC34 cells by stable transduction with lentiviruses (new Appendix Fig S1E). We have analyzed the effect on dendritic outgrowth (new Fig 2F). We observed that in both cases the percentage of cells with neurites significantly decreased (new Fig 2F). These results confirmed a...
physiological role of PDIA1 and ERp57 in neurite outgrowth and that ALS-linked mutations impair this process. We also performed western blot analysis of the total ubiquitination pattern, which showed no alterations in the CNS tissue of ERp57 deficient animals (not shown).

Importantly, we recently addressed the possible impact of ERp57 on the ER stress response on a parallel study and reported that ERp57 does not induce spontaneous ER stress or change the susceptibility of cells to ER stress in vivo and in vitro (Torres et al., 2015 JBC). We have included this information in the discussion of the new version of this manuscript.

**Point (ii):** We now have included data on SOD1 and TDP43 expression levels in cortex and spinal cord of ERp57 deficient animals. Western blot analysis revealed no clear changes in the expression levels of the wild-type proteins (new Appendix Fig. S4A, B and E). In addition, no spontaneous aggregation of these two proteins was observed after filtertrap or western blot analyses (new Appendix Fig S4C and D).

**Point (iii):** We agree we need to provide some hints on the possible mechanism leading to the neurological alterations in ERp57 animals that trigger neuronal dysfunction rather than cell toxicity. Since ERp57 is a relevant foldase of the secretory pathway, folding glycosylated substrates as part of the calnexin cycle, we explored a new avenue that gave us interesting results to propose a novel mechanism of action. We analyzed the expression of a panel of synapse-related proteins in our ERp57 deficient animals. Indeed, we found that the of level the synaptic protein SV2 is significantly decreased in the cortex of ERp57 heterozygous and knockout mice. More importantly, higher molecular weight species were detected in these experiments suggesting alterations in the folding of this protein leading to abnormal protein aggregation (new Figure 8). This phenomenon is in agreement with previous publications describing other clients of ERp57 (Solda et al. JBC 2006). Since this protein is a membrane glycoprotein synthesized through the secretory pathway, it is likely that it is a substrate of ERp57. Furthermore, we have recently observed that the loss of ERp57 in the brain also negatively affects the levels of the prion protein, another glycosylated synaptic protein (Torres et al., JBC 2015). Our results are in agreement with the functional defects in the morphology of the NMJ. This is why here we propose a scenario where alteration of ERp57 triggers neuronal dysfunction rather than toxic ER stress, which represents a novel mechanism of action that may be relevant to ALS. We have highlighted these issues in the new discussion.


We would like to thank again this reviewer for all the suggestions to improve this report.

Referee #3:
In this manuscript, the authors defined the involvement of PDIA1 and ERP57 mutations on ALS pathology. The authors performed a significant amount of genetic, biochemical, and in vivo studies to define the involvement of specific mutations in these PDIs on function and ALS pathology. All of the work in the manuscript is of high quality and the information is well presented in the manuscript.

1. I only have a few comments related to the work that I think should be addressed prior to publication. One global question is: what are the consequences of overexpressing these PDI mutants on ER function? Does overexpression induce ER stress? Do you see alterations in ER protein folding/trafficking when you overexpress these mutants? The authors need to include some experiments with these overexpression studies to address the impact on ER function. ER stress could impact ALS pathology independent of PDI function and this needs to be addressed.

Response:
We thank reviewer 3 for his/her comments and suggestions. Accordingly, we have investigated the effect of overexpressing PDIA1 and ERp57 wild-type and mutants on ER physiology using multiple approaches and included a series of new experiments that gave us a surprising answer and included the following text and experiments on a new section:

“PDIs represent an important group of ER stress-responsive genes that are thought to contribute to the recovery of proteostasis downstream of the UPR. To explore the possible impact of ALS-linked
PDI\textsubscript{s} identified here to the physiology of the ER, we first analyzed their subcellular distribution. NSC34 cells were cotransfected with V5-tagged PDI variants together with a KDEL-RFP construct to assess their subcellular distribution. In addition to showing a normal co-localization with the ER marker, mutant PDIA1 or ERp57 did not alter the morphology of this organelle (Appendix Fig S2). We next analyzed if the expression of ALS-linked PDIs alters the susceptibility of cells to ER stress. NSC34 cells expressing mutant or wild-type PDIA1 and ERp57 were treated with the ER stress agent tunicamycin (an inhibitor of N-glycosylation). The expression of the UPR mediators XBP1 and ATF4 was monitored by Western blot. No changes in the activation of the UPR were detected in these experiments (Fig EV2A). Similarly, the upregulation of the ER chaperone BiP was not enhanced in cells expressing ALS-linked PDIs compared to the wild-type forms (Fig EV2A). We also monitored the viability of cells undergoing ER stress. Treatment of cells with different doses of thapsigargin (an inhibitor of the SERCA pump) lead to equivalent percentages of cell death in cells expressing wild-type or mutant PDIs as monitored by propidium iodide staining (Fig EV2B).

Similar results were obtained when cells were treated with tunicamycin (Fig EV2C). These results are consistent with our recent findings indicating that ERp57 does not affect the susceptibility of cells to undergo ER stress (Torres et al., 2015). Finally, to monitor the physiological status of the ER with a fourth parameter, we determined the rate of secretion of two proteins that are synthesized through the secretory pathway. NSC34 cells were co-transfected with a BDNF-GFP expression construct together with our PDI variants. Western blot analysis of the supernatant of cells after 18 or 42 h of transfection indicated no alterations in the rate of BDNFGFP secretion (Fig EV2D). Similar results were obtained when we analyzed the levels of progranulin in the cell culture media using ELISA (Fig EV2E). Only PDIA1\textsubscript{R30H} showed a minor reduction in progranulin secretion (Fig EV2E). Taken together, these results suggest that the expression of ALS-linked PDIs does not trigger clear alterations to the homeostasis of the ER.

Based on suggestions from reviewer 1 and 2, we explored the possible effects of ERp57 on the expression of relevant synaptic proteins in vivo. We found, that in the cortex of ERp57 heterozygous and knockout mice the level of the synaptic protein SV2 is significantly decreased. More importantly, higher molecular weight species were detected in these experiments suggesting alterations in the folding of this protein leading to abnormal protein aggregation (new Figure 8). Our results are in agreement with the functional defects in the morphology of the NMJ. This is why here we propose a scenario where alteration of ERp57 triggers neuronal dysfunction rather than toxic ER stress, which represents a novel mechanism of action that may be relevant to ALS. We have highlighted these issues in the new discussion.

2. Furthermore, it would be useful to provide more context on how alterations in PDI activity influence ALS phenotypes. It is mentioned in the intro that PDIs can localize with ALS-associated aggregates, but how does this happen? Are these proteins in the cytosol? If they are in the cytosol, how would alterations in ER-centric functions influence disease progression? These points need to be addressed in the manuscript. In short, my main concern relates to how alterations in PDI activity influence ALS phenotypes which are often associated with the cytosol.

Response:

We now have included data on SOD1 and TDP43 expression levels in cortex and spinal cord of ERp57 deficient animals. Western blot analysis revealed no clear changes in the expression levels of the wild-type proteins (new Appendix Fig S4A and B). In addition, no spontaneous aggregation of these two proteins was observed after filter-trap or western blot analysis (new Appendix Fig S4C and D). These results reinforce the idea that PDI mutations act through a novel mechanism of action to trigger disease features. However, we are currently performing a full project addressing the role of ERp57\textsuperscript{WT} in ALS. We recently generated a transgenic mouse overexpressing ERp57\textsuperscript{WT} in the nervous system (Castillo et al., 2015 PloS One) that we are currently crossing with mutant SOD1 and TDP43 transgenic mice. This project was motivated by the unexpected observation that in cell culture ERp57\textsuperscript{WT} overexpression enhances mutant SOD1 and mutant TDP43 aggregation (Figures shown to referees but removed from this review file). This is why we believe these experiments are out of the scope of the current study and will be subject of a follow-up report using multiple in vitro and in vivo approaches where the current ERp57 mutants will be tested also. But we wanted to share this data with the reviewer to highlight the directions that we are following.

Regarding ER-related effects. The literature is very rich in reports indicating how ALS-linked genes alter ER physiology. For example, mutant SOD1 physically interacts with ERAD components inhibiting this activity triggering ER stress. Other genes alter ER to Golgi trafficking, affecting
protein maturation (see Figure 3, Hetz and Mollereau, 2014 Nat Rev Neurosci). Thus, many alterations to ER physiology can occur from the cytosolic phase of the ER. We have reinforced this concept in the discussion of the paper. Overall we believe that PDIs contribute to ALS by altering the folding of synaptic proteins and not through a chronic ER stress response, which represents a highly novel finding.

Minor comments.

1. Are the axon lengths shorter for PDIA1 WT overexpression in Fig. 1E? It looks like overexpressing PDI WT or the mutant affect length in the same way.

Response:
Yes, reviewer 3 is correct, the axon lengths for PDIA1\textsuperscript{WT} is slightly shorter compared to the Mock. This might be due to the overexpression of PDIA1\textsuperscript{WT} in general.

2. Does the increased glycosylation of the ERP57(D217) mutant explain the increased association with calnexin/calreticulin for this mutant protein? In other words, is the interaction not a functional interaction between these proteins but instead represents a non-functional interaction where calnexin/calreticulin are involved in the folding of this protein.

Response:
This is an important question, but we discarded this issue because the \textit{in vitro} binding assays performed here are done with recombinant proteins prepared in bacteria where no glycosylations are present. We cannot discard that in the immunoprecipitation experiments in living cells the gain of a glycosylation site may affect the interactions with calreticulin and calnexin.

3. It would be nice to include discussion on why overexpressing PDI WT promotes neuronal morphology in Fig. 3-4. This positive result is referenced in the manuscript, but a proposed explanation would be appropriate.

Response:
Based on the new results we have expanded the discussion of this issue to propose a mechanism explaining the effects on the structure and composition of synapses. However, other mechanisms may be relevant. For example, Erp57 may influence the release of ER calcium to the cytosol affecting the cytoskeleton and thus neurite outgrowth. Erp57 can control the activity of the SERCA pump (Li and Camacho, JCB 2004). We included this idea in the discussion and we are actually exploring this possibility as a follow-up project.

4. In Fig 4E, it would be better to present the data as K\textsubscript{D}.

Response: As requested, we have now included the K\textsubscript{D} data as Appendix Table S2 in the manuscript.
We would like to thank this reviewer for his/her attempts to help us improving our study.

2nd Editorial Decision 07 December 2015

Thank you for submitting your revised version to The EMBO Journal. The three referees have now re-evaluated your study and as you can see below they appreciate the introduced changes and support publication here. I am therefore very pleased to accept the manuscript for publication here.

Referee #3 finds the manuscript a bit too long and suggests shortening the text. I have looked at it and from my perspective I think it is fine as is. I would suggest that you look through the manuscript to see if you think it would benefit from tightening up the text.

Before we can transfer to the manuscript to our publisher there are a few things to sort out
- There is no figure legend provide for the appendix - please upload a modified appendix. - Figs 3, 5, 6, 7, EV1, EV2 are too wide and need to be resized to < 172mm wide.

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REFEREER REPORTS

Referee #1:
The authors have replied satisfactorily to most of the queries of this referee.

Referee #2:
I believe the authors did a thorough job to address comments from our first round of review.

Referee #3:
Overall, the authors have addressed all of my major concerns from the original submission. While I think that the underlying molecular mechanism(s) by which PDIs influence ALS progression remain to be further established, it is clear from their rebuttal letter that this is a focus of the lab and will be described in future publications. I do think that the manuscript is too long and could benefit from some tightening of the text, but in my opinion this manuscript is now suitable for publication in EMBO.