EMBO Molecular Medicine

Abnormal ClC-3/TMEM9-mediated endosomal ion transport in CLCN3-associated neurodevelopmental disease

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

19th May 2026

Dear Prof. Jentsch,

Thank you again for submitting your work to EMBO Molecular Medicine. We have now received feedback from the two referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees found the study interesting and relevant and expressed strong support for it. They also raised a number of relatively minor issues, which we ask you to address in your revision.

I think the referees' recommendations are clear and need not be repeated here. All of the issues raised need to be carefully addressed. As you may already know, our editorial policy allows in principle only one round of major revision, so it is essential that your response fully addresses the referees' comments. Please feel free to contact me if you would like to discuss any of the issues raised by the referees in further detail.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published, we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our author's guidelines for proper formatting of your revised article for EMBO Molecular Medicine.

I look forward to receiving your revised manuscript.

Sincerely,
Jingyi

Jingyi Hou
Senior Editor
EMBO Molecular Medicine

*****

When preparing your revised manuscript, please refer to our guidelines: https://link.springer.com/journal/44321/submission-guidelines#cms-Revised-submissions. We perform an initial quality control of all revised manuscripts before re-review; failure to include requested items will delay the evaluation of your revision.

We require:

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

2) Individual production quality figure files as .eps, .tif, .jpg (one file per figure). For guidance, download the 'Figure Guide PDF': https://media.springernature.com/original/springer-cms/rest/v1/content/27825798/data/v1.

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

4) A complete author checklist, which you can download from our author guidelines. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary

datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public.

In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.

7) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. You will receive a separate email with instructions for providing source data with your revised manuscript, including how to upload and organize the files.

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

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

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc.

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

11) The paper explained: EMBO Molecular Medicine articles are accompanied by a summary of the articles to emphasize the major findings in the paper and their medical implications for the non-specialist reader. Please provide a draft summary of your article highlighting

- the medical issue you are addressing,

- the results obtained and

- their clinical impact.

This may be edited to ensure that readers understand the significance and context of the research. Please refer to any of our published articles for an example.

12) Author contributions: You will be asked to provide CRediT (Contributor Role Taxonomy) terms in the submission system. These replace a narrative author contribution section in the manuscript.

13) A Conflict of Interest statement should be provided in the main text.

14) Every published paper includes a 'Synopsis' to further enhance discoverability. Synopses are displayed on the journal webpage and are freely accessible to all readers. They include a short stand first (maximum of 300 characters, including space) as well as 2-5 one-sentences bullet points that summarizes the paper. Please write the bullet points to summarize the key NEW findings. They should be designed to be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion of key acronyms and quantitative information (maximum of 30 words / bullet point). Please use the passive voice. Please attach these in a separate file or send them by email, we will incorporate them accordingly.

Please also provide a visual abstract to illustrate your article as a PNG file 550 px wide x 300 px high.

15) All Materials and Methods need to be described in the main text using our 'Structured Methods' format. According to this format, the Methods section includes a Reagents and Tools Table (listing key reagents, experimental models, software and relevant equipment and including their sources and relevant identifiers) followed by a Methods and Protocols section describing

the methods, ideally using a step-by-step protocol format. The aim is to facilitate adoption of the methodologies across labs.

Please download and fill our Reagents and Tools Table template (.docx), which you can find in our author guidelines: https://link.springer.com/journal/44320/submission-guidelines#structuredmethods.

When submitting your revised manuscript, please do not include the Reagents and Tools Table in the Methods section of the manuscript but upload it as a separate file choosing the file type "Reagent Table".

***** Reviewer's comments *****

Referee #1 (Remarks for Author):

This is a strong and interesting manuscript that expands the genetic and clinical spectrum of CLCN3-associated neurodevelopmental disease and provides a mechanistic re-evaluation of disease-associated ClC-3 variants in the context of the recently identified TMEM9/TMEM9B inhibitory β-subunits. The combination of clinical genetics, electrophysiology, imaging-based vacuolization assays, and structural interpretation provides a coherent framework for distinguishing likely loss-of-function, gain-of-function, and functionally unresolved variants. Overall, I find the manuscript suitable for publication after minor revision.
Moderate comments
1. The use of ClC-3c for plasma membrane electrophysiology and ClC-3a for vacuolization assays is technically well justified. However, the manuscript should more explicitly acknowledge that these two assays examine different splice variants in distinct membrane contexts. Plasma membrane current properties measured with ClC-3c should therefore be interpreted as complementary to, rather than quantitatively equivalent to, ClC-3a activity in endosomal compartments.
2. LINE367: The statement that bath pH 4.5 "mimics the acidity of endolysosomes" should be phrased more carefully. The electrophysiological assay uses ClC-3c, which is described as localizing to recycling endosomes and partially to the plasma membrane upon overexpression, whereas pH 4.5 more closely approximates late endosomal/lysosomal acidity. The authors may consider rephrasing this as "acidic luminal-like conditions" or "late endosomal/lysosomal-like acidity" to avoid implying that pH 4.5 reflects the native environment of ClC-3c.
3. LINE392: The authors refer to the vacuolization assay as "more sensitive." This is plausible, as small residual ClC-3 activity or partial escape from TMEM9-mediated inhibition may accumulate over time and produce a morphological phenotype even when plasma membrane currents remain below the detection threshold. However, the term "more sensitive" should be defined more explicitly. The vacuolization assay is an indirect, cumulative morphological readout and may be influenced by expression level, trafficking, organelle identity, and image-analysis parameters.
4. Related to this point, enlarged LysoSensor-positive compartments could potentially reflect altered endosomal recycling, internalization, trafficking, maturation, fusion/fission, or organelle homeostasis, in addition to altered ClC-3-mediated ion exchange. This is particularly relevant because ClC-3 splice variants localize to distinct endosomal compartments, and TMEM9 proteins can regulate both transport inhibition and trafficking/surface expression. I suggest that the authors discuss this limitation more explicitly and avoid presenting vacuolization as a direct or exclusive readout of ion transport gain-of-function.
5. Please clarify whether the statistical unit in the vacuolization assay is vesicle, cell, image, dish, or independent experiment, and how biological replicates were handled. This is important because vesicles within the same cell or image are not fully independent observations. Please also clarify the family of comparisons used for Benjamini-Hochberg correction.
6. Have the authors examined the time course or reversibility of vacuole formation, for example by chase/recovery experiments? Such data are not essential, but clarification would help distinguish whether the assay detects an acute increase in ClC-3 activity or a cumulative defect in endosomal/lysosomal homeostasis.

Minor points:
1. LINE139: Please define the abbreviations T9A and T9B more explicitly at first use and ensure consistent terminology throughout the manuscript. The manuscript alternates between TMEM9, T9A, T9 proteins, T9 subunits, and T9-mediated inhibition.
2. LINE193: Although all constructs were verified by Sanger sequencing of the entire ORF, providing the QuikChange mutagenesis primer sequences, or at least a full list of generated and sequence-verified constructs, in the Supplementary Information would further improve reproducibility.

Referee #2 (Comments on Novelty/Model System for Author):

The view on ClC-3 function and the consequences of mutations was dramatically altered by the recent discovery of the TMEM9 inhibitory beta-subunit. This created a significant knowledge gap concerning the interpretation of these mutations. This gap is filled here and additional mutations are described.

Referee #2 (Remarks for Author):

Polovitskaya et al. report 15 individuals with a heterogeneous neuropsychiatric disorder caused by 13 missense, 1 in-frame

deletion, and 1 splice variant. Since the inhibitory subunit TMEM9 had not been known at the time of the first description of the CLCN3-related syndrome, all 20 known variants not leading to obvious LOF were tested electrophysiologically and by a endolysosomal swelling assay upon heterologous expression in HEK293 cells. 12 of the variants showed impaired inhibition by TMEM9 and of these 8 also displayed an abnormal inward current at acidic pH. The remaining variants did not produce conclusive effects. The clinical manifestations of the 16 individuals carrying function-altering variants are discussed in detail.

General

This manuscript fills an important gap in our understanding of the CLCN3-associated neurodevelopmental disorder. It underlines the crucial role of TMEM9 in the pathogenesis and confirms the relevance of amino acid residues in ClC-3 shown to mediate the interaction with TMEM9 by previous structural analysis. The paper contains high quality phenotype and functional data, clear figures, and is well written. It will be of interest for ion channel researchers as well as for clinicians.

Major

In the abstract and throughout the text the authors forget to mention the Asp87del variant that leads to a GOF. An amino acid deletion is no missense variant.

The clinical presentation is given in great detail in Table 1 and described in paragraph 3.2. To grasp the phenotype more easily I would suggest to present the ten most frequent hallmarks of the disorder with their respective frequencies. E.g. hypotonia 8/13=63%, which is more precise than "Ten subjects exhibited abnormal findings in the neonatal period. Muscular hypotonia was the most frequent feature.". This information can be added to the table or be presented as a separate figure.

No information is given on the subcellular localisation and expression levels of the ClC-3 mutants. In Figure EV2 the Y386H mutant protein shows a quite strong Golgi localisation if co-expressed with TMEM9. I would strongly suggest to address this point in the manuscript because this could be an indication for the pathomechanism. If reproducible changes have been seen this could also be mentioned in Tables S1 and S2.

The ACMG criterium PM2 has been generally downgraded to PM2_supporting.

Minor

Dystonia was not only seen in #12 and #14, but also in #3 with the unclear homozygous p.Trp385Cys variant.

It is worth mentioning that all 4 individuals showing regression have autism of which the regression is probably a consequence.

In Table S1 the ACMG criteria PM2, PM3, PP2, PP3, PP4 are claimed for c.1155G>T p.Trp385Cys, which corresponds to 7 points and thus to likely pathogenic. However, PM3 cannot be chosen because the variant is homozygous and not in combination with a bone fide pathogenic heterozygous variant. PP4 is precluded since the phenotype is an unspecific retardation with autism that can be caused by dozens if not hundreds of disease genes. PM2 can only be counted as PM2_sup. The final verdict VUS remains the same.
While the amino acid change induced by c.1155G>T has highly pathogenic predictions c.1156T>C seems rather benign on protein level. For both variants SpliceAI predicts a mild splice defect (acceptor loss) that does not reach the -0.2 threshold. The phenotype of individual 3 harboring c.1155G>T is surprisingly mild and both, c.1155G>T and c.1156G>T, are not associated with seizures.

The statement "However, these variants are extremely rare in population databases..." is not precise since only one of the eight variants without clear functional effects is found in gnomAD. This variant c.1238C>T p.Ala413Val is a conservative exchange and shows weak bioinformatic scores. All others are absent and in those for which parents were available for analysis de novo.

Detailed response to the reviewers of EMM-2026-23864 (Polovitskaya et al.)

We thank both reviewers for their positive comments. Please find our answers and details of changes made in response to their constructive suggestions below.

Referee #1 (Remarks for Author):

This is a strong and interesting manuscript that expands the genetic and clinical spectrum of CLCN3-associated neurodevelopmental disease and provides a mechanistic re-evaluation of disease-associated ClC-3 variants in the context of the recently identified TMEM9/TMEM9B inhibitory β-subunits. The combination of clinical genetics, electrophysiology, imaging-based vacuolization assays, and structural interpretation provides a coherent framework for distinguishing likely loss-of-function, gain-of-function, and functionally unresolved variants. Overall, I find the manuscript suitable for publication after minor revision.

We thank the reviewer for these positive comments.

Moderate comments

1. The use of ClC-3c for plasma membrane electrophysiology and ClC-3a for vacuolization assays is technically well justified. However, the manuscript should more explicitly acknowledge that these two assays examine different splice variants in distinct membrane contexts. Plasma membrane current properties measured with ClC-3c should therefore be interpreted as complementary to, rather than quantitatively equivalent to, ClC-3a activity in endosomal compartments.

We agree that it is important to stress these differences more than previously done.
We now added in Results, line 277-279, the following sentence:
'These splice variants have identical ion transport properties when directed to the plasma membrane (Guzman et al, 2015). However, their localization to different membranes, which differ in lipid and phosphatidylinositide composition, may differentially affect their activity.'
We also addressed this point later in discussion, see response to point 3 that discusses the apparently higher sensitivity of the vacuolization assay.

2. LINE367: The statement that bath pH 4.5 "mimics the acidity of endolysosomes" should be phrased more carefully. The electrophysiological assay uses ClC-3c, which is described as localizing to recycling endosomes and partially to the plasma membrane upon overexpression, whereas pH 4.5 more closely approximates late endosomal/lysosomal acidity. The authors may consider rephrasing this as "acidic luminal-like conditions" or "late endosomal/lysosomal-like acidity" to avoid implying that pH 4.5 reflects the native environment of ClC-3c.

*We agree with the reviewer that the pH value (4.5) used in these experiments to reveal the pH sensitivity of ClC-3 variants is probably somewhat more acidic that the pH of late endososomes where most ClC-3/T9 is thought to reside. As suggested by the reviewer, we reworded the sentence (now line 286) to: '*Switching bath pH to 4.5, close to late endosomal/lysosomal-like acidity, moderately….'

3. LINE392: The authors refer to the vacuolization assay as "more sensitive." This is plausible, as small residual ClC-3 activity or partial escape from TMEM9-mediated inhibition may accumulate over time and

produce a morphological phenotype even when plasma membrane currents remain below the detection threshold. However, the term "more sensitive" should be defined more explicitly. The vacuolization assay is an indirect, cumulative morphological readout and may be influenced by expression level, trafficking, organelle identity, and image-analysis parameters.

Thank you for the excellent suggestion to highlight the cumulative nature of vesicle 'swelling' and the importance of subcellular localization. We changed the sentence to (line 395-398):
'The apparently higher sensitivity of the vacuolization assay may be owed to the integration of small increases in volume over time, or to differences in the membrane environment. Both types of assays only qualitatively reflect ion transport as the human variants may also affect subcellular trafficking of the transporters.'

4. Related to this point, enlarged LysoSensor-positive compartments could potentially reflect altered endosomal recycling, internalization, trafficking, maturation, fusion/fission, or organelle homeostasis, in addition to altered ClC-3-mediated ion exchange. This is particularly relevant because ClC-3 splice variants localize to distinct endosomal compartments, and TMEM9 proteins can regulate both transport inhibition and trafficking/surface expression. I suggest that the authors discuss this limitation more explicitly and avoid presenting vacuolization as a direct or exclusive readout of ion transport gain-of-function.

Good point, we addressed this issue as stated above for point 3.

5. Please clarify whether the statistical unit in the vacuolization assay is vesicle, cell, image, dish, or independent experiment, and how biological replicates were handled. This is important because vesicles within the same cell or image are not fully independent observations. Please also clarify the family of comparisons used for Benjamini-Hochberg correction.

No quantitative comparison was performed for the vacuolization assay. Vesicle area distributions were built by pooling all detected lysosomes from date-matched experiments to represent the complete dataset and avoid choosing 'representative' images. Each variant was assessed in at least three independent transfections with 4–9 images taken from each dish, each containing multiple cells, not taking into account their transfection status. Thus, these distributions are affected by cells not expressing ClC-3 in the field of view which differed between dates but largely not between conditions.
All legends read: Mann-Whitney U test; false discovery rate was controlled using the Benjamini–Hochberg procedure.

6. Have the authors examined the time course or reversibility of vacuole formation, for example by chase/recovery experiments? Such data are not essential, but clarification would help distinguish whether the assay detects an acute increase in ClC-3 activity or a cumulative defect in endosomal/lysosomal homeostasis.

We have only roughly investigated the time course of vacuole formation, which becomes apparent, depending on transfection efficiency and mutant, about one day after transfection, and increases over time. The shown images were obtained two days after transfection. Of course, the process is non-linear and will come to an end when the cells are filled with vacuoles or die. We did not observe reversibility, within this time period, but you could expect it after longer time intervals with transient, low-level expression and variants causing only moderate enlargement (thereby avoiding cell death). In our study on a CLCN6 variant (https://pubmed.ncbi.nlm.nih.gov/33217309/) we followed the increase in vesicle size over time in live cell imaging using a U2-OS cells carrying lamp2-GFP as KI, and in some experiment added

the proton pump inhibitor bafilomycin A which reversed the swelling since net CLC transport is fueled by the H^+ gradient generated by the H^+-ATPase. The amount and time course of swelling depends on transfection efficiency, which is difficult to control. Quantifying the kinetics of vacuolization is difficult and we would consider it to be pseudo-exact. The assay is very nice, but only semiquantitative.

Minor points:

1. LINE139: Please define the abbreviations T9A and T9B more explicitly at first use and ensure consistent terminology throughout the manuscript. The manuscript alternates between TMEM9, T9A, T9 proteins, T9 subunits, and T9-mediated inhibition.

We have defined T9A and T9B in line 142, and in line 146 T9 as generic T9A and T9B. We agree that it may have been confusing to switch between spelling out the TEMEM9 and T9 terminology and have now – with the exception of the title and abstract – always use the short abbreviations (which we had also used in our first paper on the subunits (Planells-Cases et al, Nature Comm 2025).

2. LINE193: Although all constructs were verified by Sanger sequencing of the entire ORF, providing the QuikChange mutagenesis primer sequences, or at least a full list of generated and sequence-verified constructs, in the Supplementary Information would further improve reproducibility.

The sequences of all these primers have now been added to the Table S3.

Referee #2 (Comments on Novelty/Model System for Author):

The view on ClC-3 function and the consequences of mutations was dramatically altered by the recent discovery of the TMEM9 inhibitory beta-subunit. This created a significant knowledge gap concerning the interpretation of these mutations. This gap is filled here and additional mutations are described.

Referee #2 (Remarks for Author):

Polovitskaya et al. report 15 individuals with a heterogeneous neuropsychiatric disorder caused by 13 missense, 1 in-frame deletion, and 1 splice variant. Since the inhibitory subunit TMEM9 had not been known at the time of the first description of the CLCN3-related syndrome, all 20 known variants not leading to obvious LOF were tested electrophysiologically and by a endolysosomal swelling assay upon heterologous expression in HEK293 cells. 12 of the variants showed impaired inhibition by TMEM9 and of these 8 also displayed an abnormal inward current at acidic pH. The remaining variants did not produce conclusive effects. The clinical manifestations of the 16 individuals carrying function-altering variants are discussed in detail.

General

This manuscript fills an important gap in our understanding of the CLCN3-associated neurodevelopmental disorder. It underlines the crucial role of TMEM9 in the pathogenesis and confirms the relevance of amino acid residues in ClC-3 shown to mediate the interaction with TMEM9 by previous structural analysis. The paper contains high quality phenotype and functional data, clear figures, and is well written. It will be of interest for ion channel researchers as well as for clinicians.

We thank the reviewer for these positive comments.

Major

In the abstract and throughout the text the authors forget to mention the Asp87del variant that leads to a GOF. An amino acid deletion is no missense variant.

Sorry for this mistake, which we have now corrected in line 178 to '1 in-frame deletion of a single amino-acid'.

The clinical presentation is given in great detail in Table 1 and described in paragraph 3.2. To grasp the phenotype more easily I would suggest to present the ten most frequent hallmarks of the disorder with their respective frequencies. E.g. hypotonia 8/13=63%, which is more precise than "Ten subjects exhibited abnormal findings in the neonatal period. Muscular hypotonia was the most frequent feature.". This information can be added to the table or be presented as a separate figure.

We thank the reviewer for this comment. Indeed, the section summarizing the clinical findings did not read well and the reader may have got lost. We have now replaced this entire section with a new, better structured and even somewhat shorter text (lines 207-262), that highlights the most frequent findings in the first paragraph we have pasted here:

'Clinical findings are summarized in Table 1 and Supplemental Table S1. Frequencies are reported as n/N, with N indicating the number of individuals for whom the respective feature was available. Among the 16 individuals carrying function-changing *CLCN3* variants or biallelic likely loss-of-function variants, the most frequent clinical hallmarks were developmental delay and/or intellectual disability (13/13, 100%), gross motor delay or impaired ambulation (13/14, 93%), intellectual disability (11/12, 92%), vision or eye abnormalities (11/13, 85%), language impairment (11/14, 79%), behavioral or psychiatric features (10/14, 71%), gastrointestinal or feeding symptoms (10/16, 63%), hypotonia (8/13, 62%), and autistic features (8/13, 62%).'
We believe that this section is now more informative.

No information is given on the subcellular localisation and expression levels of the ClC-3 mutants. In Figure EV2 the Y386H mutant protein shows a quite strong Golgi localisation if co-expressed with TMEM9. I *would strongly suggest to address this point in the manuscript* because this could be an indication for the pathomechanism. If reproducible changes have been seen this could also be mentioned in Tables S1 and S2.

The reviewer is of course absolutely right in stating that subcellular trafficking may influence the outcome of our assays. We are well aware of this issue as our previous work (Planells-Cases et al, Nature Comm 2025) had revealed that overexpression of ClC-4 and ClC-5 (without T9 proteins) does not generate large vesicles except when they are carried to the swelling-competent endolysosomal compartment by co-expression of ClC-3 and T9, respectively.

*In response to reviewer one (point 3) we have added the sentence: '*Both types of assays only qualitatively reflect ion transport as the human variants may also affect subcellular trafficking of the transporters' *which partially also addresses this point of reviewer 2.*

We would like to stress that in general we did not observe significant changes in the subcellular localization of the transporters as a function of the amino acid change. A major problem is, of course, the over-

expression of the variants in our assays, which may lead to localization in the ER or Golgi and varies from cell to cell.

In order to directly answer the concern of the reviewer concerning the localization of the Y386H mutant, we now directly compared in new experiments (with SK and RPC added as new co-authors) the subcellular localization of the Y386H variant in comparison to the Golgi marker GM130 (Fig. R1 for the reviewer). For comparison, we also included new experiments for S453R, K645E, P666A, and V772A, all of them with T9A co-expression. We failed to detect prominent Golgi localization. Neither did we detect differences between these mutants.

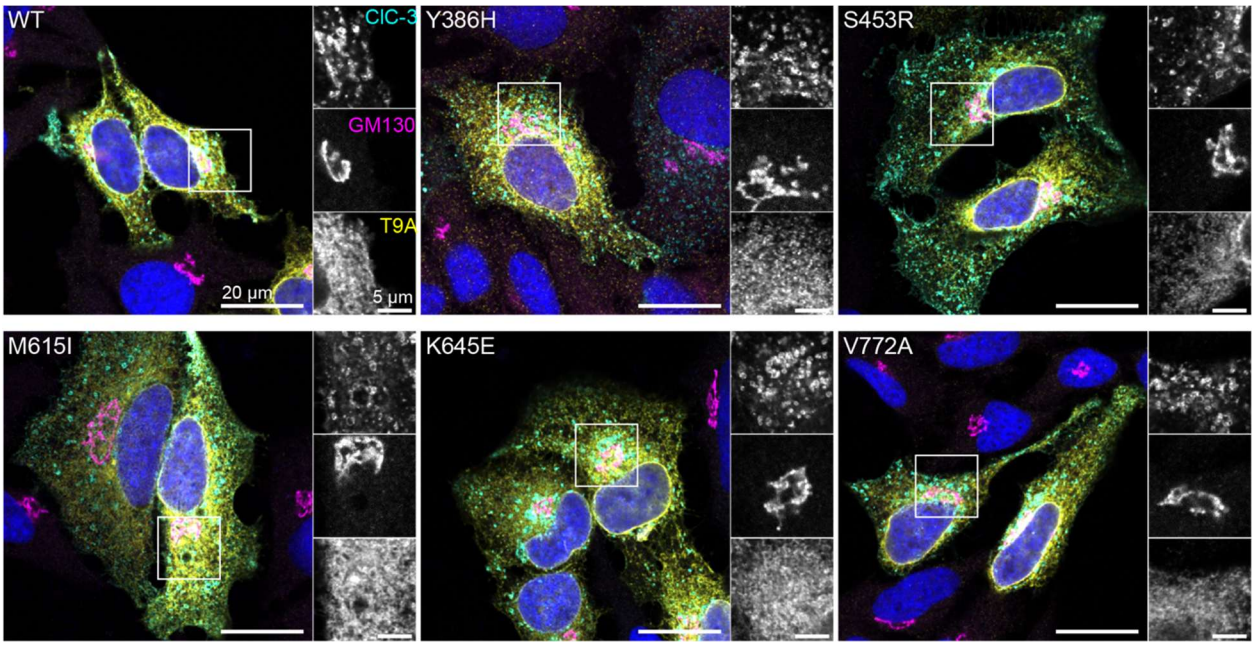

Figure R1 for the reviewer
Immunocytochemistry of HeLa cells two days after transfection of GFP-tagged ClC-3, WT or mutant, together with T9A. Cell were stained for ClC-3, T9A, or the Golgi marker GM130. No marked co-localization of ClC-3 with the Golgi marker. Staining for T9A, which is in large molecular excess, is ER-like. No marked difference between the ClC-3 mutants.

In addition to these new experiments, we also revised the other images of transfected cells we had taken previously. It became clear that the image for the Y388H variant (mentioned by the reviewer) in Figure EV2C was atypical, showing marked expression of the mutant close to the nucleus. We therefore exchanged this panel by a more representative field of view depicting two cells with differing overexpression levels in the revised Fig. EV2.

The ACMG criterium PM2 has been generally downgraded to PM2_supporting.

Thank you, we have corrected the Table.

Minor

Dystonia was not only seen in #12 and #14, but also in #3 with the unclear homozygous p.Trp385Cys variant.

Thank you, we have corrected this mistake in the revised section on clinical finding (line 225).

It is worth mentioning that all 4 individuals showing regression have autism of which the regression is probably a consequence.

Thank you for this interesting observation, which we have added in the section describing the clinical findings (line 241).

In Table S1 the ACMG criteria PM2, PM3, PP2, PP3, PP4 are claimed for c.1155G>T p.Trp385Cys, which corresponds to 7 points and thus to likely pathogenic. However, PM3 cannot be chosen because the variant is homozygous and not in combination with a bone fide pathogenic heterozygous variant. PP4 is precluded since the phenotype is an unspecific retardation with autism that can be caused by dozens if not hundreds of disease genes. PM2 can only be counted as PM2_sup. The final verdict VUS remains the same.

Thank you so much for carefully evaluating our table, which we have corrected.

While the amino acid change induced by c.1155G>T has highly pathogenic predictions c.1156T>C seems rather benign on protein level. For both variants SpliceAI predicts a mild splice defect (acceptor loss) that does not reach the -0.2 threshold. The phenotype of individual 3 harboring c.1155G>T is surprisingly mild and both, c.1155G>T and c.1156G>T, are not associated with seizures.

Both variants are located quite far from the nearest exon boundary and are unlikely to affect splicing. A tyrosine to histidine substitution retaining an aromatic side chain is potentially better tolerated than an introduction of a highly reactive cysteine residue. Mild phenotype of the W385C is consistent with no obvious gain-of-function and potentially partial loss of function without the loss of protein. No change made in the manuscript.

The statement "However, these variants are extremely rare in population databases..." is not precise since only one of the eight variants without clear functional effects is found in gnomAD. This variant c.1238C>T p.Ala413Val is a conservative exchange and shows weak bioinformatic scores. All others are absent and in those for which parents were available for analysis de novo.

*We have changed the wording to: '*these variants are extremely rare in or absent from population databases*'. (line 424) p.Pro666Ala occurs once in AllofUs and p.Met804Thr appears once in gnomAD.*

26th Jun 2026

Dear Prof. Jentsch,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We are satisifed with the revisions made and I am pleased to inform you that we will be able to accept your manuscript pending the following editorial-level amendments:

1. Please remove "Authors' contribution" from the manuscript file.

2. Please ensure that the EV figures are called out in sequential order; currently Fig EV2A is called out before Fig EV1E, Figure EV3 before Fig EV1C,D.

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4. Please move the information currently included in the Ethical Declaration section to the appropriate locations within the Methods section. The separate Ethical Declaration section should then be removed.

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Kind regards,
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The authors addressed the remaining editorial issues.

6th Jul 2026

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The data shown in figures should satisfy the following conditions:
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Please complete ALL of the questions below.
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