CIC-7 is a slowly voltage-gated 2Cl⁻/1H⁺-exchanger and requires Ostm1 for transport activity

Lilia Leisle, Carmen F. Ludwig, Florian A. Wagner, Thomas J. Jentsch and Tobias Stauber

Corresponding author: Thomas J. Jentsch, FMP/MDC

Review timeline:

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Transaction Report:

(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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 26 February 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their reports are shown below. As you will see while referees 1 and 3 are very positive and would support publication here, referee 2 raises major concerns about the functional properties you observed for CIC-7 and overall about the physiological significance of your findings. In the meantime, we have sought further expert advice on the concerns raised by referee 2 (see below). Our expert editorial advisor agrees with referee 2, but thinks that the concerns can be addressed by major revision. On balance, we have come to the conclusion that we should be able to consider a revised version of the manuscript. However, the concerns raised by referee 2 and the other referees need to be addressed in an adequate manner. We would like to specify that patch-clamp data with lysosomes will not be required.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees’ comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.
Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This manuscript by Leisle et al. describes some very well-executed experiments on ClC-7, making use of a mutant that is distributed in part to the plasma membrane, which makes the protein amenable for the first time to electrophysiological characterization. There are several novel findings including a description of properties that are unique to ClC-7, amongst the ClC-3 to 7 sub-family of voltage-gated 2Cl-/1H+ transporters: a strict requirement for assembly with Ostm1 for exchange activity and slow voltage-dependent activation which possibly requires conformational changes in protein structure. The studies include the first functional characterization of human disease-causing mutations in ClC-7. Thus in this single manuscript we have a substantial increase in understanding of the functional properties of an important lysosomal ion transporter, which would not have been discernable using previous indirect approaches. The paper would be strengthened by experiments that identify the conditions under which Cl-/H+ exchange takes place in intact lysosomes and cells, but the initial functional characterization of ClC-7 and disease-causing mutations in the present work make a distinct and very interesting series of studies.

This referee finds no obvious major weakness in this manuscript with respect to the experimental approach, presentation of the results, and their interpretation. There are however a number of points that require clarification or consideration, listed below:

1. The citation of "(Zerangue et al, 1999)" on p.5 is ambiguous and in its present form the sentence implies that the data on ClC-7 trafficking can be found therein. Since the paper is cited in the Materials & Methods section this citation can be omitted here in the Results.

2. It is not clear on p.5 as to in which expression system human and rat ClC-7/Ostm1 currents are indistinguishable. Does human ClC-7(PM) behave similarly in mammalian cells?

3. A explanation of the results employing BCECF imaging is required in the Results section.

4. Please describe how the "activation rate constants" and Q10 values given on p.6 were calculated from the fitted time constants.

5. The findings with non functional, yet correctly localized, osteopetrosis mutants on p.9 (V297 et al) could be explained by mechanisms in addition to effects on ion transport: expression levels may be low (as a result of diminished stability) or they may interrupt functional interaction with Ostm1, which is required for transport activity.

6. Figures 5 and S4 and the interpretation could be improved by generating a homology model of ClC-7, using CmClC. If this is technically unfeasible please indicate in the main text how well these regions are conserved and how the equivalent residues were identified.

7. Source of anti-HA antibody should be Covance (p.20)

8. P.21 "time constants" (tau) should be used here instead of "rate constants", or explain how the underlying rates were determined (see point 4).

9. Supplementary Information p.1: indicate corresponding author with correct symbol.
Referee #2 (Remarks to the Author):

Jentsch and collaborators address in this ms gating properties of the lysosomal CIC-7/Ostm1 ion transporter, a member of the CIC ion-channel/transporter family. Members of this family are associated with a variety of diseases and have, therefore, been well characterized. Yet the CIC7 CI/H exchanger, which normally does not reach the plasma membrane, has been difficult to characterize by electrophysiological means. Now Jentsch et al. have used a previously published mutation in CIC-7 to investigate CIC-7 gating properties. The mutants disrupted a sorting motif enabling the CIC-7 exchanger to reach the plasma membrane. Tissue culture cells or Xenopus oocytes expressing the mutant CIC-7 were then used for current recordings. The recordings were done either in the two-electrode voltage-clamp or in the whole-cell patch-clamp configuration. The results showed that outwardly-rectifying CIC-7 currents activated slowly at voltages more positive than +20 mV. Yet full activation of CIC-7 was not obtained. This uncomfortable situation is nicely circumscribed as measuring 'pseudo steady-state current'. Using tail current protocols ('open exchanger' currents) a plot of tail current amplitudes against voltage, however, showed no voltage-dependence (Fig. 3A). But voltage-gated ion channels are defined by a voltage, where they are half-maximally activated. This important information is completely missing.

In a second set of experiments the authors investigated which parts of Ostm1, a b-subunit of CIC-7, are required for functional CIC-7 expression. Dividing the Ostm1 protein into three parts indicated that the membrane spanning part and the N-terminus of Ostm1 are necessary for functional CIC-7 expression. The studies are based on immuno-cytochemical localization studies combined with current measurements. How the results relate to protein-protein interactions and/or processing remains unclear.

Finally, the authors investigated the effect of CIC-7 mutations associated with osteopetrosis and lysosomal storage disease. Many of the mutations seem to yield dysfunctional exchangers which are still normally localized to the lysosome. Yet some mutations apparently left the CIC-7 currents unchanged or even increased CIC-7 activity. An explanation, what this means for the phenotype/genotype correlation of CIC-7 'disease causing mutations', is not discussed.

The ms shows that CIC-7 is a 2Cl-/H+ exchanger sharing most of its properties with those of the other CIC-transporters. The claim, that the CIC-7 exchanger gates voltage-dependent, is based on preliminary data. The authors should show that the exchanger has a genuine voltage-dependence of gating. This requires full current activation, not a 'pseudo steady state' and a current-voltage relation that has been derived from a tail current protocol. The analysis of the R76Q mutant may help. More importantly, Jentsch and collaborators should carry out inside-out patch-clamp or cut-open experiments for CIC-7 current characterization to have a better control over confounding effects. These may include a voltage-dependent process at the plasma membrane which indirectly changes the gating properties of the CIC-7 exchanger, for example activation of a voltage-dependent phosphatase. Finally, it remains unclear from the data what the physiological significance of the voltage-dependence, which the authors observed above +20 mV, really is. To address this point would significantly improve the interest of this ms for the general readership.

Referee #3 (Remarks to the Author):

Endosomal/lysosomal CLC anion transport proteins regulate vesicular proton and chloride concentrations. Gene mutations of human CLC-5 are associated with Dent's disease whereas mutations in CLC-7 gene (CLCN7) cause osteopetrosis and lysosomal storage disease. In a previous article, the authors of the submitted manuscript have identified a mutated CLC-7 that is stabilized at the plasma membrane. Here, they use this mutant to carry out the electrophysiological characterization of CLC-7. When co-expressed with its beta subunit Ostm1, CLC-7 produces outwardly rectifying currents. This rectification is due to slow gating of the ion exchange that is not voltage-dependent by itself. This result is original and extends the concept of voltage-gating beyond channels to ion exchangers. Several disease-causing CLCN7 mutations affect the gating supporting its physiological relevance. In this manuscript, the authors also show that the transmembrane segment of Ostm1 is required for the CLC-7 Cl-/H+ exchange. The stoichiometry of anion exchange is 2 Cl- for a H+. The technical quality of this work is high, and the results are convincing and
important. This article will be of interest for a large readership.

Expert editorial advice

This MS presents a nice analysis CIC7 which normally resides in lysosomes and traffics due to a mutant now to the plasma membrane. Data are impressive. However, I suggest the authors considering the following concerns:

CIC7 currents are not measured in the steady state, described as "pseudosteady state". Tail current analysis requires back step from a steady state. This could be the reason that authors obtain here a linear, i.e. "voltage independent" CIC7 behavior, which is interpreted as "open exchanger". I would expect to see for a description of voltage dependence a steady state Boltzmann curve. It is strongly suggested to reach steady state by using longer pulses. Can inside out patches be used to prevent possible clamp/accumulation artifacts?

It is always a problem to study properties of a channel/exchanger and interacting proteins in another environment, in this case in the PM and not in the lysosomal membrane. In this MS, mutation data obtained from the exchanger residing in the PM are interpreted as identical (?) with the lysosomal location. Several groups have succeeded to patch lysosomes. Would this have been a possible direct approach?

Figure 3 is a crucial figure and should be improved (e.g. noise figure 3A).

16 March 2011

Detailed answers to the reviewers’ comments:

Referee #1:

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We appreciate the positive comments of the reviewer.

This referee finds no obvious major weakness in this manuscript with respect to the experimental approach, presentation of the results, and their interpretation. There are however a number of points that require clarification or consideration, listed below:

1. The citation of "(Zerangue et al, 1999)" on p.5 is ambiguous and in its present form the sentence implies that the data on CIC-7 trafficking can be found therein. Since the paper is cited in the Materials & Methods section this citation can be omitted here in the Results.

We have removed the reference from p.5.
2. It is not clear on p.5 as to in which expression system human and rat ClC-7/Ostm1 currents are indistinguishable. Does human ClC-7(PM) behave similarly in mammalian cells?

Yes, human and rat ClC-7(PM) behave the same in both oocytes and HeLa cells. We have now specified this at the end of the first paragraph on page 5. However, we feel there is no need to show example traces of human ClC-7(PM) in HeLa cells.

3. A explanation of the results employing BCECF imaging is required in the Results section.

We added two sentences on page 6: ‘In these ‘Fluorocyte’ experiments, the pH-dependent fluorescence of BCECF previously injected into oocytes provides a semi-quantitative measure of cytosolic pH changes in response to depolarizing voltage-steps. Depolarization not only activates ClC-7(PM)/Ostm1, but also provides a driving force for coupled H+-exit/Cl- entry.’

4. Please describe how the "activation rate constants" and Q10 values given on p.6 were calculated from the fitted time constants.

We have now explained the calculations in Materials and Methods, page 23: ‘…activation rate constants R = 1/τ…’ and ‘… Q10 values were determined by Q10 = (R2/R1)10(T2−T1), with R1 and R2 being the activation rate constants at temperatures T1 (37°C) and T2 (21°C), respectively.’

5. The findings with non functional, yet correctly localized, osteopetrosis mutants on p.9 (V297 et al) could be explained by mechanisms in addition to effects on ion transport: expression levels may be low (as a result of diminished stability) or they may interrupt functional interaction with Ostm1, which is required for transport activity.

We agree that reduced expression levels of the mutants may contribute to the failure to observe currents. We have examined their expression levels in oocytes by Western blot analysis and found that indeed one of them (L490F) consistently showed markedly reduced expression levels. However, also consistently found in several experiments, the functionally ‘dead’, but correctly localized V297M and F318L mutants did not show reduced expression levels. We now include a representative Western blot in the new Supplementary Figure S6 and discuss this issue at the end of Results on page 10.

On the other hand, the interaction of these mutants with Ostm1 seemed unchanged because these mutants were still able to carry Ostm1 to lysosomes in transfected cells. Of course, this does not exclude that the interaction with the N-terminal part of Ostm1, which is needed for ClC-7 activation, is changed. We discuss this issue also in the same paragraph.

Added text: ‘…or with the mechanism by which Ostm1 activates ClC-7. One should note, however, that these mutants were able to carry Ostm1 to lysosomes. A reduction in the expression level due to limited stability may also contribute to reduced currents. Western blot analysis showed that this was not the case for the V297M and F318L mutants, but protein levels were markedly reduced with the L490F mutant (Supplementary Figure S6).’

6. Figures 5 and S4 and the interpretation could be improved by generating a homology model of ClC-7, using CmClC. If this is technically unfeasible please indicate in the main text how well these regions are conserved and how the equivalent residues were identified.

We believe that generating a homology model may introduce larger uncertainties than showing the amino-acids directly in the structure of CmClC. We used exactly the alignment as published by Feng et al. (it’s shown in their paper). Although the amino-acids mutated in ClC-7 are not conserved in CmClC, the alignment both in the TMD portion and in the CBS domain is ‘fixed’ by unambiguous homology between different CLCs. For this reviewer, we attach alignments of the respective regions (Appendix 1: Alignment of CmClC and hClC-7), but do not include these as the alignment has been published by Feng et al. (2010). However, we have included the correspondence
of CmClC and CIC-7 residues in the legend to Fig. 5B to unambiguously identify the positions we have highlighted in that panel.

7. Source of anti-HA antibody should be Covance (p.20)

We have corrected the spelling error (now page 22).

8. P.21 "time constants" (tau) should be used here instead of "rate constants", or explain how the underlying rates were determined (see point 4).

We have explained it now. See our answer to point 4.

9. Supplementary Information p.1: indicate corresponding author with correct symbol.

We have removed the address and name symbols from the first page of Supplementary Information.

Referee #2:

(Remarks to the Author)

Jentsch and collaborators address in this ms gating properties of the lysosomal CIC-7/Ostm1 ion transporter, a member of the CIC ion-channel/transporter family. Members of this family are associated with a variety of diseases and have, therefore, been well characterized. Yet the CIC7 Cl/H exchanger, which normally does not reach the plasma membrane, has been difficult to characterize by electrophysiological means. Now Jentsch et al. have used a previously published mutation in CIC-7 to investigate CIC-7 gating properties. The mutants disrupted a sorting motif enabling the CIC-7 exchanger to reach the plasma membrane. Tissue culture cells or Xenopus oocytes expressing the mutant CIC-7 were then used for current recordings. The recordings were done either in the two-electrode voltage-clamp or in the whole-cell patch-clamp configuration. The results showed that outwardly-rectifying CIC-7 currents activated slowly at voltages more positive than +20 mV. Yet full activation of CIC-7 was not obtained. This uncomfortable situation is nicely circumscribed as measuring 'pseudo steady-state current'. Using tail current protocols ('open exchanger' currents) a plot of tail current amplitudes against voltage, however, showed no voltage-dependence (Fig. 3A). But voltage-gated ion channels are defined by a voltage, where they are half-maximally activated. This important information is completely missing.

Voltage-dependent ion channels are not defined by the voltage of half-maximal activation, but this voltage is one out of several parameters that may be used to biophysically characterize an ion channel. A ‘complete’ biophysical characterization of CIC-7/Ostm1 is clearly beyond the scope of our work. As the slow activation of CIC-7/Ostm1 does not allow us to reach steady-state, a determination of \( p_{\text{open}} \) and \( V_{1/2} \) by tail current analysis is not possible; however, following the suggestion of this reviewer, we have characterized a faster mutant (see below).

In a second set of experiments the authors investigated which parts of Ostm1, \( \beta \)-subunit of CIC-7, are required for functional CIC-7 expression. Dividing the Ostm1 protein into three parts indicated that the membrane spanning part and the N-terminus of Ostm1 are necessary for functional CIC-7 expression. The studies are based on immuno-cytochemical localization studies combined with current measurements. How the results relate to protein-protein interactions and/or processing remains unclear.

There is no evidence from our work or the work of others that CIC-7, the ion transporting subunit, is processed. Ostm1, by contrast, is processed by proteolytic cleavage in, or on its way to, lysosomes, as shown by us previously (Lange et al., 2006). However, the cleavage products are still associated by disulfide bonds. It has remained unclear whether the proteolytic cleavage is necessary for the ion transport activity of CIC-7/Ostm1. Since CIC-7\(^{\text{PM}}\) most likely travels to the plasma membrane
without passing a prelysosomal or lysosomal compartment, we assume that CIC-7 \(^{PM}\)/Ostm1 contains the uncleaved, immature precursor form of Ostm1. Thus, our study provides circumstantial evidence that processing (cleavage) of Ostm1 is not needed for its ability to enable CIC-7 ion transport. We have added two sentences to this respect in the discussion on page 14-15. In the same paragraph, we have also discussed that the TMDs of CIC-7 and Ostm1 may bind each other, which is the basis for CIC-7 carrying Ostm1 to lysosomes, and that there is another interaction by which the N-terminus of Ostm1 activates CIC-7 ion transport. None of our experiments, however, has investigated direct binding (even co-IP may not exclude a ‘bridging’ protein).

Finally, the authors investigated the effect of CIC-7 mutations associated with osteopetrosis and lysosomal storage disease. Many of the mutations seem to yield dysfunctional exchangers which are still normally localized to the lysosome. Yet some mutations apparently left the CIC-7 currents unchanged or even increased CIC-7 activity. An explanation, what this means for the phenotype/genotype correlation of CIC-7 ‘disease causing mutations’, is not discussed.

We had discussed this issue in the original version of the manuscript: no obvious genotype/phenotype correlation is evident with the human CIC-7 mutations we have analyzed (first sentence of second paragraph on human mutations in Results, and second sentence in the corresponding paragraph in Discussion). We have also discussed that trafficking and protein stability may be different in vivo, as exemplified by our previous work on the R762Q mutant (page 15-16). No change made.

The ms shows that CIC-7 is a 2Cl-/H\(^+\) exchanger sharing most of its properties with those of the other CIC-transporters. The claim, that the CIC-7 exchanger gates voltage-dependent, is based on preliminary data. The authors should show that the exchanger has a genuine voltage-dependence of gating. This requires full current activation, not a ‘pseudo steady state’ and a current-voltage relation that has been derived from a tail current protocol. The analysis of the R762Q mutant may help. More importantly, Jentsch and collaborators should carry out inside-out patch-clamp or cut-open experiments for CIC-7 current characterization to have a better control over confounding effects. These may include a voltage-dependent process at the plasma membrane which indirectly changes the gating properties of the CIC-7 exchanger, for example activation of a voltage-dependent phosphatase.

There is no evidence from any CLC that its voltage-dependence (or run-down, run-up) is owed to a voltage-dependent phosphatase, and we are not aware of work showing that voltage-dependent phosphatases play a role in the voltage-dependence of ion channels heterologously expressed in HeLa cells or Xenopus oocytes. Moreover, to be voltage-dependent, such a phosphatase should have a voltage-sensor in the membrane and thus be an integral membrane protein (like the voltage-dependent phosphatase Ci-VSP from worms). Integral membrane proteins, however, will not be lost when examining CIC-7/Ostm1 in excised patches (which may have other problems like run-downs or run-ups). Therefore, we did not perform excised patch measurements.

We believe that voltage-dependence of gating was evident from our results also without determining \(V_{1/2}\) from tail currents. Because tail current analysis for \(V_{1/2}\) requires steady-state currents that we cannot achieve with CIC-7/Ostm1, we followed the valuable suggestion of the reviewer and include new data on the ‘fast’ R762Q mutant of CIC-7 (new Fig. 3C,D). With this mutant, steady-state currents are reached after about 400 ms. Tail current analysis yielded \(V_{1/2} \approx 82\) mV and an apparent gating charge \(z_n \approx 1.32\). These new results are now described on page 7, last paragraph, and are discussed on pages 11 and 13 in Discussion.

Finally, it remains unclear from the data what the physiological significance of the voltage-dependence, which the authors observed above \(+20\) mV, really is. To address this point would significantly improve the interest of this ms for the general readership.

The functional role of the voltage-dependence of endosomal/lysosomal CLC proteins has been obscure and a matter of discussion for many years. We do not wish to repeat this discussion in our paper, but we have added the following text in Discussion (p. 13 and 14):

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‘It is intriguing that ClC-7/Ostm1, just like the other vesicular CLC Cl\(^{-}/H^{+}\)-exchangers (Friedrich et al, 1999; Li et al, 2002; Neagoe et al, 2010; Steinmeyer et al, 1995), displays such strong voltage dependence. This rectification is not an inevitable consequence of the Cl/H\(^{+}\)-exchange per se, as our measurements of ‘open transporter’ currents have shown. This suggests that the rectification might have a physiological importance, which, however, remains obscure. The strong outward rectification of ClC-4 and -5 has puzzled the field for a long time because it implies that these transporters are almost inactive in inside-positive endosomes (Jentsch, 2007; Jentsch et al, 2002). However, recent model calculations have shown that vesicles may attain an inside-negative voltage through the activity of 2Cl/H\(^{+}\)-exchangers (Weinert et al, 2010).’

**Referee #3:**

(Remarks to the Author)

*Endosomal/lysosomal CLC anion transport proteins regulate vesicular proton and chloride concentrations. Gene mutations of human CLC-5 are associated with Dent’s disease whereas mutations in CLC-7 gene (CLCN7) cause osteopetrosis and lysosomal storage disease. In a previous article, the authors of the submitted manuscript have identified a mutated CLC-7 that is stabilized at the plasma membrane. Here, they use this mutant to carry out the electrophysiological characterization of CLC-7. When co-expressed with its beta subunit Ostm1, CLC-7 produces outwardly rectifying currents. This rectification is due to slow gating of the ion exchange that is not voltage-dependent by itself. This result is original and extends the concept of voltage-gating beyond channels to ion exchangers. Several disease-causing CLCN7 mutations affect the gating supporting its physiological relevance. In this manuscript, the authors also show that the transmembrane segment of Ostm1 is required for the CLC-7 Cl-/H\(^{+}\) exchange. The stoichiometry of anion exchange is 2 Cl\(^{-}\) for a H\(^{+}\). The technical quality of this work is high, and the results are convincing and important. This article will be of interest for a large readership.*

We thank this reviewer for carefully evaluating our manuscript and for his/her positive comments. No changes necessary.

**Expert editorial advice**

*This MS presents a nice analysis ClC7 which normally resides in lysosomes and traffics due to a mutant now to the plasma membrane. Data are impressive.*

We appreciate the positive comment.

*However, I suggest the authors considering the following concerns:*

*ClC7 currents are not measured in the steady state, described as “pseudosteady state”. Tail current analysis requires back step from a steady state. This could be the reason that authors obtain here a linear, i.e. "voltage independent" ClC7 behavior, which is interpreted as "open exchanger". I would expect to see for a description of voltage dependence a steady state Boltzmann curve. It is strongly suggested to reach steady state by using longer pulses. Can inside out patches be used to prevent possible clamp/accumulation artifacts?*

Tail current analysis of instantaneous ‘open exchanger (channel)’ currents does not require steady-state currents, and therefore our analysis of the ‘voltage-independent’ ‘open exchanger’ is correct (see Appendix 2: Remarks on tail current analysis).

However, in response to reviewer 2, we have now performed tail current analysis for \(V_{1/2}\) and the gating charge \(z_{n}\) with the much faster R762Q mutant, as suggested by reviewer 2. Here steady-state currents (i.e. \(p_{\text{open}}\)) are reached. Although these experiments yield values for the mutant, they are
technically solid and are likely not to differ much in the WT. Please see our response to reviewer 2 for more details.

We believe that these additional experiments have added an interesting point.

It is always a problem to study properties of a channel/exchanger and interacting proteins in another environment, in this case in the PM and not in the lysosomal membrane. In this MS, mutation data obtained from the exchanger residing in the PM are interpreted as identical (?) with the lysosomal location. Several groups have succeeded to patch lysosomes. Would this have been a possible direct approach?

Patching of lysosomes is technically very demanding and is certainly beyond the scope of this paper (although the comparison suggested by the expert would be interesting, of course). Our plasma-membrane localized ClC-7 mutant is a breakthrough that facilitates studies of ClC-7/Ostm1 enormously.

Figure 3 is a crucial figure and should be improved (e.g. noise figure 3A).

We have performed further measurements and now show a representative curve with less noise in Figure 3A. These new experiments also increased the number of measurements for the current/voltage curve to \( n = 8 \) for the ‘open exchanger’, further decreasing the error bars.

Appendix 1: Alignment of CmClC and hClC-7 with other CLC proteins

Alignment of CmClC, vertebrate CLCs, and S. cerevisiae Gef1p in the regions encompassing membrane-embedded helices D – G (top) and the cytoplasmic CBS2 domain (bottom) to show the correspondence of mutated ClC-7 residues highlighted in Fig. 5B to those of CmClC. Although (with the exception of L213/L174) residues are not conserved at the equivalent positions in CmClC, the alignment indicates sufficient homology to assign equivalent residues with a high degree of confidence.

Appendix 2: Remarks on tail current analysis

The total current \( I_{\text{tot}} \) across the cell membrane is given by:
\[ I_{tot} = n \cdot i(V) \cdot p_{\text{open}}(V,t), \]

With \( n \) = number of channels (or transporters), \( i(V) \) current through an individual channel or transporter, and \( p_{\text{open}}(V,t) \) the open probability of the channel (or transporter) (\( p_{\text{open}} \) can vary between 0 and 1 (fully closed and open, respectively)).

The voltage dependence of total currents mediated by a channel or transporter can thus arise from voltage-dependent gating that changes \( p_{\text{open}}(V,t) \), from an intrinsic voltage-dependence of the current through the open channel or transporter, or a combination of both. Both mechanisms are found with ion channels in nature.

Whereas the current \( i(V) \) through an open channel changes instantaneously (for all practical purposes) upon changing the voltage \( V \), voltage-dependent changes in \( p_{\text{open}}(V,t) \) take time as it generally involves a conformational change of the protein, often over a rather large energy barrier. Hence, upon jumping to a different voltage, currents ‘relax’ in a time-dependent manner until \( p_{\text{open}}(V,t) \) has reached its steady-state value at that particular voltage, giving rise to current relaxations. These current relaxations, as found in ClC-7/Ostm1, are already by itself a strong indication for voltage-dependent gating. If these current relaxations are sufficiently slow to be measurable, tail current analysis can provide information on the open channel (transporter) current \( i(V) \) and the voltage-dependence of \( p_{\text{open}}(V) \).

There are two types of tail current analysis:

**Tail currents to measure ‘instantaneous’ currents flowing through ‘open channels’ or ‘open transporters’**:
Here the channel (transporter) is activated by voltage to a certain \( p_{\text{open}} = a \) (yielding a certain total current) and then stepped, in sequential sweeps, to different voltages. When the change of voltage is fast compared to the change of \( p_{\text{open}} \) (as is the case in our experiments), the channel (transporter) still has the same value of \( p_{\text{open}} \) at the beginning of the test pulse. Therefore, when stepping to different voltages, \( I_{tot} \) is directly proportional to the open channel (transporter) current \( i(V) \) because \( p_{\text{open}} \) is the same for each ‘jump’. This is the analysis we have performed in our manuscript (Fig. 3A,B). This analysis clearly does not require \( I_{tot} \) (or \( p_{\text{open}} \)) to be at steady state. The prerequisite for this analysis is that we have the same value of \( p_{\text{open}} = a \) at the beginning of each voltage-jump to different voltages. We have made sure that this is the case, as evident in Fig. 3A,B from the same value of \( I_{tot} \) at the beginning of voltage-jumps.

**Tail currents to measure voltage-dependence of \( p_{\text{open}} \)**:
In this type of analysis, tail currents are measured by jumping from different voltages and are measured at the same voltage. Current measurements at the same voltage ensure that \( n \cdot i(V) = \text{const} \). Tail currents only differ due to different values of \( p_{\text{open}} \), and are relaxing to a new value as \( p_{\text{open}} \) reaches its new steady-state value at that new value of voltage. Extrapolating back to the time of voltage-jump gives you the current determined by the value of \( p_{\text{open}} \) that was obtained at the voltage before jumping to the test voltage where tail currents were measured, and thus allow to determine \( p_{\text{open}}(V) \). This analysis clearly requires that \( p_{\text{open}} \) (i.e. \( I_{tot} \)) has reached steady-state before jumping to the constant test pulse.

Because we could not reach clean steady-state currents with the very slow activation of WT ClC-7/Ostm1 in either expression system, we have now performed this type of analysis with the ClC-7\textsuperscript{PM}(R762Q)/Ostm1 mutant which activates much faster, reaching steady-state currents within 400 ms (Fig. 3C,D).

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Thank you for sending us your revised manuscript. I am sorry for the delay in getting back to you with a decision. We experienced difficulties with the availability
of the reviewers at the time of resubmission. In the meantime, referee 3 and our expert editorial advisor have now seen the manuscript again, and you will be pleased to learn that in their view you have addressed all criticisms in a satisfactory manner. The paper will now be publishable in The EMBO Journal and you will receive a formal acceptance letter shortly.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #3 (Remarks to the Author):

The rebuttal to reviewers' comments is convincing, in particular the response concerning the voltage-dependence of activation and its study. I do not feel that recording of CIC-7 in lysosomes is mandatory for publication of this work.

Expert editorial advisor:

Authors have very carefully responded to all my concerns. I appreciate the additional experiments and I also understand that the certainly justified concern to study a channel/transporter in its physiological environment is difficult and probably out of the scope of this MS. The quality of the shown traces is substantially improved!

I recommend accepting this MS.