ENDOLYSOSOMAL CLC CHLORIDE/PROTON ANTIPORTERS - FROM BIOPHYSICS TO HUMAN GENETIC DISEASES

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Endosomes and lysosomes are intracellular vesicular organelles with important roles in cell functions such as protein homeostasis, clearance of extracellular material, and autophagy. Endolysosomes are characterized by an acidic luminal pH that is critical for proper function. Five members of the gene family of dimeric voltage-gated ChLoride Channels (CLC proteins) are localized to endolysosomal membranes, carrying out anion/proton exchange activity and thereby regulating pH and chloride concentration[1]. Mutations in the genes encoding these vesicular CLCs (vCLCs) cause global developmental delay, intellectual disability, various psychiatric conditions, lysosomal storage diseases, and neurodegeneration, resulting in severe pathologies or even death[1-5]. Currently, there is no cure for any of these diseases. Based on sequence homology and functional properties, vCLCs fall into two classes: the first comprises endosomal ClC-3, ClC-4, and ClC-5 that share a high sequence homology (the genes encoding these transporters are denoted for example as *CLCN3* for ClC-3). Among these, ClC-5 is kidney specific, while ClC-3 and ClC-4 are most important in neurons[1]. While ClC-3 is stable in homodimeric form, ClC-4 requires ClC-3 to form stable heterodimers[6, 7]. We characterized the first disease-mutations found in *CLCN3* and discovered a novel gain-of-function (GoF) effect associated with dramatically enhanced inward currents at acidic extracellular/luminal pH[4]. In parallel, we investigated a large number of X-linked *CLCN4* variants, some of which exhibited a similar GoF, while others induced a shift of the voltage-dependence to more positive voltages[5]. Both effects are expected to be dominant, explaining that such variants cause disease also in females[5]. Very recently, we discovered that the transmembrane protein of unknown function, TMEM9B strongly suppresses currents carried by ClC-3 and ClC-4, but not that of ClC-1 and ClC-7. We further showed physical interaction of TMEM9B with ClC-3 and ClC-4 using FLIM-FRET microscopy[8].

ClC-6 and ClC-7 for a separate branch and are located in late endosomes and lysosomes, respectively. Recently, we discovered that ClC-6 requires very positive voltages for transport activation, with slow activation kinetics, similar to ClC-7[9]. The GoF disease mutation Y553C[3] exhibited a leftward shift of the voltage-dependence of activation[9]. Recently we further characterized the luminal chloride and pH dependence of ClC-6 and ClC-7 and discovered that the two transporters exhibit opposite pH and chloride dependence: ClC-6 is activated, whereas ClC-7 is inhibited by acidic pH and high chloride. This shows that the two transporters play non-overlapping

physiological role in late endosomes and lysosomes, respectively[10]. Finally, we propose that inhibition of ClC-7 by high chloride serves to limit accumulation of lysosomal chloride in order to prevent an osmotic disaster.

1. Jentsch, T.J. & Pusch, M. CLC chloride channels and transporters: structure, function, physiology, and disease. *Physiol. Rev.* **98**, 1493-1590 (2018).
2. Nicoli, E.R. et al. Lysosomal storage and albinism due to effects of a de novo CLCN7 variant on lysosomal acidification. *Am. J. Hum. Genet.* **104**, 1127-1138 (2019).
3. Polovitskaya, M.M. et al. A Recurrent Gain-of-Function Mutation in CLCN6, Encoding the ClC-6 Cl(-)/H(+)-Exchanger, Causes Early-Onset Neurodegeneration. *Am. J. Hum. Genet.* **107**, 1062-1077 (2020).
4. Duncan, A.R. et al. Unique variants in CLCN3, encoding an endosomal anion/proton exchanger, underlie a spectrum of neurodevelopmental disorders. *Am. J. Hum. Genet.* (2021).
5. Palmer, E.E. et al. Functional and clinical studies reveal pathophysiological complexity of CLCN4-related neurodevelopmental condition. *Mol. Psychiatry* **28**, 668-697 (2023).
6. Guzman, R.E., Bungert-Plumke, S., Franzen, A. & Fahlke, C. Preferential association with ClC-3 permits sorting of ClC-4 into endosomal compartments. *J. Biol. Chem.* **292**, 19055-19065 (2017).
7. Weinert, S. et al. Uncoupling endosomal CLC chloride/proton exchange causes severe neurodegeneration. *EMBO J.*, e103358 (2020).
8. Festa, M. et al. TMEM9B Regulates Endosomal ClC-3 and ClC-4 Transporters. *Life (Basel)* **14** (2024).
9. Zifarelli, G., Pusch, M. & Fong, P. Altered voltage-dependence of slowly activating chloride-proton antiport by late endosomal ClC-6 explains distinct neurological disorders. *J. Physiol.* (2022).
10. Coppola, M.A. et al. Distinct ClC-6 and ClC-7 Cl(-) sensitivities provide insight into ClC-7's role in lysosomal Cl(-) homeostasis. *J. Physiol.* (2023).