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COMMENTARY

Not so transport incompetent after all: Revisiting a CLC-7 mutant sheds new mechanistic light on lysosomal physiology

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Lysosomes have long been regarded as cellular garbage disposal compartments; acidic organelles filled with enzymes that break down and recycle macromolecules into reusable small molecules (Hamer et al., 2012; Appelqvist et al., 2013). In recent years however, our understanding of the lysosomes' roles in physiology has evolved as they emerged as central regulators of a wide range of cellular metabolic pathways. Mutations in lysosome-specific proteins are associated with >70 recessive genetic diseases, collectively known as lysosomal storage disorders (LSD; Platt et al., 2018). Optimal function of lysosomal enzymes is dependent on a highly acidic lumenal environment and on the precise control of the ionic composition of the intravesicular compartment (Xiong and Zhu, 2016). While lysosomal acidification is mediated by V-ATPases that import protons at the expense of ATP hydrolysis, the molecular mechanisms maintaining charge balance and regulating ionic homeostasis remain poorly understood and controversial. The charge-neutralizing ion fluxes enabling proton accumulation by the ATPase have been proposed to be mediated by the CLC-7 transporter (Kornak et al., 2001; Kasper et al., 2005; Graves et al., 2008), cation channels (Steinberg et al., 2010), and by a proton-activated chloride channel (Yang et al., 2019). In a previous issue of the *Journal of General Physiology*, Pusch and Zifarelli (2020) evaluate the currents associated with transport and gating of CLC-7 and their findings challenge past conclusions on the roles of this transporter in lysosomal physiology (Weinert et al., 2014) and shed new light into the gating mechanism of CLC-type exchangers.

Unique among known CLC exchangers, CLC-7 requires assembly with the single-pass membrane protein Ostm1 for trafficking and stability (Lange et al., 2006). Recent structural work revealed that Ostm1 forms a glycosylated cap, likely to protect the lumenal face of the transporter from attack by lysosomal enzymes (Schrecker et al., 2020; Zhang et al., 2020). The groundbreaking discovery that CLC-7 and the other vesicular CLCs are 2 Cl^-:1 H^+ exchangers rather than Cl^- channels (Picollo and Pusch, 2005; Scheel et al., 2005; Graves et al., 2008; Matsuda et al., 2008; Alekov and Fahlke, 2009; Leisle et al., 2011) induced a rethinking of their roles in endolysosomal physiology. This finding was surprising as a Cl^- channel might appear better suited to shunt the activity of the vacuolar proton ATPase than a Cl^-:H^+ exchanger, since in the latter Cl^- import is coupled to dissipation of the proton gradient. However, quantitative models of lysosomal acidification suggest that a 2 Cl^-:1 H^+ exchanger can enable the formation of a more acidic intralumenal environment than a passive Cl^- channel (Weinert et al., 2010; Ishida et al., 2013; Marcoline et al., 2016), providing a plausible rationalization of their exchange activity.

Mutations in CLC-7 are associated with multiple genetic disorders, such as osteopetrosis, neurodegeneration, LSD, and albinism (Jentsch and Pusch, 2018; Nicoli et al., 2019), and its genetic ablation in model organisms causes dominant osteopetrosis, pigmentation defects, and neurodegeneration (Jentsch and Pusch, 2018). Reduced acidification has been observed in the osteoclastic resorption lacunas of Clcn7$^{-/-}$ mice (Kornak et al., 2001). However, there is contrasting data on whether its defective function is associated with impaired lysosomal acidification. Some reports showed that lysosomes from Clcn7$^{-/-}$ and Ostm1$^{-/-}$ mice have normal acidification but altered Cl^- levels (Kornak et al., 2001; Kasper et al., 2005; Steinberg et al., 2010), leading to the proposal that CLC-7 might use the pH gradient established by the proton ATPase to increase luminal Cl^-. Others reported that knockdown of CLC-7 leads to lysosomal alkalinization (Graves et al., 2008) and that a CLC-7 gain of function mutant is associated with lysosomal hyper-acidification (Nicoli et al., 2019), consistent with the idea that CLC-7 provide the main shunt conductance for the proton ATPase. Thus, the role of CLC-7 in lysosomal acidification remains an unanswered riddle.

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The Cl^-:H^+ exchange mechanism is well-preserved among CLC transporters from prokaryotes and eukaryotes. Despite low overall sequence conservation, key residues for H^+ transport are conserved in sequence, function, and structure (Accardi, 2015; Jentsch and Pusch, 2018). One exception to this evolutionarily harmonious picture is the role of the so-called "proton-glutamate," an intracellular-facing glutamic acid that, in many CLC transporters, plays a critical role in enabling proton shuttling between the intra- and extracellular solutions (Accardi et al., 2005; Zdebik et al., 2008; Lim and Miller, 2009; Neagoe et al., 2010; Lim et al., 2012; Guzman et al., 2013). This glutamic acid is, however, not required for coupling; the algal cmCLC mediates coupled 2 Cl^-:1 H^+ exchange despite having a neutral threonine at this position (Feng et al., 2010), and replacement of the proton-glutamate with any protonatable side chain preserves partial coupling in bacterial and mammalian CLCs (Zdebik et al., 2008; Lim and Miller, 2009). Disruption of coupling arises because mutations at the proton-glutamate can differentially affect the rates of Cl^- and H^+ transport, depending on the specific physicochemical properties of the introduced side chain (Zdebik et al., 2008; Lim and Miller, 2009). In some homologues, such as the *Escherichia coli* CLC-ec1, H^+ transport is more strongly affected, such that Cl^-:H^+-coupling is severely weakened or abolished (Accardi et al., 2005; Lim and Miller, 2009; Chavan et al., 2020). In contrast, in mammalian CLCs, including CLC-7, such mutations impair transport altogether (Zdebik et al., 2008; Neagoe et al., 2010; Leisle et al., 2011; Guzman et al., 2013). It was thus surprising that CLC-7 knock-in mice bearing a charge neutralizing mutation at this position (E312A) exhibit a mixed phenotype of severe osteopetrosis, like that of Clcn7$^{-/-}$ animals, but milder neurodegeneration and no pigmentation defect (Weinert et al., 2014). This finding led to the proposal that the ClC-7–Ostm1 complex might also have transport-independent functions, such as participating in a macromolecular complex with other lysosomal proteins (Weinert et al., 2014). In a previous issue of the *Journal of General Physiology*, Pusch and Zifarelli (2020) revisit the biophysical properties of the E312A mutant, using a mammalian expression system in place of the previously used *Xenopus laevis* oocyte system (Leisle et al., 2011). Unexpectedly, they find that in HEK293 cells this mutant mediates currents that are strongly rectifying, like those of the WT exchanger, but are smaller in amplitude and have different kinetics than those of the parent transporter. This finding provides a simple explanation for the intermediate phenotype of the knock-in animals (Weinert et al., 2014), as the—presumably H^+-uncoupled—residual Cl^- transport could be enough to enable lysosomal acidification and/or maintain sufficient Cl^- levels for function of the lysosomal enzymes. The apparent contrast with a previous report that indicated that the E312A mutant was transport incompetent (Leisle et al., 2011) could be due to different translation efficiency of the mutant in the two heterologous expression systems. Alternatively, recent structural work showed that CLC-7 binds soluble Mg^{2+} and ATP molecules in the cytosolic domain and the phosphatidylinositol (3,4,5)-trisphosphate lipid in the transmembrane region (Schrecker et al., 2020), raising the possibility that the transporter is regulated by exogenous factors that might be present at different abundance levels in the diverse cellular expression systems, possibly accounting for some of the reported differences with regards to its function.

The second key finding of the work from Pusch and Zifarelli (2020) is that the transient currents mediated by CLC-7 are associated with the transport cycle rather than with the activation gating process. The activity of most transporters is controlled by substrate availability and by the rates of substrate-induced conformational rearrangements that occur during the transport cycle (Drew and Boudker, 2016). In contrast, mammalian CLC exchangers are also regulated by a voltage-dependent conformational rearrangement that switches them between a transport-incompetent (inactive) and a transport-competent (active) conformation (Zdebik et al., 2008; Alekov and Fahlke, 2009; Leisle et al., 2011; Orhan et al., 2011; De Stefano et al., 2013; Ludwig et al., 2013). This process is thought to act on both subunits in the CLC dimer, and it is reminiscent of the common-pore gating mechanism that controls activation of CLC channels (Miller, 1982; Accardi, 2015; Jentsch and Pusch, 2018). Voltage-dependent activation of endosomal CLC exchangers, such as CLC-3, -4, and -5, is fast and occurs in a few milliseconds (Zdebik et al., 2008; Alekov and Fahlke, 2009; Orhan et al., 2011; De Stefano et al., 2013). In contrast, the lysosomal CLC-7 is a slowly activating transporter; its activation is incomplete after several seconds (Leisle et al., 2011; Ludwig et al., 2013). In addition to steady-state currents associated with their exchange activity, CLC-3, -4, and -5 also mediate transient currents (Smith and Lippiat, 2010; Zifarelli et al., 2012; Guzman et al., 2013). A priori, these transient currents could arise from the rearrangements that occur during the transport cycle or from those associated with voltage-dependent activation gating. Initial work suggested that transient currents arise from the movement of a conserved glutamic acid, the so-called "gating glutamate," within the Cl^- pathway during transport, as mutating the gating glutamate eliminates these currents (Smith and Lippiat, 2010; Zifarelli et al., 2012; Guzman et al., 2013). However, in CLC-3, -4, and -5, the transient currents and activation gating take milliseconds whereas their transport cycle is thought to occur on the microsecond timescale (Zdebik et al., 2008; Zifarelli et al., 2012). This kinetic discrepancy suggested that the transient currents could arise from rearrangements occurring during gating, especially since mutations of the gating glutamate eliminate voltage-dependent activation gating in addition to coupled exchange (Smith and Lippiat, 2010; Zifarelli et al., 2012; Guzman et al., 2013). In an elegant series of experiments, Pusch and Zifarelli (2020) take advantage of the slow activation gating kinetics of CLC-7 to show that the transient currents are indeed associated with the transport cycle. First, they find that the transient currents occur on the millisecond timescale while activation requires several seconds. Second, they show the charge movement associated with these transient currents is independent of the fraction of activated transporters. Third, they identify a mutant with accelerated gating kinetics but WT-like transient currents. Together, these observations argue that the transient currents are indeed associated with the transport cycle rather than with gating. Since the transient currents are mediated by all CLC-7 transporters, regardless of their activation state, they

conclude that the underlying rearrangements can occur in both transport-competent and -incompetent conformations. As these currents depend on the internal chloride concentration (and on the external concentration in CLC-5) and on the external pH, Pusch and Zifarelli come to the remarkable conclusion that inactivation of the transporter entails a rearrangement that specifically prevents exchange of protons with the intracellular milieu.

In sum, the work of Pusch and Zifarelli is important for our understanding of physiology and mechanism (Pusch and Zifarelli, 2020). It indicates that the physiological role of CLC-7 is likely just to transport ions in and out of lysosomes, rather than to serve as a scaffolding platform for interacting partners. Further, the insight that activation gating in CLC-7 entails a conformational rearrangement that prevents exchange of intracellular protons provides a guidelight for the design of experiments aimed at revealing the structural underpinnings of CLC activation gating, rearrangements that remain elusive despite nearly two decades of intense structural scrutiny (Accardi, 2015; Jentsch and Pusch, 2018).

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References

Accardi, A. 2015. Structure and gating of CLC channels and exchangers. *J. Physiol.* 593:4129–4138. https://doi.org/10.1113/JP270575

Accardi, A., M. Walden, W. Nguitragool, H. Jayaram, C. Williams, and C. Miller. 2005. Separate ion pathways in a Cl^-/H^+ exchanger. *J. Gen. Physiol.* 126:563–570. https://doi.org/10.1085/jgp.200509417

Alekov, A.K., and C. Fahlke. 2009. Channel-like slippage modes in the human anion/proton exchanger ClC-4. *J. Gen. Physiol.* 133:485–496. https://doi.org/10.1085/jgp.200810155

Appelqvist, H., P. Wäster, K. Kågedal, and K. Öllinger. 2013. The lysosome: from waste bag to potential therapeutic target. *J. Mol. Cell Biol.* 5:214–226. https://doi.org/10.1093/jmcb/mjt022

Chavan, T.S., R.C. Cheng, T. Jian, I.I. Mathews, R.A. Stein, A. Kohel, H.S. Mchaourab, E. Tajkhorshid, and M. Maduke. 2020. A CLC-ec1 mutant reveals global conformational change and suggests a unifying mechanism for the CLC Cl^-/H^+ transport cycle. *eLife.* 9:e53479. https://doi.org/10.7554/eLife.53479

De Stefano, S., M. Pusch, and G. Zifarelli. 2013. A single point mutation reveals gating of the human ClC-5 Cl-/H+ antiporter. *J. Physiol.* 591:5879–5893. https://doi.org/10.1113/jphysiol.2013.260240

Drew, D., and O. Boudker. 2016. Shared Molecular Mechanisms of Membrane Transporters. *Annu. Rev. Biochem.* 85:543–572. https://doi.org/10.1146/annurev-biochem-060815-014520

Feng, L., E.B. Campbell, Y. Hsiung, and R. MacKinnon. 2010. Structure of a eukaryotic CLC transporter defines an intermediate state in the transport cycle. *Science.* 330:635–641. https://doi.org/10.1126/science.1195230

Graves, A.R., P.K. Curran, C.L. Smith, and J.A. Mindell. 2008. The Cl-/H+ antiporter ClC-7 is the primary chloride permeation pathway in lysosomes. *Nature.* 453:788–792. https://doi.org/10.1038/nature06907

Guzman, R.E., M. Grieschat, C. Fahlke, and A.K. Alekov. 2013. ClC-3 is an intracellular chloride/proton exchanger with large voltage-dependent nonlinear capacitance. *ACS Chem. Neurosci.* 4:994–1003. https://doi.org/10.1021/cn400032z

Hamer, I., G. Van Beersel, T. Arnould, and M. Jadot. 2012. Lipids and lysosomes. *Curr. Drug Metab.* 13:1371–1387. https://doi.org/10.2174/138920012803762684

Ishida, Y., S. Nayak, J.A. Mindell, and M. Grabe. 2013. A model of lysosomal pH regulation. *J. Gen. Physiol.* 141:705–720. https://doi.org/10.1085/jgp.201210930

Jentsch, T.J., and M. Pusch. 2018. CLC Chloride Channels and Transporters: Structure, Function, Physiology, and Disease. *Physiol. Rev.* 98:1493–1590. https://doi.org/10.1152/physrev.00047.2017

Kasper, D., R. Planells-Cases, J.C. Fuhrmann, O. Scheel, O. Zeitz, K. Ruether, A. Schmitt, M. Poët, R. Steinfeld, M. Schweizer, et al. 2005. Loss of the chloride channel ClC-7 leads to lysosomal storage disease and neurodegeneration. *EMBO J.* 24:1079–1091. https://doi.org/10.1038/sj.emboj.7600576

Kornak, U., D. Kasper, M.R. Bösl, E. Kaiser, M. Schweizer, A. Schulz, W. Friedrich, G. Delling, and T.J. Jentsch. 2001. Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. *Cell.* 104:205–215. https://doi.org/10.1016/S0092-8674(01)00206-9

Lange, P.F., L. Wartosch, T.J. Jentsch, and J.C. Fuhrmann. 2006. ClC-7 requires Ostm1 as a beta-subunit to support bone resorption and lysosomal function. *Nature.* 440:220–223. https://doi.org/10.1038/nature04535

Leisle, L., C.F. Ludwig, F.A. Wagner, T.J. Jentsch, and T. Stauber. 2011. ClC-7 is a slowly voltage-gated 2Cl(-)/1H(+)-exchanger and requires Ostm1 for transport activity. *EMBO J.* 30:2140–2152. https://doi.org/10.1038/emboj.2011.137

Lim, H.H., and C. Miller. 2009. Intracellular proton-transfer mutants in a CLC Cl^-/H^+ exchanger. *J. Gen. Physiol.* 133:131–138. https://doi.org/10.1085/jgp.200810112

Lim, H.H., T. Shane, and C. Miller. 2012. Intracellular proton access in a Cl(-)/H(+) antiporter. *PLoS Biol.* 10:e1001441. https://doi.org/10.1371/journal.pbio.1001441

Ludwig, C.F., F. Ullrich, L. Leisle, T. Stauber, and T.J. Jentsch. 2013. Common gating of both CLC transporter subunits underlies voltage-dependent activation of the 2Cl-/1H+ exchanger ClC-7/Ostm1. *J. Biol. Chem.* 288:28611–28619. https://doi.org/10.1074/jbc.M113.509364

Marcoline, F.V., Y. Ishida, J.A. Mindell, S. Nayak, and M. Grabe. 2016. A mathematical model of osteoclast acidification during bone resorption. *Bone.* 93:167–180. https://doi.org/10.1016/j.bone.2016.09.007

Matsuda, J.J., M.S. Filali, K.A. Volk, M.M. Collins, J.G. Moreland, and F.S. Lamb. 2008. Overexpression of CLC-3 in HEK293T cells yields novel currents that are pH dependent. *Am. J. Physiol. Cell Physiol.* 294:C251–C262. https://doi.org/10.1152/ajpcell.00338.2007

Miller, C. 1982. Open-state substructure of single chloride channels from *Torpedo* electroplax. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 299:401–411. https://doi.org/10.1098/rstb.1982.0140

Neagoe, I., T. Stauber, P. Fidzinski, E.Y. Bergsdorf, and T.J. Jentsch. 2010. The late endosomal ClC-6 mediates proton/chloride countertransport in heterologous plasma membrane expression. *J. Biol. Chem.* 285:21689–21697. https://doi.org/10.1074/jbc.M110.125971

Nicoli, E.R., M.R. Weston, M. Hackbarth, A. Becerril, A. Larson, W.M. Zein, P.R. Baker II, J.D. Burke, H. Dorward, M. Davids, et al. Undiagnosed Diseases Network. 2019. Lysosomal Storage and Albinism Due to Effects of a De Novo CLCN7 Variant on Lysosomal Acidification. *Am. J. Hum. Genet.* 104:1127–1138. https://doi.org/10.1016/j.ajhg.2019.04.008

Orhan, G., C. Fahlke, and A.K. Alekov. 2011. Anion- and proton-dependent gating of ClC-4 anion/proton transporter under uncoupling conditions. *Biophys. J.* 100:1233–1241. https://doi.org/10.1016/j.bpj.2011.01.045

Picollo, A., and M. Pusch. 2005. Chloride/proton antiporter activity of mammalian CLC proteins ClC-4 and ClC-5. *Nature.* 436:420–423. https://doi.org/10.1038/nature03720

Platt, F.M., A. d'Azzo, B.L. Davidson, E.F. Neufeld, and C.J. Tifft. 2018. Lysosomal storage diseases. *Nat. Rev. Dis. Primers.* 4:27. https://doi.org/10.1038/s41572-018-0025-4

Pusch, M., and G. Zifarelli. 2020. Large transient capacitive currents in wild-type lysosomal Cl-/H+ antiporter ClC-7 and residual transport activity in the proton glutamate mutant E312A. *J. Gen. Physiol.* 153:e202012583. https://doi.org/10.1085/jgp.202012583

Scheel, O., A.A. Zdebik, S. Lourdel, and T.J. Jentsch. 2005. Voltage-dependent electrogenic chloride/proton exchange by endosomal CLC proteins. *Nature.* 436:424–427. https://doi.org/10.1038/nature03860

Schrecker, M., J. Korobenko, and R.K. Hite. 2020. Cryo-EM structure of the lysosomal chloride-proton exchanger CLC-7 in complex with OSTM1. *eLife.* 9:e59555. https://doi.org/10.7554/eLife.59555

Smith, A.J., and J.D. Lippiat. 2010. Voltage-dependent charge movement associated with activation of the CLC-5 2Cl-/1H+ exchanger. *FASEB J.* 24:3696–3705. https://doi.org/10.1096/fj.09-150649

Steinberg, B.E., K.K. Huynh, A. Brodovitch, S. Jabs, T. Stauber, T.J. Jentsch, and S. Grinstein. 2010. A cation counterflux supports lysosomal acidification. *J. Cell Biol.* 189:1171–1186. https://doi.org/10.1083/jcb.200911083

Weinert, S., S. Jabs, S. Hohensee, W.L. Chan, U. Kornak, and T.J. Jentsch. 2014. Transport activity and presence of ClC-7/Ostm1 complex account for

different cellular functions. *EMBO Rep.* 15:784–791. https://doi.org/10.15252/embr.201438553

Weinert, S., S. Jabs, C. Supanchart, M. Schweizer, N. Gimber, M. Richter, J. Rademann, T. Stauber, U. Kornak, and T.J. Jentsch. 2010. Lysosomal pathology and osteopetrosis upon loss of H^+-driven lysosomal Cl^- accumulation. *Science.* 328:1401–1403. https://doi.org/10.1126/science.1188072

Xiong, J., and M.X. Zhu. 2016. Regulation of lysosomal ion homeostasis by channels and transporters. *Sci. China Life Sci.* 59:777–791. https://doi.org/10.1007/s11427-016-5090-x

Yang, J., J. Chen, M. Del Carmen Vitery, J. Osei-Owusu, J. Chu, H. Yu, S. Sun, and Z. Qiu. 2019. PAC, an evolutionarily conserved membrane protein, is a proton-activated chloride channel. *Science.* 364:395–399. https://doi.org/10.1126/science.aav9739

Zdebik, A.A., G. Zifarelli, E.Y. Bergsdorf, P. Soliani, O. Scheel, T.J. Jentsch, and M. Pusch. 2008. Determinants of anion-proton coupling in mammalian endosomal CLC proteins. *J. Biol. Chem.* 283:4219–4227. https://doi.org/10.1074/jbc.M708368200

Zhang, S., Y. Liu, B. Zhang, J. Zhou, T. Li, Z. Liu, Y. Li, and M. Yang. 2020. Molecular insights into the human CLC-7/Ostm1 transporter. *Sci. Adv.* 6: eabb4747. https://doi.org/10.1126/sciadv.abb4747

Zifarelli, G., S. De Stefano, I. Zanardi, and M. Pusch. 2012. On the mechanism of gating charge movement of ClC-5, a human Cl(-)/H(+) antiporter. *Biophys. J.* 102:2060–2069. https://doi.org/10.1016/j.bpj.2012.03.067