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Mutant PDIA3 causes neurodevelopmental impairment by disturbing endoplasmic reticulum proteostasis

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Dear Claudio,

Thank you for submitting your manuscript to The EMBO journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from these comments the referees appreciate the analysis. However, they also indicate that further analysis is needed. The genetics expert, ref #3, find that further human data is needed to show that PDIA3 is indeed the relevant gene. While referees #1 and 2 find that further molecular insight into PDIA3-C57Y causes disease should be added. Should you be able to address the raised concerns then I would like to invite a revised version. I am happy to discuss the raised points further and maybe it would be most helpful to do so via phone/email or video. Let me know what works best for you.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Thank you for the opportunity to consider your work for publication. I look forward to further discuss the revisions.

Yours sincerely,

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

The paper outlines the identification of a disease causing mutation causing severe mental retardation and developmental impairment. The mutation was mapped to a protein involved in protein folding namely a member iof the protein disulfide isomerase family PDIA3. The mutation is at one of the active site thiols with the likely consequence of a diminished enzymatic activity. As the phenotype is only seen in homozygotes it was classified as a recessive loss of function mutation. The paper then goe son to describe a series of experiments carried out in vitro and in vivo to look at the consequence of protein expression on development, protein aggregation and function. Most notably overexpression of the mutant protein has relatively severe consequences on vertebrate development, alters the proteome in brain tissue, and disturbs cell adhesion. In addition, the mutant protein form aggregates when exogenously expressed in cells, elicits abnormal interaction with calnexin and calreticulin and has impaired enzymatic activity.

Taken together the results show a clear link between PDIA3 function and the disease phenotype. It would have been useful for the authors to have discussed the lack of a phenotype in heterozygotes as their experiments involving expression of the mutant in the presence of wild type protein show a dominant negative phenotype. While it is clear that this particular mutation causes the disease , and that the mechanism is related to cell adhesion leading to impaired development, they could have extended to work to show that the C57Y mutation affected protein folding (for example of the integrins). This would complete the paper by linking the mutation to a defect in the folding and secretion of a specific protein involved in cell adhesion. It would have also been nice to see whether any of the ER chaperones or substrate proteins are in the cellular aggregates. This would provide some indication why the exogeneous expression of the mutant is giving the dominant negative phenotype (due to higher levels of expression than the endogenous protein).

In summary, this is a nice paper that could be improved by the addition of a couple of simple experiments. It provides an indication of function for one of the PDI family members. Such functional analysis has proved difficult due to supposed redundancy in this large protein family.

One very minor issue is that figure 4B does not contain any information on immune function or MHC Class 1 as stated in the text.

Referee #2:

- general summary and opinion about the principal significance of the study, its questions and findings.

The paper presents a genetic analysis of a recessive, consanguineous developmental disorder that results in intellectual disability and skeletal abnormalities. The authors attribute the cause to a homozygous mutation in PDIA3 (ERp57), resulting in compromised catalytic activity of the oxidoreductase; and the authors provide evidence of a "gain-of-dysfunction" phenotype in the zebrafish and mouse models, which leads to compromised proteostasis.

Identifying the genetic causes of complex intellectual illness is difficult, and the finding that defects in protein quality control contribute to these conditions is an important and novel step forward. The work is topical and relates well to other recent studies, including one from Hetz and colleagues showing that PDIA1 and PDIA3 mutants contribute to motor dysfunction in ALS.

The majority of the experiments appear technically well performed. A major strength of this study is that it is wide-ranging, drawing on genetics, whole organism studies, cell biology and biochemistry.

- specific major concerns essential to be addressed to support the conclusions

Despite the impressive breadth of the work, in my opinion the cell biological studies do not identify exactly how PDIA3-C57Y causes disease at a molecular level. It remains possible that defective proteostasis contributes to the patient phenotype, but is not the primary cause, and I am not convinced that the PDIA3 "loss of function" vs "gain of dysfunction" question has been fully resolved. A key experiment is to show how misfolded/misoxidised PDIA3-C57Y causes cytoskeletal (e.g. vimentin) disruption at the molecular/cell biological level.

In earlier studies, some published in EMBO (e.g. Antoniou EMBO J 21:2655-2663 (2002); Peaper et al EMBO J 24:3613-3623 (2005)), PDIA3 was identified as a part of the MHC class I loading complex and as a functional interaction partner with tapasin - so is MHC class I cell surface expression normal in patient or C57Y-expressing cells?

This is an important point because there is evidence that MHC class I plays a role in human brain development (e.g. Huh GS, et al. Science 290:2155-2159 (2000); Goddard CA, Butts DA, Shatz CJ Proc Natl Acad Sci USA 104:6828-6833 (2007) and others). I wonder whether dysregulation of MHC quality control contributes to the patient phenotype - something that may not be apparent in the zebrafish or mouse injection model.

A related point is that there is a notable difference in the interaction of the PDIA3 C57Y mutant protein with several immune and

interferon-inducible proteins, compared to wild-type PDIA3 (supplementary tables). Since patient 501 died having had recurrent infections, has immunity of the surviving individuals been investigated? A full immunological analysis is clearly beyond the scope of the current manuscript, but please could the authors comment on this in the text.

For the zebrafish and transfection experiments with PDIA3-V5 and PDIA3C57Y-V5, please could the authors confirm where the V5 tag was positioned - it should be upstream of the KDEL retention-retrieval motif. This is particularly important when considering the appearance of intracellular puncta in Fig 6.

I would like to see further controls and discussion of the caveats for the murine transgene expression experiments- although the V5-tagged PDIA3 protein expression control looks equal in Fig EV2, the IF in panel B suggests that expression of C57Y is less than wild type. I would also like to see a blotting and IF control for endogenous PDIA3, in addition to the V5 blot, because it is possible that misfolded C57Y induces the degradation or mis-localisation of endogenous PDIA3 in this essentially heterozygous situation.

It is not clear to me why the calnexin/calreticulin IP in Fig EV4 panel H was not also performed with the C57Y R282A mutant. This is needed to evaluate any indirect role of C57Y in lectin chaperone association, in light of the recombinant protein work in Fig 6.

- minor concerns that should be addressed

The data in Fig EV4A suggest that C57Y does not, by itself, cause an unfolded protein response, yet the C57Y protein does aggregate - this merits further discussion in the text.

There are some minor typographical, grammatical and spelling errors requiring further proofreading.

Referee #3:

This is a potentially interesting manuscript that describes the discovery of a homozygous variant in the PDIA3 gene in 4 relatives in 2 sibships from a highly inbred family. There is extensive work done that aims to characterize this missense variant Cys57Tyr in zebrafish.

While the work looks interesting there seem to be 2 major concerns:
1. Because of the complex inbreeding, the authors were unable to assess the mapping information that was derived from SNP typing in a single linage analyis. The authors therefore used a stepwise analysis that started with 3 affected (from 2 sibships), then took the candidate regions forward and tested all 3 affected in the second sibship while leaving out the first affected, and then checked which of the remaining candidate regions was homozygous in all 4 affected. This strategy is not unreasonable, but it is complicated and the result is not entirely clear to me.

The authors then performed a single exome, and apparently checked only the candidate region for potential homozygous variants. The only variant that looked promising from this region was in PDIA3.

This is problematic, because in such a highly inbred family, there are likely multiple homozygous variants whether disease-causing or not. I am not sure how much of the genome was effectively excluded by the stepwise linkage that the authors employed here.

As a solution, I believe that the authors need to perform at least one more exome (in patient 401), in order to compile a list of homozygous loss of function, and likely damaging missense variants that are shared between these 2 individuals. That would allow a more targeted analysis of all possible candidate genes, which can then be compared with the linkage data (or checked by Sanger) in the other two affected. This is essentialy the opposite strategy (candidate variants first, and then confirm/exclude by linkage) to what is in the manuscript now. Having the two strategies side by side could strengthen confidence in the validity of the final result that points to PDIA3 as the culprit gene.
Note that we need to be extra critical here since there is only a single allele, without independent confirmation in another pedigree.

2. Given that parents were apparently normal, and inheritance appears as a classical autosomal recessive, I am puzzled that there should be consistent effects of expression of the allele in normal cells from mouse and in zebrafish embryos. Surely that indicates a dominant effect.
While I am aware of situations where an allele that works through haploinsufficiency in humans acts as a true recessive null in mice, th observation of oerexpression effects appears to suggest the opposite situation. This is puzzling to me.
Somewhat reassuring in this respect is that the phenotype of knockout of PDIA3 in mouse embryonic fibroblasts, can be rescued by the (human) wildtype PDIA3 but not the mutant allele. Also, overexpression of wildtype PDIA3 enhances neurite number and length but expression of the the variant does not. So some of the experiments do support a loss of function scenario.

Overall, I would have liked to see a critical discussion of how we should intepret these findings in different systems, and how we may understand effects that seem to be dominant in some paradigms and recessive (loss of function) in others.

Referee #1:

The paper outlines the identification of a disease causing mutation causing severe mental retardation and developmental impairment. The mutation was mapped to a protein involved in protein folding namely a member of the protein disulfide isomerase family PDIA3. The mutation is at one of the active site thiols with the likely consequence of a diminished enzymatic activity. As the phenotype is only seen in homozygotes it was classified as a recessive loss of function mutation. The paper then goes on to describe a series of experiments carried out in vitro and in vivo to look at the consequence of protein expression on development, protein aggregation and function. Most notably overexpression of the mutant protein has relatively severe consequences on vertebrate development, alters the proteome in brain tissue, and disturbs cell adhesion. In addition, the mutant protein form aggregates when exogenously expressed in cells, elicits abnormal interaction with calnexin and calreticulin and has impaired enzymatic activity.

Point 1: *Taken together the results show a clear link between PDIA3 function and the disease phenotype. It would have been useful for the authors to have discussed the lack of a phenotype in heterozygotes as their experiments involving expression of the mutant in the presence of wild type protein show a dominant negative phenotype.*

Response:

We thank the reviewer for acknowledging that we were able to establish a clear link between PDIA3 function and the disease phenotype. The reviewer raises an interesting point as to whether the toxicity of the mutant protein manifests by a dominant negative mechanism. This is a valid interpretation of our overexpression experiments in the presence of the endogenous protein. However, the heterozygous carriers would be expected to manifest mild disease symptoms. Instead, the deleterious effects obtained in overexpression experiments may be interpreted by a toxic gain-of-function due to the C57Y mutation or the competition between mutant and endogenous PDIA3, with the mutant protein concentration reaching a threshold for toxic properties and/or hindrance of endogenous activity to manifest at the phenotypic level. Nevertheless, we predict that overexpression of the human form will compete with the endogenous wild-type protein, saturating the system with an excess of the mutant form that results in a condition where the functional output is mostly dependent on mutant PDIA3, mimicking a homozygous condition.

Since comparison of human and mouse protein levels by Western-blot or immunofluorescence is difficult due to different affinities of antibodies for each protein form, we performed relative quantification of human to endogenous PDIA3 levels from proteomic data by measuring intensity of shared peptides in mouse and human

proteins. This analysis showed that human PDIA3 is overexpressed 2.6- (wild-type) and 2.1-fold (mutant) relative to endogenous PDIA3, sustaining the argument that human PDIA3 may effectively outcompete the endogenous protein for substrates and chaperones in the ER milieu. In addition, AAV only transduces a fraction of cells in the hippocampus, suggesting even higher expression levels at single cell level. This point was added to the discussion of results as follows:

'We previously identified dominant variants in PDIA3 as risk factors for amyotrophic lateral sclerosis (Woehlbier et al, 2016). Here we report a homozygous variant that causes severe syndromic ID. This finding offered the opportunity to further understand the biological function of a major ER oxidoreductase in the nervous system using various biochemical, cellular and whole organism experiments. We provide compelling evidence supporting pathogenicity of the variant, which may act by both loss- and gain-of-function mechanisms.

Haploinsufficiency or dominant negative effect resulting from the C57Y variant are unlikely since heterozygous individuals are unaffected. The substitution of a catalytic cysteine strongly suggests loss-of-function as the pathogenic mechanism. On the other hand, PDIA3^{C57Y} may acquire deleterious features that surpass a toxicity threshold for clinical manifestation upon expression of two mutant PDIA3 alleles. The loss- and gain-of-function mechanisms are not mutually exclusive. Indeed, our data show that the C57Y mutation causes PDIA3 aggregation and abnormal interactions with CNX/CRT while greatly compromising enzymatic activity. Overexpression experiments both in vivo and in vitro also corroborate this notion. Zebrafish development and cognitive function of mice may be affected by gain-of-toxic properties of PDIA3^{C57Y}. Alternatively, when overexpressed the mutant protein may outcompete endogenous PDIA3 for substrates and chaperones in the ER milieu, generating nonproductive enzymatic cycles that disturb folding of specific clients.'

Point 2: *While it is clear that this particular mutation causes the disease, and that the mechanism is related to cell adhesion leading to impaired development, they could have extended to work to show that the C57Y mutation affected protein folding (for example of the integrins). This would complete the paper by linking the mutation to a defect in the folding and secretion of a specific protein involved in cell adhesion. It would have also been nice to see whether any of the ER chaperones or substrate proteins are in the cellular aggregates. This would provide some indication why the exogeneous expression of the mutant is giving the dominant negative phenotype (due to higher levels of expression than the endogenous protein).*

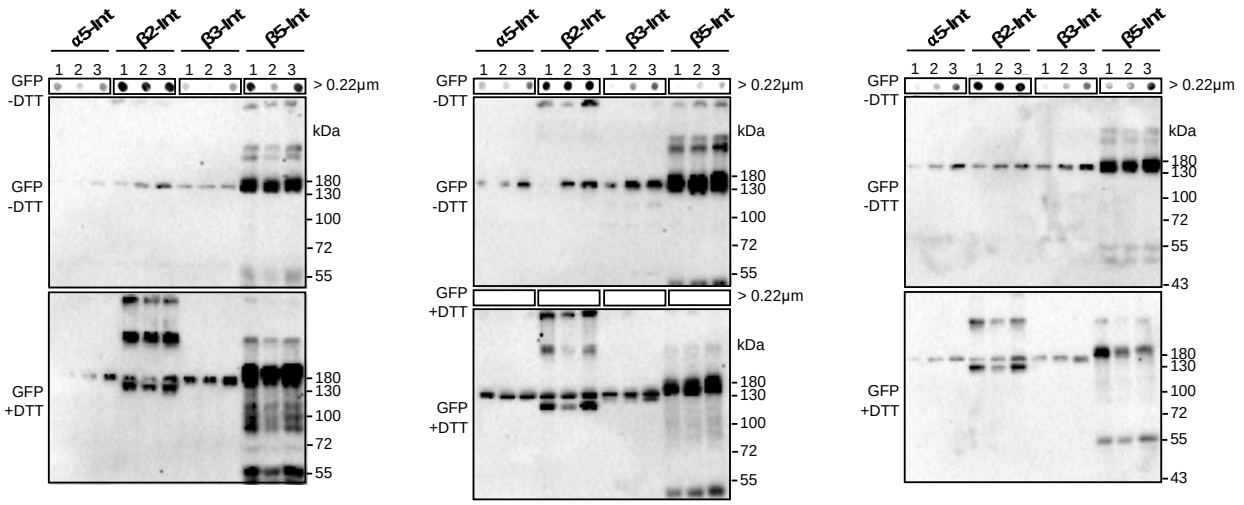
Response:

We thank the reviewer for this relevant suggestion to improve our study. We took these comments very seriously and obtained several constructs to setup and perform these

experiments. To screen possible candidates, we expressed a panel of four integrins, including α5-integrin (α5-int), β2-integrin (β2-int), β3-integrin (β3-int) and β5-integrin (β5-int) fused to either GFP or YFP, in stable NSC-34 cell lines expressing PDIA3 or PDIA3^{C57Y}. Next, we performed Western blot and filter-trap analysis under reducing and non-reducing conditions to investigate abnormal species or aggregates containing disulfide crosslinks (included as **Figure 5I and Figure EV3C**. Data also presented below as **Letter Figure 1**). The fusion proteins α5-int-GFP, β2-int-YFP and β3-int-YFP migrate in the reducing SDS-PAGE between the 130 and 180 kDa markers, while the β5-int-2xGFP migrates slightly above due to an extra GFP unit. The molecular weight observed for this band is consistent with the glycosylated monomer. β2-int-YFP and β5-int-2xGFP showed a complex band pattern, with additional species migrating slower than the monomer, located at the interface between the stacking and resolving gels and trapped at the stacking gel. Finally, β5-int-2xGFP also displays species migrating faster than the monomer, possibly reflecting proteolytic processing of the 2xGFP tag.

Importantly, we found two distinctive band patterns between PDIA3 and PDIA3^{C57Y} while analyzing this panel of integrins in three independent experiments. β2-int-YFP presents a faster migrating species that is decreased upon expression of PDIA3, but not PDIA3^{C57Y}. Interestingly, this lower band is only detectable under reducing conditions. A close inspection of β3-int-YFP signal reveals possible partition of the monomer into a faster migrating species upon expression of PDIA3^{C57Y}. As observed for β2-int-YFP, this lower band is only detectable under reducing conditions. Surprisingly, the non-reducing Western blot of the panel of integrins has a simpler band pattern than the reducing condition, also revealing a monomer migrating between 130 and 180 KDa. No particular signal in the non-reducing Western blot suggestive of disulfide-crosslinked high-molecular-weight species or aggregates of integrins was detected. On the contrary, species detected in the reducing condition were actually absent in the non-reducing analysis of β2-int-YFP and β3-int-YFP. This observation suggests that such species may correspond to disulfide-crosslinked aggregates undetectable by non-reducing Western blot.

We also analyzed samples using filter-trap to detect large aggregates that may be not seen in Western blot analysis. Indeed, the filter-trap analysis revealed the existence of integrin aggregates that disappear under reducing conditions, suggestive of disulfide-crosslinked species. Across the panel of integrins examined, PDIA3^{C57Y} cells presented higher levels of aggregated species compared to those in PDIA3 cells. This result was further corroborated by expression of β5-int-2xGFP in *Pdia3*^{KO} MEFs and reconstitution with wild-type or mutant PDIA3, with higher levels of disulfide-crosslinked aggregates accumulating in PDIA3^{C57Y}-expressing cells (new **Figure EV3D**). Together, our data suggests that PDIA3^{C57Y} may alter the folding or quality control of integrins. Further studies based on pulse-chase experiments and analysis of posttranslational modifications are warranted in the future, but are beyond the scope of this manuscript.



Letter Figure 1. Biochemical analysis of mutant PDIA3 impact on integrin expression in cell culture. A panel of integrin paralogs was overexpressed in NSC-34 cell lines stably overexpressing PDIA3 or PDIA3^{C57Y}. The cells were transfected with constructs for overexpression of α5-integrin (α5-int) fused to GFP, β2-integrin (β2-int) fused to YFP, β3-integrin (β3-int) fused to YFP and β5-integrin (β5-int) fused to 2xGFP and analyzed under non-reducing (-DTT, dithiothreitol) or reducing (+DTT) conditions by Western blot and filter-trap (cut-off 0.22 μm). A main band consistent with the glycosylated monomer of the different paralogs is detected between 130 and 180 KDa. Three independent experiments are shown. 1, mock; 2, PDIA3; 3, PDIA3^{C57Y}.

We have also probed the presence of ER chaperones in PDIA3^{C57Y} aggregates. To this end, we performed immunoprecipitation of PDIA3 from uncleared cell extracts followed by filter-trap analysis under native conditions to maintain non-covalent interactions. This approach showed that calnexin forms a complex with PDIA3^{C57Y} aggregates (new **Figure 6F**). Moreover, immunofluorescence analysis indicates that the ER chaperone BiP co-localizes with mutant PDIA3 inclusions (new **Figure 6G**).

Point 3: *In summary, this is a nice paper that could be improved by the addition of a couple of simple experiments. It provides an indication of function for one of the PDI family members. Such functional analysis has proved difficult due to supposed redundancy in this large protein family.*

Response:
We thank the reviewer for the positive response and enthusiasm about our study. The manuscript has been significantly improved by your suggestions.

Point 4: *One very minor issue is that figure 4B does not contain any information on immune function or MHC Class 1 as stated in the text.*

Response:

We have corrected this mistake by referring only to new Appendix Tables S5 and S6, as follows:

'In addition, proteins related to the MHC-I pathway, such as tapasin, antigen peptide transporter 1 and 2, β2-microglobulin and H2 class I histocompatibility antigen, were also down-regulated (Appendix Tables S5 and S6), consistent with the role of PDIA3 as a scaffold that regulates antigen presentation (Garbi et al, 2006).'

Referee #2:

The paper presents a genetic analysis of a recessive, consanguineous developmental disorder that results in intellectual disability and skeletal abnormalities. The authors attribute the cause to a homozygous mutation in PDIA3 (ERp57), resulting in compromised catalytic activity of the oxidoreductase; and the authors provide evidence of a "gain-of-dysfunction" phenotype in the zebrafish and mouse models, which leads to compromised proteostasis.

Identifying the genetic causes of complex intellectual illness is difficult, and the finding that defects in protein quality control contribute to these conditions is an important and novel step forward. The work is topical and relates well to other recent studies, including one from Hetz and colleagues showing that PDIA1 and PDIA3 mutants contribute to motor dysfunction in ALS.

The majority of the experiments appear technically well performed. A major strength of this study is that it is wide-ranging, drawing on genetics, whole organism studies, cell biology and biochemistry.

***Point 1:** Despite the impressive breadth of the work, in my opinion the cell biological studies do not identify exactly how PDIA3-C57Y causes disease at a molecular level. It remains possible that defective proteostasis contributes to the patient phenotype, but is not the primary cause, and I am not convinced that the PDIA3 "loss of function" vs "gain of dysfunction" question has been fully resolved. A key experiment is to show how misfolded/misoxidised PDIA3-C57Y causes cytoskeletal (e.g. vimentin) disruption at the molecular/cell biological level.*

Response:

We thank the reviewer for acknowledging the relevance and quality of our study and agree that functional experiments to link PDIA3 substrate alterations and cellular phenotypes were missing. Regarding this point, integrins are key proteins linking the actin cytoskeleton to the extracellular matrix via signaling that shape cell morphology and is likely mediating the biological effects of PDIA3 on cell adhesion and neuritogenesis. Indeed, these adhesion molecules have been previously identified as PDIA3 substrates (Jessop et al, EMBO J, 2007). In the revised version of the manuscript, we provide new data showing that PDIA3^{C57Y} expression may alter folding or quality control of integrins (**Figure 5I and Figure EV3C and D**). To investigate whether integrins contribute to enhanced neuritogenesis upon PDIA3 overexpression, we have performed experiments in cell culture using Echistatin, a potent and selective antagonist of integrins signaling (Kapp *et al*, 2017). Echistatin treatment totally blunted the effects of PDIA3 overexpression on neuritogenesis, whereas it did not exert a

significant action on cells expressing mutant PDIA3 (new **Figure 5H**). Together, our results indicate that overexpression of PDIA3 may improve integrins quality control, resulting in enhanced neuritogenesis, whereas overexpression of mutant PDIA3 does not.

The discussion about integrins was also extended as follows:

'Remarkably, expression of PDIA3^{C57Y} led to down-regulation of members of the Integrin family, a class of cell adhesion proteins that are obligate substrates for PDIA3/CNX/CRT pathway in the ER (Jessop et al, 2007). Accordingly, inhibition of integrin signaling suppressed the effects of PDIA3 expression enhancing neuritogenesis while not significantly impacting cells expressing PDIA3^{C57Y}. Moreover, biochemical analysis of a panel of integrins indicate that mutant PDIA3 may disturb their folding and/or quality control mechanisms. We reason that the failure to induce signaling events initiated by these adhesion molecules results in altered actin cytoskeleton dynamics as a downstream response, impacting neuronal connectivity and function.'

Point 2: *In earlier studies, some published in EMBO (e.g. Antoniou EMBO J 21:2655-2663 (2002); Peaper et al EMBO J 24:3613-3623 (2005)), PDIA3 was identified as a part of the MHC class I loading complex and as a functional interaction partner with tapasin - so is MHC class I cell surface expression normal in patient or C57Y-expressing cells? This is an important point because there is evidence that MHC class I plays a role in human brain development (e.g. Huh GS, et al. Science 290:2155-2159 (2000); Goddard CA, Butts DA, Shatz CJ Proc Natl Acad Sci USA 104:6828-6833 (2007) and others). I wonder whether dysregulation of MHC quality control contributes to the patient phenotype - something that may not be apparent in the zebrafish or mouse injection model.*

Response:

We thank the reviewer for this important observation. While the possible participation of MHC-I in the clinical phenotype merits to be investigated, we respectfully argue that this point is out of the scope of the present manuscript. Mechanistically, we focused the current study on intrinsic effects on neurons and its relation to integrin signaling.

Regarding this very intriguing point raised by the reviewer, our proteomic data indicates possible effects of mutant PDIA3 on MHC-I pathway in the hippocampus with downregulation of several components such as tapasin, antigen peptide transporter 1 and 2, β2-microglobulin, and H2 class I histocompatibility antigen. This information is now better described in the results section. We believe this question warrants further exploration in the future as a full study, and the present investigation cannot address whether alterations in the MHC-I pathway is epiphenomena or causative of the cognitive and electrophysiological impairment resulting from mutant PDIA3 expression. Importantly, we have succeeded in generating a knockin mouse model to properly

investigate this important point, planning on a full characterization in the future to assess immune cell function and its relation to possible cognitive phenotypes. This study will take at least 2 more years of research. The results section was modified as follows:

'In addition, proteins related to the MHC-I pathway, such as tapasin, antigen peptide transporter 1 and 2, β2-microglobulin and H2 class I histocompatibility antigen, were also down-regulated (Appendix Tables S5 and S6), consistent with the role of PDIA3 as a scaffold that regulates antigen presentation (Garbi et al, 2006). We are currently generating PDIA3^{C57Y} knockin mice to study the possible connection between MHC-I biology and cognitive impairment'.

Point 3: *A related point is that there is a notable difference in the interaction of the PDIA3 C57Y mutant protein with several immune and interferon-inducible proteins, compared to wild-type PDIA3 (supplementary tables). Since patient 501 died having had recurrent infections, has immunity of the surviving individuals been investigated? A full immunological analysis is clearly beyond the scope of the current manuscript, but please could the authors comment on this in the text.*

Response:

We thank the referee for this observation. The immunity of patients was not investigated. Despite the death of patient 501 due to recurrent infection, the other have never presented signs of compromised immune system. We have added a note to the results section as follows:

'Despite the death of one patient due to recurrent infections (Appendix Text), the other cases did not present signs of compromised immunity as it would be expected if MHC-I pathway was disrupted.'

We have also extended the discussion about the possible role of this immune pathway during neurodevelopment as follows:

'The MHC-I pathway has been shown to regulate synaptic plasticity, promoting signaling from post- to presynaptic terminal (Shatz, 2009). It has also been suggested to mediate crosstalk between immune cells and neurons, contributing to synaptic pruning by microglia, a possibility not yet proven (Elmer & McAllister, 2012). Whether mutant PDIA3 impairs MHC-I activity in the nervous system leading to altered synaptic refinement and cognitive dysfunction in ID is an important point to be addressed in future studies.'

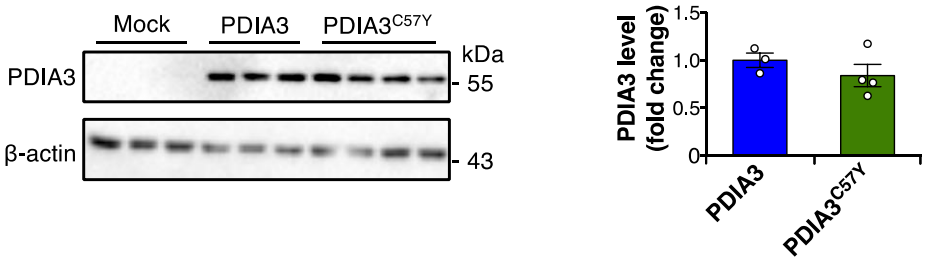
Point 4: *For the zebrafish and transfection experiments with PDIA3-V5 and PDIA3C57Y-V5, please could the authors confirm where the V5 tag was positioned - it should be upstream of the KDEL retention-retrieval motif. This is particularly important when considering the appearance of intracellular puncta in Fig 6.*

Response:
We confirm that the V5 tag sequence is upstream of the KDEL retention sequence.

Point 5: *I would like to see further controls and discussion of the caveats for the murine transgene expression experiments- although the V5-tagged PDIA3 protein expression control looks equal in Fig EV2, the IF in panel B suggests that expression of C57Y is less than wild type. I would also like to see a blotting and IF control for endogenous PDIA3, in addition to the V5 blot, because it is possible that misfolded C57Y induces the degradation or mis-localization of endogenous PDIA3 in this essentially heterozygous situation.*

Response:
We appreciate the rigorous analysis of the referee. We have modified the methods section to clarify that human PDIA3 overexpression was employed in this study. The comparison of human and mouse protein levels by Western blot or immunofluorescence is difficult due to different affinities of antibodies for each protein. We do not have an antibody specific for the mouse protein. Here we provide Western blot analysis of hippocampal tissue overexpressing human PDIA3 to illustrate this technical limitation (see **Letter Figure 2**). To solve this question, we performed relative quantification of human to endogenous PDIA3 levels from proteomic data by measuring intensity of shared peptides in mouse and human proteins. This analysis showed that human PDIA3 is overexpressed 2.6 (wild-type) and 2.1 (mutant) fold relative to endogenous PDIA3. Thus, the referee is correct in pointing out that the levels of wild-type protein are slightly higher than those of the mutant form (about 1.2 fold). Of note, Western blot analysis of relative levels of human wild-type and mutant PDIA3 corroborated the proteomics result (**Letter Figure 2**). The lower levels of the mutant protein may be due to its increased degradation, but it is still enough to cause detrimental effects to mouse brain function when compared to the group overexpressing the wild-type form. The variation of endogenous PDIA3 is minimal, around 10%, between groups overexpressing human wild-type and mutant protein according to quantification of mouse only peptides from the proteomics data. Actually, endogenous PDIA3 levels are the same in PDIA3^{C57Y} and mock control. These results are presented in the manuscript as follows:

'Quantification of PDIA3 overexpression by measuring shared peptides in mouse and human proteins showed 2.6- and 2.1-fold the endogenous levels for the wild-type and mutant forms, respectively (Appendix Table S7). According to quantification of peptides that are exclusive of the mouse protein, there is minimal variation in the levels of endogenous PDIA3 between AAV-PDIA3 and AAV-PDIA3^{C57Y} groups (Appendix Table S7).'



Letter Figure 2. Western blot analysis of human PDIA3 overexpression. Young mice at P55 received bilateral stereotaxic injection of adeno-associated virus serotype 9 (AAV9) to express human wild-type PDIA3-V5 or PDIA3^{C57Y}-V5 and GFP or GFP alone (Mock) into the hippocampus. Western blot analysis against PDIA3 detected the human form, but not endogenous PDIA3. Graph shows quantification of protein expression by band densitometry revealing that mutant PDIA3^{C57Y} levels are approximately 0.84 fold those of wild-type PDIA3. β-actin was employed as loading control. Rabbit anti-PDIA3 (ERp57) from Santa Cruz Biotechnology H-220, cat. #28823.

Point 6: *It is not clear to me why the calnexin/calreticulin IP in Fig EV4 panel H was not also performed with the C57Y R282A mutant. This is needed to evaluate any indirect role of C57Y in lectin chaperone association, in light of the recombinant protein work in Fig 6.*

Response:

We thank the reviewer for suggesting this complete experimental set. This experiment was performed as control to verify loss of interaction with calnexin and calreticulin due to the R282A mutation in PDIA3. As requested, here we provide an additional co-IP experiment to replace the initial control showing that PDIA3$^{C57Y/R282A}$ also loses interaction with calnexin (**Figure EV4H**). The Western blot against calreticulin was not sensitive enough to detect the protein in the co-IP fraction of this new experiment.

Point 7: *The data in Fig EV4A suggest that C57Y does not, by itself, cause an unfolded protein response, yet the C57Y protein does aggregate - this merits further discussion in the text.*

Response:

We thank the referee for this interesting comment and have added discussion about this point as follows:

'*The accumulation of mutant PDIA3^{C57Y} aggregates may be limited by an interaction with calnexin or calreticulin interaction directing their lysosomal degradation. Since alterations of PDIA3/CNX/CRT network may affect only a subset of ER cargo, no overt ER stress appears to result from mutant PDIA3 expression.*'

Point 8: *There are some minor typographical, grammatical and spelling errors requiring further proofreading.*

Response:
We thank the reviewer for pointing out language issues. We performed proofreading of the manuscript to correct these errors.

Referee #3:

This is a potentially interesting manuscript that describes the discovery of a homozygous variant in the PDIA3 gene in 4 relatives in 2 sibships from a highly inbred family. There is extensive work done that aims to characterize this missense variant Cys57Tyr in zebrafish.

While the work looks interesting there seem to be 2 major concerns:

Point 1: *Because of the complex inbreeding, the authors were unable to assess the mapping information that was derived from SNP typing in a single linage analysis. The authors therefore used a stepwise analysis that started with 3 affected (from 2 sibships), then took the candidate regions forward and tested all 3 affected in the second sibship while leaving out the first affected, and then checked which of the remaining candidate regions was homozygous in all 4 affected. This strategy is not unreasonable, but it is complicated and the result is not entirely clear to me.*

Response:

We thank the referee for acknowledging the relevance of our study and for the careful interpretation of our experimental design. We have performed a thorough genetic analysis using a general strategy for large families in inbred communities. Pedigree was too large for the program employed such that including all individuals with SNP genotype data would have exceeded the capacity of the program. We understand that the strategy was not clear and thus added a summary before the details. In this revised version of the manuscript, we provide a complete explanation of our experimental strategy to identify the mutant gene. We mapped the disease locus with a multipoint LOD score of 3.85 assuming a simplified pedigree, which means that the probability that we found such a locus by chance (and that it might not in fact be the real locus) is 1 in 7,000. No other possible candidate locus was found, as would be expected for such a large pedigree.

The added summary follows: '*To summarize the 3-step linkage analysis to search for a unique region where the homozygous SNP genotype was shared by affected individuals only and thus homozygosity was possibly due to identity by descent: first analysis A was performed assuming a partial pedigree and detected ten regions yielding maximal LOD scores >2.8, on chromosomes 1, 6, 7, 8, 13, 15, 17, 19 and 21. Another partial pedigree was assumed in the second analysis B, and the regions on chromosomes 1, 8, 13, 17 and 21 were eliminated due to decreased LOD scores. Region on chromosome 7 was eliminated due to small size. We investigated the genotypes in the remaining homozygous regions and found that in the two regions on chromosome 15 but not in regions on chromosomes 6 and 19, exclusively patients shared the homozygosity. We performed a detailed linkage analysis C for those regions*

(15q15.1-21.1 and 15q22.31) using all SNP markers and a simplified pedigree that included all participants with SNP genotype data but assumed closer kinship. Both regions yielded high LOD scores, but the latter region was excluded because exome data were not homozygous. We thus identified 15q15.1-21.1 as the disease locus.'

We hope that the screening strategy is now clearer. This paragraph would be useful for non-geneticists, and we thank the referee for alerting us.

Point 2: *The authors then performed a single exome, and apparently checked only the candidate region for potential homozygous variants. The only variant that looked promising from this region was in PDIA3. This is problematic, because in such a highly inbred family, there are likely multiple homozygous variants whether disease-causing or not. I am not sure how much of the genome was effectively excluded by the stepwise linkage that the authors employed here.*

As a solution, I believe that the authors need to perform at least one more exome (in patient 401), in order to compile a list of homozygous loss of function, and likely damaging missense variants that are shared between these 2 individuals. That would allow a more targeted analysis of all possible candidate genes, which can then be compared with the linkage data (or checked by Sanger) in the other two affected. This is essentially the opposite strategy (candidate variants first, and then confirm/exclude by linkage) to what is in the manuscript now. Having the two strategies side by side could strengthen confidence in the validity of the final result that points to PDIA3 as the culprit gene.

Response:

We appreciate the rigor of the referee. Indeed, we had already performed a SNP genotype-based analysis for the same purpose as part of our routine exome data evaluation strategy. There were many other rare (frequency <0.01) homozygous variants listed in the exome file, 20 other exonic variants to be precise (new **Appendix Table S4**). Not all those variants were predicted deleterious. Nonetheless, none of those was in a region of homozygosity shared exclusively by all affected individuals. We are now presenting this information in a new table to clarify the question. A second file would eliminate only half of those homozygous variants, as siblings share on the average half of their autosomal information. Thus, no other exome file is needed according to our criteria.

We can explain the rationale behind our strategy: To avoid missing a variant in regions outside the candidate region (i.e., a very small region of homozygosity shared by affected individuals only as judged by SNP data), we selected from the exome file all rare homozygous variants that could possibly alter protein sequence, and then utilized SNP genotypes of all participants to investigate whether any of those variants fell in a

region of homozygosity shared by all patients only (**Appendix Table S4**). We found 20 such variants besides the one in *PDIA3*. As expected, all of them, except for the one in *MUC4*, were in regions of homozygosity in patient 502 (with exome data). The novel homozygous variant in *MUC4* located in a heterozygous region was considered unreal, most likely an artefact due to misalignment of a paralog sequence (only 2 reads were aligned), and thus eliminated. All other 17 variants on the autosomes plus the 2 on the X-chromosome were in non-linked regions, where either one or more patients do not share the homozygosity or one or more unaffected relatives also share the homozygosity (**Appendix Table S4**), and thus eliminated as potential causative mutations. Since all 20 variants were excluded, we concluded that a second exome file was not needed.

In addition, the exome file was evaluated for any exonic deletions and duplications within the gene locus, as mentioned in methods. Finally, the identified gene locus was investigated thoroughly for possibly another variant that could underlie the disease, but no such candidate was found (**Appendix Table S4**).

In summary, by linkage mapping and exome sequence analysis, we identified a single candidate variant. The variant was validated as the causative gene defect via other cellular and molecular studies.

Point 3. *Given that parents were apparently normal, and inheritance appears as a classical autosomal recessive, I am puzzled that there should be consistent effects of expression of the allele in normal cells from mouse and in zebrafish embryos. Surely that indicates a dominant effect.*
While I am aware of situations where an allele that works through haploinsufficiency in humans acts as a true recessive null in mice, the observation of overexpression effects appears to suggest the opposite situation. This is puzzling to me.
Somewhat reassuring in this respect is that the phenotype of knockout of PDIA3 in mouse embryonic fibroblasts, can be rescued by the (human) wildtype PDIA3 but not the mutant allele. Also, overexpression of wildtype PDIA3 enhances neurite number and length but expression of the variant does not. So some of the experiments do support a loss of function scenario.
Overall, I would have liked to see a critical discussion of how we should interpret these findings in different systems, and how we may understand effects that seem to be dominant in some paradigms and recessive (loss of function) in others.

Response:
We thank the reviewer for this critical interpretation. As discussed above with reviewer 1, the deleterious effects obtained in overexpression experiments may be interpreted by a toxic gain-of-function due to the C57Y mutation or the competition between mutant and endogenous PDIA3, with the mutant protein concentration reaching a threshold for

toxic properties and/or hindrance of endogenous activity to manifest at the phenotypic level. These possibilities are not mutually exclusive. We reason that overexpression may saturate the system where mutant PDIA3 competes with the endogenous form. Further analysis of the proteomic data showed that human PDIA3 is overexpressed at least 2-fold relatively to the endogenous protein in the mouse hippocampus where only a subpopulation of neurons expresses the transgene, supporting the argument that mutant PDIA3 can effectively compete with the endogenous PDIA3 for interaction with substrates and other chaperones in the ER milieu. For the zebrafish experiment, we estimate the overexpression of PDIA3 according to the following considerations:

1) average dry weight of zebrafish embryo between 0 and 48 hpf: 70 μg (PMID: 26292096).

2) about 50% of the yolk mass is consumed within 48 hpf, resulting in approximately 35 μg of dry weight of cells (PMID: 8589427).

3) the average composition of an eukaryotic cell is approximately (% of dry weight): proteins, 50; lipids, 10; RNA, 8; DNA, 1. (https://bionumbers.hms.harvard.edu/; ID 111209).

4) about 5% of total RNA is mRNA (from yeast). (https://bionumbers.hms.harvard.edu/; ID 100264).

Thus, a zebrafish embryo at 48 hpf has approximately 140 ng or 140,000 pg of mRNA.

5) considering that the pool of mRNA in a developing embryo represents a universe of 1,000 transcripts on average for the distinct developmental stages (PMID: 29144233), that *pdia3* transcript has the size of an average transcript, and that the amount of mRNA is rather similar for different transcripts, there should be approximately 140 pg of pdia3 mRNA at 48 hpf.

6) given that 70 pg of PDIA3 mRNA were injected per embryo and considering that it is evenly distributed upon cell division, it is expected that overexpression levels are within a concentration range that enables the competition between the human and the endogenous PDIA3 for substrates and interacting chaperones in the ER. While some degradation of the injected mRNA is expected by 48 hpf, we point out that the calculation for overexpression presented is very conservative. Actually, pdia3 mRNA at earlier developmental stages is expected to be much lower, while the amount of exogenous mRNA degradation is probably negligible, what would translate in much higher overexpression levels and efficient competition of the human protein displacing the endogenous counterpart from the proteostasis network.

These arguments about loss- and gain-of-function and competition between human and endogenous proteins were further expanded in the discussion as follows:

'We previously identified dominant variants in PDIA3 as risk factors for amyotrophic lateral sclerosis (Woehlbier et al, 2016). Here we report a homozygous variant that causes severe syndromic ID. This finding offered the opportunity to further understand the biological function of a major ER oxidoreductase in the nervous system

using various biochemical, cellular and whole organism experiments. We provide compelling evidence supporting pathogenicity of the variant, which may act by both loss- and gain-of-function mechanisms.

Haploinsufficiency or dominant negative effect resulting from the C57Y variant are unlikely since heterozygous individuals are unaffected. The substitution of a catalytic cysteine strongly suggests loss-of-function as the pathogenic mechanism. On the other hand, PDIA3^{C57Y} may acquire deleterious features that surpass a toxicity threshold for clinical manifestation upon expression of two mutant PDIA3 alleles. The loss- and gain-of-function mechanisms are not mutually exclusive. Indeed, our data show that the C57Y mutation causes PDIA3 aggregation and abnormal interactions with CNX/CRT while greatly compromising enzymatic activity. Overexpression experiments both in vivo and in vitro also corroborate this notion. Zebrafish development and cognitive function of mice may be affected by gain-of-toxic properties of PDIA3^{C57Y}. Alternatively, when overexpressed the mutant protein may outcompete endogenous PDIA3 for substrates and chaperones in the ER milieu, generating nonproductive enzymatic cycles that disturb folding of specific clients.'

We would like to thank again the constructive comments from this reviewer to improve the clarity, descriptions and interpretations of the genetic experiments. We hope to have satisfied the reviewer's concerns.

Dear Claudio.

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by referees #2 and 3.

While referee #2 is satisfied with the introduced changes, referee #3 has some remaining concerns regarding the genetic analysis. I should also add that this is the expertise of the referee. Do you have access to another patients from the family? The strength of the analysis lies in the combination of approaches used, but it is also important that the genetic analysis stands on its own.

Let's discuss further one can address the raised concern.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

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https://emboj.msubmit.net/cgi-bin/main.plex

Referee #2:

Medinas et al have thoughtfully and thoroughly revised their manuscript in the light of suggestions made by the referees.

The additional controls, along with the deeper functional consideration of integrin quality control, have strengthened the manuscript and resulted in a topical piece of work that should be widely read.

I have no further concerns to be addressed.

Referee #3:

The genetics situation has improved a bit. Nonetheless, there is really no reason why the authors would not run an exome on another patient from the family as I suggested. This by now is no longer difficult, and as I stated in my review it provides a complementary strategy for gene identification to the rather complex (because of consanguinity loops) linkage strategy that they employed. Please note that the genetics community has become quite critical of papers that present a single deleterious allele. In fact, the American journal of Human Genetics will not consider any such manuscript. The reason of course being that among a sea of variants, one can easily go for the one that is a red herring.

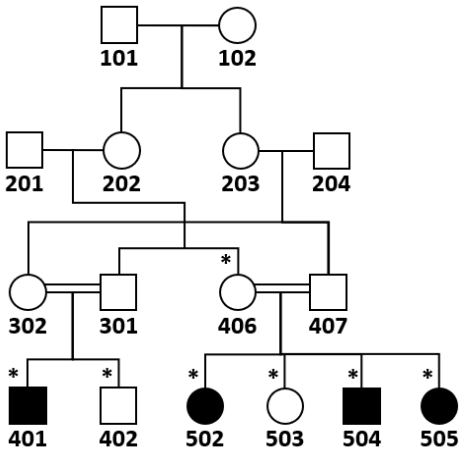
The answers to the question that each of us reviewers raised concerning loss versus gain of function are not within my area of maximum expertise. Nonetheless, I do feel that the scenario that they now present where overexpression creates a situation that is different from that which actually occurs in the carrier parents would lead me to look quite critically at their relevance for the paper. Perhaps doing overexpression experiments was not such a good idea after all.

Referee #3:

Point 1: *The genetics situation has improved a bit. Nonetheless, there is really no reason why the authors would not run an exome on another patient from the family as I suggested.*

Response:

Based on the results provided and the bioinformatics analysis, the exome file of another patient would not provide any useful data and thus would not add essential data to the study. This request would unnecessary delay the publication as we would have to get exome service from Yale University Center for Genome Analysis (YCGA). In brief, we had performed linkage analysis and evaluated SNP genotypes at all loci yielding relatively high LOD scores. Modified from page 22 of main text: Only two candidate regions remained in Linkage analysis B (15q15.1-21.1 and 15q22.31). For the last analysis (C), we included all available SNP data but again had to assume a simplified pedigree (presented below) to not exceed the capacity of program SimWalk. Both loci yielded LOD scores >3.8. At 15q15.1-21.16 maximal homozygosity was approximately 2.2 Mb. At 15q22.31 patients shared homozygosity in a 665 kb-region, but exome sequence data revealed that all three rare variants in the region were heterozygous, indicating that the SNPs in the region were non-informative and the homozygosity was not due to identity by descent. Hence, we eliminated the latter locus and identified the disease locus as the 2.2-Mb region at 15q15.1-21.16. The maximal LOD score was 3.85 for the simplified pedigree assumed.



Simplified pedigree used for linkage analysis C.
SNP genotype data of individuals marked with asterisks were used.

Conclusion: There is only a single region where homozygosity is shared by affected members of the family only, and that small region has been analysed extensively for genetic variation. Nonetheless, let us assume that we have an exome file for distant uncle 401. The file is expected to contain 4 of the other rare autosomal variants in the file of 502 (according to **Table S3**), but other patients are not homozygous at the loci of those genes according to SNP genotype data (**Table S4**). As for the X-chromosome variant, unaffected relatives also share the haplotype. Patient 401 is expected to have many rare variants not present in patient 502. So, the conclusion of the study would be the same as the present one which is based on the data we have provided.

Point 2: *This by now is no longer difficult, and as I stated in my review it provides a complementary strategy for gene identification to the rather complex (because of consanguinity loops) linkage strategy that they employed.*

Response:

Linkage results are robust, considering that we mapped the disease locus using **simplified pedigrees with a highly significant LOD score to a small region** harbouring a few genes and found no other region of homozygosity shared by patients only. We certainly understand the concern about excess parental consanguinity, 304-305 and 303-306. However, the simplified pedigrees assumed in linkage analyses should compensate for those consanguinities. Parents 406 and 407, who are double-second cousins, are not included in any analysis but it was assumed that their siblings are born a single-first cousin marriage. In other words, we assumed grandparents to be parents of affected siblings. Also, affected distant uncle 401 was assumed to be a double-nephew to parents 406 and 407 rather than double-cousin, a more distant kinship, but his relation as a double second cousin to parents had to be ignored to stay within the capacity of the program. SimWalk can handle a larger family than EasyLINKAGE, albeit with very slow pace. The obtained LOD score of about 4 is much higher than the acceptable score of 3.

Our team is one of the few who still performs linkage analysis rather than just filtering several patient exomes, which is much simpler to do. We want to make sure that there is no second locus that could contribute to the disease, as we are working on families with unusual phenotypes, as in the presented family with neurodevelopmental impairment and skeletal anomalies.

Point 3: *Please note that the genetics community has become quite critical of papers that present a single deleterious allele. In fact, the American journal of Human Genetics*

will not consider any such manuscript. The reason of course being that among a sea of variants, one can easily go for the one that is a red herring.

Response:
We have identified the disease gene locus with a highly significant LOD score. There is no other candidate variant in the gene region, i.e., any rare (frequency <0.01) SNV or indel that are deleterious to protein structure/function (**Table S3**). Four of the rare variants at other loci are expected to be present in exome file for 401, but not in all of the affected siblings.

We hope that our explanations would convince the referee that another exome file is not necessary. We tried to make it clearer to the reader with revisions in both main text and supplementary materials.

2nd Revision - Editorial Decision **17th Sep 2021**

Dear Claudio,

Thanks for sending me your response to the comments by referee #3 regarding the genetics situation and the need to carry out exome sequencing on another patient.

I have sought further advice on this issue from a good expert in the field. The advisor agrees with the reviewer that a single allele in a single family, no matter how strong the linkage and functional studies, is weak. However, the advisor also doesn't see much advantage to do exome sequencing of another person. It would be good though to include the sequence data from the other affected cases into the paper.

Please also make sure that you have a balanced discussion on the genetics part and that you don't overstate the findings.

When you submit the revised version will you also take care of the following points:

- "Competing Interests" should be called "Conflict of Interest"

- The reference list - for articles with more than 10 authors the author list should be cut after 10 authors followed by et al.

- please make sure that the funding information matches between the MS and the online submission system

- Appendix file: Please label figures as 'Appendix Figure S#' or 'Appendix Table S#' and correct callouts in text. Regarding Appendix Tables 3-7 please include them in the Appendix. If you want to keep them as individual tables or if they don't fit the appendix format they should be renamed as 'Dataset EV#' tables and the legends added to the individual files.

- Source data needs to be split into one file per figure. If there are multiple file types for a figure they can be ZIPed together.

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please look at the word file and the comments regarding the figure legends and respond to the issues.

- I think the image in Fig 1C is reused in S1A - please mention that in the figure legends.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

That should be all - you can use the link below to upload the revised version.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 16th Dec 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Dr. Karin Dumstrei
Senior Editor
EMBO Journal

Re: EMBOJ-2020-105531

Dear Dr. Dumstrei,

Thank you very much for consulting with another expert on human genetics to make a decision regarding further revision of our manuscript entitled **'Mutant PDIA3 causes neurodevelopmental impairment by disturbing endoplasmic reticulum proteostasis'** by Danilo B. Medinas, Sajid Malik, Esra Yıldız-Bölükbaşı, Janina Borgonovo, Mirva J. Saaranen, Hery Urra, Eduardo Pulgar, Muhammad Afzal, Darwin Contreras, Madison T. Wright, Felipe Bodaleo, Gabriel Quiroz, Pablo Rozas, Sara Mumtaz, Rodrigo Díaz, Carlos Rozas, Felipe Cabral, Ricardo Piña, Vicente Valenzuela, Ozgun Uyan, Christopher Reardon, Ute Woehlbier, Robert H. Brown, Miguel Sena-Esteves, Christian Gonzalez-Billault, Bernardo Morales, Lars Plate, Lloyd W. Ruddock, Miguel L. Concha, and ourselves under consideration for publication in *EMBO Journal*.

Regarding the question of this expert, if we have sequenced the mutation in all affected members of the family, we have performed Sanger sequencing only in patients 401 and 502, in addition to unaffected subjects 402 and 406. The results validated the mutation as homozygous in patients from both affected sibships, in addition to identifying unaffected subject 406, mother of 502, as a heterozygote. Electropherograms are now presented as Appendix Figure S3 and enclosed in this letter. In the revised manuscript, we also added a new table displaying SNP genotypes in the disease gene region as Appendix Table S3 to show segregation in the family.

We appreciate your support during the revision and thank you for the time and effort in handling the manuscript. We hope that the additional data provided will make the manuscript suitable for publication in *EMBO Journal*.

The authors state that the material in the manuscript corresponds to original research, has not been previously published and has not been submitted for publication elsewhere while under consideration by *EMBO Journal*. The authors declare no conflict of interest.

Consultation with another human genetics expert:

Point 1: *The expert had one question and that is if you sequenced the mutation in all the affected cases in the family?*

Response:
We have validated the mutation by Sanger sequencing as homozygous in patient 502 and distant affected uncle 401, heterozygous in unaffected subject 406 and not present in unaffected subject 402. Electropherograms are now presented in the revised manuscript as Appendix Figure S3 and below as Figure 1.

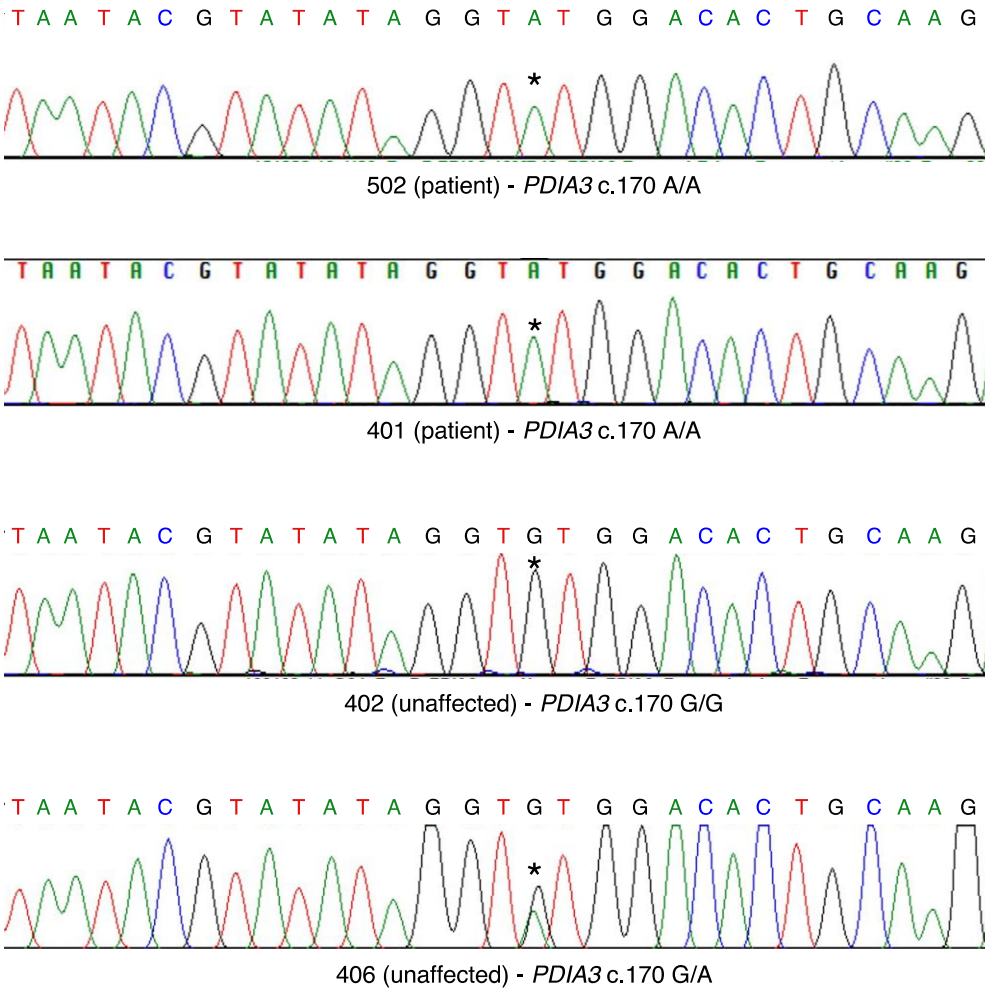

Figure 1. Sanger sequencing of mutation region in *PDIA3* of patients and unaffected relatives. Electropherograms show that patients 502 and 401 are homozygous for mutation c.170G>A while unaffected subject 406 is a heterozygous carrier and unaffected subject 402 is a non-carrier. Asterisks mark the position of the base substitution.

3rd Revision - Editorial Decision **14th Oct 2021**

Dear Claudio,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a careful look at everything and all looks good. I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Corresponding Author Name: Claudio Hetz and Aslihan Tolun
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2020-105531

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A- Figures

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The data shown in figures should satisfy the following conditions:

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- 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.

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- 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?	Mouse studies. For behavioral analysis, sample size was estimated for measurement of a continuous variable following the model n=1+2C(s/d)^2 (n is the number of animals; C is a constant that depend on the power and statistical differences; s is the standard deviation of the measurement and d is the differences expected in each condition; see "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research"). Assuming statistical power of 0.9 and significance level of 0.05, considering the mean standard deviation of 50% for behavioral tests in our animal facility, and expecting to observe experimental differences of approximately 80% between groups expressing the wild-type and mutant proteins, a total of ten male mice was calculated to obtain significant results. The same experimental cohort used for behavioral studies was latter submitted to electrophysiological and proteomic analyses, with number of samples defined based on previous experience of the investigators. Zebrafish and cell culture studies. No pre-specified effect size was estimated.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For mouse studies, sample size was estimated as described above. For zebrafish studies, sample size was defined based on previous experience of the investigators.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Exclusion criteria were established for different experiments. In behavioral analysis, if a data point varyied two times the standard deviation from the mean, the Grubb's test was performed to identify a possible outlier. In proteomic analysis, the data was manually inspeceted. The samples were divided into three mas spectrometry runs. We noticed some inconsistencies in quantification of major differentially expressed hits in two samples of run 1, named PDIA3WT1 and PDIA3WT2. Albeit very unlikely, an experimental noise introducing biological variability was deemed possible since these two animals were housed in the same cage. Thus, the bioinformatics analysis was performed considering only samples from run 2 and 3. In zebrafish studies, unfertilized embryos were removed during blastula stage and later larvae with no evident anterior-posterior axis (<7%) were excluded from further analyses.
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.	In mouse studies, littermates were assigned by chance to receive injection of adeno-associated viral particles to express either wild-type or mutant PDIA3, in addition to a third group not expressing PDIA3 (Mock). In zebrafish studies, one-cell stage eggs from ten couples were randomly pooled in three groups for the injection of either wild-type or mutant PDIA3 mRNA or uninjected for the control group.
For animal studies, include a statement about randomization even if no randomization was used.	In all animals experiments, individuals were randomly allocated into three groups, mock, PDIA3 and mutant PDIA3.
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.	The investigators performing behavioral tests and electrophysiological measurements in mice were blinded to the experimental groups until data acquisition was completed. Cell culture experiments involving image quantification (axonal length, neuritogenesis, lamellipodia, aggregates) were also performed in a blinded fashion.
4.b. For animal studies, include a statement about blinding even if no blinding was done	In mouse studies, investigators were blinded to experimental groups in behavioral tests, electrophysiological measurements ex vivo and proteomics analysis.
5. For every figure, are statistical tests justified as appropriate?	Yes. All statistical tests were selected considering specifics of experimental design and number of groups.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jji.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	D'Agostino and Pearson and/or Shapiro-Wilk normality tests were employed to verify normal distribution.
Is there an estimate of variation within each group of data?	The standard deviation provided an estimate of variation within each experimental group.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	anti-acetylated tubulin (Sigma, cat. T6793, clone 6-11B-1), anti-Tau-1 (Millipore, cat. MAB3420, clone PC1C6), anti-V5 tag (Invitrogen, cat. R960-25), anti-beta-actin (MP Biomedicals, cat. 8691001, clone C4), anti-calnexin (Enzo Life Sciences, cat. ADI-SPA-860-F), anti-calreticulin (Enzo Life Sciences, cat. ADI-SPA-600-F), anti-calponin-3 (abcam, cat. ab151427), anti-Psmb10 (abcam, cat. ab183506), anti-PDIA3 (Santa Cruz Biotechnology, cat. sc-28823), anti-GFP (Santa Cruz Biotechnology, cat. sc-9996, clone B-2), anti-GFP (abcam, cat. ab6556), anti-BiP (abcam, cat. ab21685).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Mouse embryonic fibroblasts (MEF) from Marek Michalak laboratory, NSC-34 cell line from Neil Cashman laboratory, HEK-293 from ATCC. Wild-type and knock-out MEF for PDIA3 were verified by Western blot. NSC-34 and HEK-293 cell lines were previously published by our group and routinely examined for expected morphology and growth rate. No further authentication was performed. All cell lines were free of mycoplasma contamination as routinely assessed using nuclear staining with Hoechst 33342 and EZ-PCR Mycoplasma Test Kit (Biological Industries).

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mus musculus, C57BL/6 strain, male, 2 months old when submitted to stereotaxic injection, 3-4 months old during behavioral assessment and 6 months old when submitted to euthanasia, obtained from Institute of Public Health of Chile, housed in standard facility in cages with lid and filter, water and food ad libitum, 12h dark/light cycle and 25°C. Zebrafish (Danio rerio), wild-type Tübingen and transgenic Tg(Huc:mCherry), Tg(sox17:GFP) and Tg(actb1:mCherry-utrCH) lines, male and female at larvae stage.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal procedures were approved by the Bioethics Committee of the Faculty of Medicine of University of Chile.
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.	We confirm compliance to all guidelines and recommendations for animal use and care.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The Ethical Review Committee of Quaid-i-Azam University and the Boğaziçi University Institutional Review Board for Research with Human Participants.
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.	We confirm that informed consent was obtained from all subjects and experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Please note that no experiment was done on humans but only clinical investigations and genetic studies using DNA extracted from peripheral blood samples.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	We confirm that consent to publish photos was obtained.
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	Variant (PDIA3 c.170G>A; NM_005315) has been submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) with Submission ID SUB3488668. Proteomics dataset has been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al, 2019) partner repository with accession number PXD026507.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Data of all quantifications are provided as supplementary information in spreadsheets according to figure panels.
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).	All clinical data collected from patients are either described in materials and methods section or provided as supplementary information.
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA