Biochemical and biophysical approaches to study the structure and function of the chloride channel (CIC) family of proteins

Priyanka D. Abeyrathne^{1,2*}, Mohamed Chami¹ and Henning Stahlberg¹

¹ Center for Cellular Imaging and NanoAnalytics, Biozentrum, University of Basel, WRO-1508 Mattenstrasse 26, CH-4058 Basel, Switzerland.

² Howard Hughes Medical Institute, Janelia Research Campus, 19700 Helix Drive, Ashburn, VA, USA (current address)

* to whom correspondence should be addressed:
Email abeyrathnep@janelia.hhmi.org

1 ABSTRACT

2 The chloride channel (CIC) protein family comprises both chloride (Cl^-)
3 channels and chloride/proton (Cl^-/H^+) antiporters. In prokaryotes and eukaryotes,
4 these proteins mediate the movement of Cl^- ions across the membrane. In
5 eukaryotes, CIC proteins play a role in the stabilization of membrane potential,
6 epithelial ion transport, hippocampal neuroprotection, cardiac pacemaker activity
7 and vesicular acidification. Moreover, mutations in the genes encoding CIC
8 proteins can cause genetic disease in humans. In prokaryotes, the Cl^-/H^+
9 antiporters, such as CIC-ec1 found in *Escherichia coli* promote proton expulsion
10 in the extreme acid-resistance response common to enteric bacteria. To date,
11 structural and functional studies of the prokaryotic protein have revealed unique
12 structural features, including complicated transmembrane topology with 18 α -
13 helices in each subunit and an anion-coordinating region in each subunit. Several
14 different approaches such as X-ray crystallography, NMR, biochemical studies,
15 and molecular dynamics simulations have been applied to the study of CIC
16 proteins. Continued study of the unique structure and function of this diverse
17 family of proteins has the potential to lead to the development of novel
18 therapeutic targets for neuronal, renal, bone, and food-borne diseases.

19

20 **Keywords:** channel, antiporter, chloride ion, proton, acid resistance, membrane
21 protein

22

23

1. Introduction

Chloride (Cl^-) is the most abundant ion in nature. It is transported by several distinct families of chloride transporting proteins including chloride channels (CIC), ligand gated GABA and glycine receptors, cystic fibrosis conductance transmembrane regulators, cation chloride transporters, and chloride/bicarbonate exchangers. These proteins reside in both the plasma membrane and in the membranes of intracellular organelles, and contribute to a variety of essential processes. Members of the CIC protein family in particular, which includes both Cl^- channels and chloride/proton (Cl^-/H^+) antiporters, participate in many different physiological functions within prokaryotic and eukaryotic organisms. In this review we focus on recent structural and functional insights pertaining to the CIC proteins in particular.

The first CIC protein to be identified was CIC-0, found in the electric organ of the *Torpedo marmorata*, also known as the torpedo fish [1] [2]. Since then, CIC proteins have been identified in numerous species of bacteria, plants, and mammals. In the first part of this review, we discuss the mammalian CIC proteins, which have been shown to be vital to cellular function and to play a role in human disease. However, the mechanisms underlying their roles are not well understood. Much of what we know about CIC proteins, their structures and their function, comes from the study of prokaryotic CIC proteins. Thus, in the second part of this review, we discuss the prokaryotic CIC proteins, specifically CIC-ec1, the protein found in *Escherichia coli* (*E. coli*) that plays a critical role in the bacteria's ability to survive in highly acidic environments for short periods of time.

Finally, we review several different approaches currently used to study the structure of CIC-ec1.

2. The Mammalian CIC Family

2.1 Function and distribution.

To date, there are nine known members of the human CIC family. These are categorized into three groups based on their sequence homology (Figure 1) [1].

The first group comprises the Cl⁻ channels: CIC-1, CIC-2, and the kidney-specific chloride channels CIC-Ka and CIC-Kb [1]. All four of these proteins are found predominantly in the plasma membrane where they function as gated Cl⁻ channels and act to stabilize the membrane potential or to mediate epithelial transport [1].

The remaining proteins, classified as group 2 and 3, are electrogenic Cl⁻/H⁺ antiporters, whose distribution and physiological functions are distinct from the Cl⁻ channel proteins [1, 3-5]. These five proteins (CIC-3, CIC-4, and CIC-5 in group 2; and CIC-6 and CIC-7 in group 3) are predominantly found in intravascular membranes of the endosomal-lysosomal pathway and are only expressed to a limited extent at the plasma membrane [1]. Currently, little is known about the physiological function of CIC-3, CIC-4, and CIC-6. CIC-3 knockout mice exhibit retinal and neuronal degeneration indicating that CIC-3-mediated acidification of synaptic compartments may play a significant role in neuronal stability [6-8]. Moreover, studies in cultured fibroblasts show that CIC-4 is essential for proper trafficking and function of the transferrin receptor [9, 10].

The two best-characterized Cl^-/H^+ antiporters so far are CIC-5 and CIC-7. CIC-5 is predominantly expressed in the proximal tubule of the kidney, where it is localized to apical endosomes. CIC-3, CIC-4, and CIC-5 cooperate with the vacuolar-type proton-ATPase (V-ATPase) to facilitate the acidification of endosomes [6, 11, 12]. Endosomal acidification mediated by group 2 CIC proteins is essential to promote ligand/receptor dissociation, enable early-to-late endosomal maturation, and activate hydrolytic enzymes [13-15]. The precise mechanism of CIC-mediated acidification remains controversial. One proposed scenario is that antiporters may mediate the entry of Cl^- into the endosome, thereby dissipating the positive charge generated by the V-ATPase [6, 11, 12, 15, 16]. The group 3 antiporter proteins, CIC-6 and CIC-7, are thought to facilitate protein degradation by regulating Cl^- ion concentration to ensure optimal function of the hydrolytic enzymes of late endosomes and lysosomal compartments [17].

Evidence of a role for CIC proteins in human disease comes from genetic studies linking mutations in these proteins to the occurrence of many hereditary diseases. For example, malfunction of CIC-5 leads to Dent's disease, an X-chromosome-linked disorder associated with proteinuria and the development of kidney stones. Mutations in CIC-7 can cause impaired bone resorption and are linked with several variants of osteopetrosis (Table 1) [18]. Clearly, mammalian CIC channels and antiporters play a vital role in cellular function, but details of the mechanisms involved remain elusive. Clarification of these processes requires a thorough understanding of the behavior of CIC proteins at the molecular level. However, to date most of the molecular level work has focused

on prokaryotic proteins, as they are relatively easy to express and purify in milligram quantities compared to their eukaryotic counter parts. Clearly, more studies are needed in order to fully appreciate the role that CIC proteins play in mammalian physiology and pathophysiology.

2.2 CIC channels: structure and gating properties.

The structural organization of CIC proteins varies somewhat between protein types found in prokaryotes and eukaryotes, but several key features are conserved. All CIC proteins have a transmembrane catalytic domain and most (all eukaryotic types and a few prokaryotic types) have a cytoplasmic regulatory domain [19]. The cytoplasmic domain appears to be involved in diverse regulatory mechanisms, including oligomerization, protein sorting, channel gating, and ligand binding [20]. In the eukaryotic proteins, the cytoplasmic domain is crucial for protein function [21, 22], as mutations in this domain are associated with hereditary diseases, such as myotonia, Dent's disease, osteopetrosis, and Bartter syndrome [23].

Insights into the conductance and gating properties of group 1 CIC proteins came from electrophysiological experiments studying the eukaryotic muscle-type channels CIC-0 (a CIC protein variant found in *T. marmorata*) and CIC-1 [24]. The gating mechanisms of CIC-0 have also been studied via analysis of the behavior of single channels reconstituted in artificial lipid membranes [24]. This work revealed the surprising result that the channel has two equally spaced, binominally distributed conductance states, indicative of two identical,

independently-gated pores [24]. Additionally, this study showed that individual gates act on each pore separately, while a separate common gating mechanism can affect both pores simultaneously. We now know that gate opening and closing is determined by three factors: pH, Cl^- concentration, and membrane potential, where pore opening is favored by low pH, high Cl^- concentration, and depolarization of the membrane. While the channel is more likely to open at more depolarized membrane potentials, movement of Cl^- ions into the transmembrane electric field during the opening step can locally alter membrane potential, thereby shifting the voltage-dependence of channel gating [25]. This permeant ion concentration-dependent gating mechanism is profoundly different from that found in classical voltage-gated cation channels. In those channels, gating is rendered voltage-dependent by a “voltage sensor” domain containing several positively charged residues that move within the transmembrane electric field [26].

In CIC channels, the structural determinant of the individual gating process is a single glutamate (E) residue that alternates between occupying the extracellular site for Cl^- , thereby prohibiting ion flow in the closed state, or moving away from this site allowing Cl^- permeation through the selectivity filter when the channel is open. Less is known about the specific structural determinants of the common gating mechanism in this channel. Several experiments have suggested that additional conformational changes underlie the concerted closing of both pores during common gating [27-29]. Biochemical studies have indicated that mutations at the dimer interface of the catalytic

domains [30], as well as those occurring in the cytoplasmic domains can influence the functioning of the common gate [31]. Finally, mutations that are known to disrupt common gating have been associated with congenital myotonia in humans, indicating that further study of this unique feature of the CIC channel is warranted.

2.3 CIC antiporters.

Based on their homology to CIC-0, all CIC proteins were initially assumed to also function as Cl^- channels. However, the electrophysiological characterization of the prokaryotic homolog CIC-ec1 showed that this protein is in fact an antiporter, not a channel, raising the possibility that other proteins in the CIC protein family may be antiporters as well. Indeed, we now know that the group 2 and group 3 mammalian CIC proteins, CIC-3, CIC-4, CIC-5, CIC-6, and CIC-7, also function as antiporters [3, 4, 32]. These proteins, like CIC-ec1, are highly electrogenic antiporters [4, 33, 34], with a stoichiometry of ion transport of two Cl^- to one H^+ . The passage of ions is mediated by two gates that lie on opposite sides of the membrane, through which ions pass in opposite directions. The gates effectively prevent free diffusion of ions by alternately opening and closing.

3. The prokaryotic CIC family

The eukaryotic CIC proteins are homologs to several prokaryotic proteins with related structures and function. These prokaryotic proteins are relatively easy to express and purify compared to their eukaryotic counterparts. Thus, prokaryotic

CIC proteins have served as model systems to study the structure and function of the CIC protein family. In addition to facilitating high-resolution structural data relevant to many members of the CIC protein family, functional investigations of prokaryotic CIC proteins showed them to be Cl^-/H^+ antiporters. Sequence alignment of CIC in a strongly conserved region commonly known as the “PIGG-pen” revealed that a key glutamate residue (E203) is uniformly present in Cl^-/H^+ antiporters, but absent in Cl^- channels [35]. This results supports the idea that there are structural differences between the two kingdoms, and comparison studies are likely to reveal key features underlying their divergent functions. For example, we now know that prokaryotic CIC proteins play a role in conferring acid resistance in many different species of bacteria. Elucidation of the structural features that underlie these functions has not only informed our ideas of the structure and function of related eukaryotic proteins, but has shed light on this unique property that is shared by some of the most pathogenic species of bacteria.

3.1 CIC proteins and bacterial acid resistance

Researchers first encountered CIC proteins in bacteria while studying the bacterial acid resistance mechanism [36]. Although the stomach is highly acidic (pH 1.5-2.5), many pathogenic bacteria successfully navigate this extremely hostile environment, surviving to replicate and ultimately cause disease [36]. To accomplish this, some bacterial species, such as *Vibrio cholera* and *Salmonella spp*, launch an assault that involves huge numbers of infecting microorganisms,

1 after that a few ultimately survive and enter the intestine [36]. Others, such as
2 *Helicobacter pylori* (*H. pylori*), have evolved counter defenses such as acid
3 neutralization mechanisms, that allow them to survive under extreme acid stress.

4 Interestingly, *E. coli*, an intestinal microorganism that prefers to be at
5 neutral pH, displays neither of these survival mechanisms. Instead, it employs a
6 strategy for increasing acid tolerance and enabling resistance to acid stress for a
7 few hours [37-40]. Under highly acidic conditions, protons likely enter the *E. coli*
8 inadvertently, through protein channels, or directly across the cell membrane
9 without an associated Cl^- . This flux increases the positive charge inside the cell
10 [36], and causes the internal pH of the bacterial cell to decrease (from
11 approximately pH 7.5 to 4.5) [36]. The bacteria deal with the influx of ancillary
12 protons through the decarboxylation of amino acids. However, while this process
13 removes the protons, positive charges remain as an end-product of the
14 decarboxylation process. It has been proposed that CIC proteins, specifically the
15 Cl^-/H^+ antiporters, then facilitate the movement of negative charges into the cell,
16 while simultaneously removing excess protons to compensate for the excess of
17 intracellular positive charges [36]. As the external pH become less acidic and the
18 proton intrusion slows, decarboxylases continue to remove internal protons,
19 increasing the internal pH and ultimately inverting the membrane potential to
20 allow *E. coli* to resume its normal cellular functions [36].

21 There is a growing body of evidence to support the role of CIC-ec1 in the
22 mechanism described above. Studies have shown that the *E. coli* genome
23 includes two genes (*eriC*, also known as *CIC-ec1*, and *mriT*) that are homologues

to the CIC family of proteins [41-43]. Deletion of either gene has no detectable effect on acid resistance. However, knocking out both genes critically impairs the ability of the bacteria to cope with an extremely acidic environment [44]. Furthermore, acid resistance is rescued in the double-knockout strain by complementation *in trans* with CIC-ec1 in a low-copy plasmid [36]. The acid sensitivity of the double knockout arises because the CIC proteins cannot deliver the necessary substrates to, and remove the by-products from, the proton-consuming decarboxylases [36]. Iyer and co-workers [44] proposed that the counter transport processes are electrogenic, effectively moving the positive charge outwards. Thus, to halt extravagant inner-membrane hyperpolarization, an electric shunt is needed. In the HCl-rich stomach, it would make physiological sense for CIC proteins to fulfill this role.

To further understand the behavior of the *E. coli* CIC-ec1 protein under acid shock conditions, Iyer and coworkers examined whether the CIC-ec1 is activated by strong acid [44]. They reconstituted purified CIC-ec1 into liposomes and measured electrogenic Cl^- influx as a function of pH. Around physiological pH, the channel mediated a low, but non-zero uptake of Cl^- . Interestingly, this rate increased approximately tenfold as the pH was lowered below pH 5. Thus, they were able to demonstrate that CIC-ec1 is activated under acid-shock conditions, validating the predicted biological function of CIC-ec1 [44]. Moreover, the acid resistance mechanism employed by pathogenic *E. coli* strain O157:H7 is identical to those described for the wild type *E. coli* laboratory strain MG1655, mentioned above [36]. Similar mechanisms may be present in other enteric

1 pathogens such as *Shigella*, *Vibrio*, *Yersinia* or *Salmonella*, all of which possess
2 genes for CIC proteins [44]. Considering this, lyre and coworkers proposed that
3 pathogenic bacteria might rely on CIC proteins as a means to survive acid shock,
4 thereby increasing their virulence [44].

6 **3.2 CIC-ec1: Channel or Transporter?**

7 The CIC-ec1 protein mediates the movement of Cl^- ions across the membrane.
8 Both biochemical and structural studies have helped determine whether it
9 functions as a channel or transporter. Accardi and Miller reconstituted purified
10 CIC-ec1 into planar lipid bilayer membranes and recorded the Cl^- -selective
11 current [33, 45]. They observed macroscopic current that was voltage-dependent
12 and maximally activated at low pH, suggesting that CIC-ec1 functions as a
13 proton-activated electrical leak for Cl^- . Moreover, when they recorded the current
14 under a salt gradient, the current-voltage relationship indicated selective Cl^-
15 permeation. However, they also found that the reversal potential was below the
16 Nerst equilibrium, indicating the movement of an additional ion along with Cl^- .
17 Indeed, they determined that protons also cross the membrane, and proposed a
18 2:1 stoichiometric exchange of Cl^- and H^+ .

19 Structural data also support the idea that CIC-ec1 does not conform to
20 common ion channel characteristics. The data indicate that CIC-ec1 is a
21 homodimer with two-fold symmetry, and that it is mostly membrane-embedded
22 with short cytoplasmic N and C termini [46]. Finally, CIC family proteins have a
23 highly conserved region around position 203 in the *E. coli* sequence, the so-

called “PIGG-pen” signature sequence. Interestingly, this sequence varies depending on whether the protein is a channel or transporter; residue 203 is a valine in channel proteins, but glutamate in antiporters. In CIC-ec1 there is a glutamate residue at this site (E203) [47]. Indeed, it has been proposed that the conserved glutamate residue at this position may be involved in the H⁺ conduction pathway [48].

3.3 Structural features of CIC-ec1

Understanding structure and function of CIC-ec1 is important not only to gain insight into its role in facilitating bacterial acid resistance, but also to gain a foothold for the study of related eukaryotic proteins that may be involved in human disease. As discussed above, the gross structural features of CIC-ec1 support the idea that this protein functions as a transporter of Cl⁻ and H⁺ in *E. coli*. For more than a decade, researchers have explored biophysical and biochemical approaches to obtain more detailed structural information about this protein in order to understand its role in bacterial acid resistance. Here, we discuss data from X-ray crystallography, biochemical studies, molecular dynamics simulations, and NMR studies that shed light on the detailed structural characteristics of CIC-ec1.

3.3.1. X-ray crystallography

In 2002, Dutzler and coworkers resolved the first high-resolution X-ray structures of a prokaryotic CIC protein, CIC-ec1, providing insight into the architectural

characteristics of the family and the chemistry of Cl^- binding [46]. They determined that CIC-ec1 has a complicated transmembrane topology with 18 α -helices (A-R) in each subunit (Table 2) [46, 49]. Each subunit of CIC-ec1 has an active site consisting of an anion-coordinating region that is occupied by a pair of Cl^- ions (Fig. 2A). Three Cl^- -binding sites located within the ion conduction pathway of each monomer were identified from the X-ray structure of CIC-ec1, including one in the central part of the membrane and one each on the internal and external sides of the membrane [46, 49]. The external Cl^- -binding site is located between the N termini of helices F and N, where amide nitrogen atoms form a cage surrounding the ion. When an anion is at this site, it can contact main-chain amide atoms, suggesting that this external site is important for selective ion conduction through the open state. The key residue E148 of helix F, located in the exofacial region of the membrane, serves as an external gate for Cl^- ions (Fig. 2A). The central Cl^- -binding site is located mid-way through the membrane. There, the positively charged N termini of helices D, F and N point towards the central site. This creates an electrostatically favourable environment for anion binding. The Cl^- ion therefore coordinates the main-chain amide nitrogen atoms from the end of helix N and from the side chain of conserved amino acids serine 107 (S107) and tyrosin 445 (Y445) (Fig. 2A). The internal site is adjacent to the protein's cytoplasmic surface proximate to helix D and tyrosin 445 (Y445) of helix R (Fig. 2A).

The structure of CIC-ec1 also revealed how the residues involved Cl^- -binding might mediate Cl^- permeation. Specifically, the side chain of E148 at the

external Cl⁻-binding site projects into the ion pathway, thereby blocking the entry of ions from the external environment [46]. Once the side chain is protonated at low pH, it moves away from the pore, allowing anions to enter. Mutation studies have shown that neutralization of E148 to alanine (E148A) or glutamine (E148Q) completely abolishes proton flux while maintaining Cl⁻ transport [50]. This mechanism appears to be conserved to some extent in mammalian CIC proteins (CIC-4 and CIC-5); in these proteins the same mutation removed sensitivity to extracellular acidity and rendered them voltage dependent [3, 4].

3.3.2 Biochemical studies

Data from the high-resolution X-ray structure of CIC-ec1 can then be combined with data from biochemical studies to better understand which residues are important in ion transport mechanisms. For example, a combination of structural and biochemical data originally identified E203 as a key residue in CIC-ec1. As discussed above, It appears to be required for proton transport and to act as an internal proton transfer site [47]. Biochemical studies show that mutation of E203 abolishes proton transport while preserving the pH activation mechanism. Structural data reveal that E203 is the second glutamate residue of helix H and that it is located near the intracellular surface of CIC transporter proteins. This residue is highly conserved among prokaryotic CIC antiporters and has been identified in mammalian CIC antiporters, further supporting the critical role of E203 in antiporter function [51].

Several other insights into the structure and function of transporters have

stemmed from biochemical studies. For example, key residues involved in the dual gating mechanism of CIC-ec1 were identified based on studies of proteins in which both E148 and Y445 were mutated. These mutant proteins display uncoupled, ungated channel-like properties with increased conductance leading to conclude that E148 appears to form the extracellular gate, while Y445 functions as the intracellular gate [52].

The identification of key residues involved in gating is the first step towards understanding how these proteins function. Given that transporters work through a series of protein conformational changes with every round of ion transport, researchers next started to investigate whether such conformational changes occur in CIC-ec1 during its ion transport cycles. The conserved E148 and Y445 residues, residing on the exterior and interior sides of the CIC-ec1, respectively, block Cl^- from escaping from the protein. Mutating E148 to alanine (E148A) or glutamine (E148Q), abolishes proton transport but maintains Cl^- transport, resulting in Cl^- movement that is completely uncoupled from its electrochemical gradient [33]. Decreasing the side chain volume at Y445 by replacing it with serine (Y445S) also partially disrupts proton transport, and leads to partial uncoupling [53]. Combining the two mutations (E148A/Y445S) results in dramatic enhancement of Cl^- fluxes (on the order of $36,000 \text{ s}^{-1}$) 3-fold faster than the fastest known transporter [28].

Biochemical studies have also been essential in elucidating the conformational changes of CIC-ec1 during permeation. Using site-directed fluorescence labelling, Bell and co-workers investigated whether regions of CIC-

ec1 beyond E148 are moving during ion permeation [54]. Specifically, they introduced labeled cysteines at several positions in helix R of CIC-ec1 and examined the fluorescence changes in response to acidification, over the same pH range that is known to activate transport. They observed changes in fluorescence upon acidification, suggesting that the helix R or other nearby parts of the protein (such as helix J or D) experience a conformational change, and confirming that conformational changes in CIC-ec1 extend beyond E148.

Basilio and co-workers performed experiments to see if CIC-ec1 involves conformational rearrangements outside the Cl⁻ pathway [55]. Using crosslinking experiments, they focused on the helical bundle formed by the helices J, O and Q. They introduced cysteine pairs at helical bundle contact points and crosslinked them with divalent mercury, a bi-functional thiol-specific cross-linker. The crosslinked protein adopts a wild type like conformation, indicating that the disulfide bond does not distort the structure of the protein. However, they did find that crosslinking the intracellular portion of helix O to helix Q or J reduces Cl⁻ transport by 30-100 fold, suggesting that movement of helix O is required for transport. Finally, their approach also allowed them to deduce that movement of helix O is transduced to the Cl⁻ pathway via a direct contact between its C-terminal end and Tyrosine 445.

3.3.3. Molecular dynamics simulations

Biochemical studies, such as those described above, can reveal the occurrence of conformational changes in functioning proteins, but often cannot

1 provide information about how the protein actually moves. Moreover, even high-
2 resolution structural data from X-ray crystallographic studies cannot address the
3 nature of conformational changes, as these data represent a snapshot of the
4 motionless protein [56]. For example, the crystal structure of CIC-ec1 depicts a
5 closed state, in which a bound Cl^- ion does not have access to either the
6 extracellular or intracellular solutions. In order for transport to occur, additional
7 protein conformations must exist, which allow the transporter to pick up and
8 subsequently deliver Cl^- from one side of the membrane to the other [56].
9 However, despite the use of crystallization conditions spanning a wide range of
10 Cl^- and proton concentrations, only the closed conformation has been observed
11 in the wild-type protein to date [46, 47, 49, 56, 57]. This drawback is not unique
12 to the CIC proteins. Although the growing database of high-resolution membrane
13 protein structures provides crucial molecular details that are fundamental to
14 understanding membrane protein function [58], it also highlights the necessity for
15 complementary studies to reveal the dynamics of protein conformational changes
16 [56].

17 Computational techniques have the potential to utilize data from both
18 structural and biochemical studies to study how proteins move and change over
19 time. Molecular dynamics simulations (MD) are one such technique that is used
20 to calculate the time-dependent behavior of a molecular system. MD simulations
21 have provided detailed information on the fluctuation and conformational
22 changes of proteins and nucleic acids. This method is now routinely used to
23 investigate the structure, dynamics, and thermodynamics of biological molecules

1 and their complexes.

2 Faraldo-Gomez and Roux investigated the electrostatics of ion
3 stabilization in CIC-ec1 using MD simulations [59]. While biochemical studies
4 have highlighted the importance of the S107 and Y445 residues in regulating Cl⁻
5 permeation, their study revealed that there is also direct involvement of several
6 other residues around the known Cl⁻-binding sites, indicating the possibility of
7 fourth Cl⁻-binding site in CIC-ec1. One of the residues highlighted in these
8 studies, lysine131 (K131), is located in helix E and plays an important structural
9 role in stabilizing the geometry of the central and internal Cl⁻-binding site by
10 forming hydrogen bonds with nearby glycine 106 (G106), and S107 in the GSGIP
11 motif (Fig. 2B,C). Interestingly, both K131 and the GSGIP motif are conserved
12 among nearly all of the CIC proteins [59]. Another residue, arginine 147 (R147),
13 located towards the extracellular side of the permeation pathway, contributes to
14 the external Cl⁻-binding site (Fig. 2B,C). A third residue, R340, is located in helix
15 M and interacts with the loop connecting helix K and helix L, specifically the
16 GGGFN motif within this loop that functions like the GSGIP motif (Fig. 2B, C)
17 [59]. Based on the above observations, Faraldo-Gomez and Roux proposed that
18 the R340-GGGFN region might serve as an additional extracellular binding site
19 (fourth Cl⁻ binding site), where the G315 and G316 interact with Cl⁻. Specifically,
20 according to their proposed model, R340 would fix the orientation of the carbonyl
21 groups of G314 and G315 and interact favorably with the bound Cl⁻ [59] (Fig. 2B,
22 C). G315, G316 and R147 are also stabilized at this site (Fig. 2B-D). They further
23 concluded that the binding of Cl⁻ ions to this forth site was as energetically

1 favored, as is the case for the other three Cl⁻ binding sites.

2 Crystallographic data show no visible Cl⁻ at this location in the protein,
3 though water molecules are observed, indicating the site is accessible (Fig. 2B,
4 C) [46, 49]. It was only through MD simulation studies that this is low-affinity site,
5 similar to the internal Cl⁻ binding sites in other proteins (CIC-0, CIC-1, and CIC-2)
6 where a similar structural motif is likely to occur, could be appreciated [59]. The
7 existence of a fourth binding site has also been proposed in the conduction pore
8 of the voltage-dependent, fast-gating CIC-0 channel [60]. Examination of single
9 channel gating kinetics in this channel indicated that external Cl⁻ ions bind to the
10 extracellular side of the channel in an essentially voltage-independent manner,
11 after which channel opening results from the voltage-dependent inward
12 movement of Cl⁻ through the conduction pore.

13 X-ray crystallographic data identified E203 as a proton transfer site in CIC-
14 ec1, and that this is likely the residue that mediates proton transfer between the
15 intracellular solution and the protein interior [46]. Specifically, in the crystal
16 structure, E203 formed a salt bridge with R28 of helix A and a hydrogen bond
17 with E113 of helix D [61]. When E203 accepts a proton from the intracellular side
18 of the membrane, the salt bridge and hydrogen bond interactions would be
19 disrupted, forming the basis of the proton transfer mechanism. Wang and Voth
20 were able to further elucidate the proton conduction pathway of CIC-ec1 using
21 MD simulations [61]. They identified a conformational change that allows the
22 protonated side chain of E203 to carry a proton from the intracellular surface to
23 the core of the protein [61]. The conformational change of the E203 side chain

1 involves a change of χ^2 from 60° to 180° . Other residues are also involved,
2 specifically the hydrophobic side chain of phenylalanine 199 (F199 in helix H)
3 and two other glutamate residues (E113 and E148). The aromatic ring of F199
4 initially blocks the proton pathway but F199 flips its side chain to a different
5 position once E203 is protonated, and later reorients during the transport cycle.
6 Wang and Voth also showed evidence of alteration between the protonated
7 states of E113 and E203 during the proton transport cycle, as well as
8 coordinated activity between the negatively charged E148 and the central Cl^- ion,
9 that drives protons to the extracellular side of the membrane [61].

10 Han and co-workers performed extended equilibrium MD simulations to
11 evaluate whether water wires might mediate proton transport between E148 and
12 E203, and to characterize factors that support water wire formation [62]. They
13 found that under equilibrium, the central hydrophobic region can accommodate
14 six water molecules. Additionally, they observed the spontaneous formation of
15 continuous water wires connecting E148 and E203. The wires form frequently
16 and last up to 2.4 ns long enough for H^+ to be exchange between E148 and E203
17 through the Grotthuss mechanism [62]. Further they demonstrated that water
18 molecules hydrating the central region come from bulk solvent on the
19 cytoplasmic side, not the extracellular side, and that they enter the central region
20 via at the dimer interface lined by E202 and A404. Data from the simulation and
21 site-directed mutagenesis showed that the small size of A404 is a critical factor in
22 allowing water to efficiently access the central region.

3.3.4. Nuclear magnetic resonance studies

Today researchers use both solid-state and solution-state nuclear magnetic resonance (NMR) techniques to investigate the structure of membrane proteins [63]. Recent advances in these techniques allow membrane proteins to be examined while inserted in a lipid membrane or solubilized in detergent micelles [63]. This approach has the advantage that structural constraints imposed by crystallization are avoided. Indeed, solution-state NMR has the potential to provide tremendous insight into protein dynamics [64-66]. In the case of CIC-ec1, the X-ray structure of one conformation is already known. Incorporating this structural information, NMR has the potential to identify regions of conformational change as well as to study the dynamics of these changes as the protein transitions through other states [67].

Elvington and coworkers utilized solution-state ^{19}F -NMR to pinpoint regions of conformational change in CIC-ec1 [56]. In their approach, the small nucleus ($1.35\text{\AA}$ radius) of the fluorine atom does not perturb protein structure [68], confers a high sensitivity to the chemical environment [68-70], and is easily incorporated [56], making it ideally suited for these studies [71]. They observed changes in the ^{19}F -NMR spectrum of fluorotyrosine labeled CIC-ec1, while varying either the H^+ or Cl^- concentration in order to observe the functioning protein. Their data confirm that structural changes occur near the subunit interface and, therefore, that substrate-driven conformational change is not restricted to the Cl^- permeation pathway alone. They also found that removal of the protein's proton transport ability does not eliminate the proton-dependent

protein movement observed in the intact antiporter. Lastly, they demonstrated that the CIC-ec1 channel-like mutant [52] does not undergo the same substrate-dependent conformational changes that occur in the CIC-ec1 antiporters. The F-NMR approach has revealed many different types of conformational changes not detected via other approaches. However, more work is needed in order to understand how these movements are involved in the ion transport cycles.

4. Conclusions

The CIC proteins have been known for many years and have been implicated in a variety of human diseases. These proteins have unique structural features leading to exciting questions about potentially unique functional mechanisms underlying their diverse physiological roles, including membrane potential stabilization, trans-epithelial transport, and vesicular acidifications. The first structural information came from the study of the prokaryotic protein CIC-ec1. Its structure revealed a complex fold of 18 α -helices per subunit with two Cl^- ions bound in the center of each pore. A critical glutamic acid residue was identified whose side-chain seems to occupy a third Cl^- ion binding site in the closed state and that moves away to allow Cl^- binding. While the overall architecture and pore structure is conserved from bacteria to humans, the prokaryotic proteins that were crystallized are actually not Cl^- channels, but coupled Cl^-/H^+ antiporters. Structural information is now available for two other eukaryotic Cl^-/H^+ antiporter proteins (from *Cynidioschyzon merolae* and *Synechocystis* sp. Pcc 6803) in addition to CIC-ec1 (Table 2).

Despite the availability of detailed structural information, it is not clear whether Cl^-/H^+ antiporters undergo conformational changes during their ion transport cycles. Such conformational changes are likely to have significant biological implications. Moreover, whether there any missing key residue(s), which might act as a regulators of the transporter gating remains unclear. Thus, there are important open questions that will require the implementation of alternative approaches to complement X-ray crystallography, biochemical studies, MD simulations, and NMR. These alternative approaches need to have the capability to detect and confirm conformational changes as the protein functions. For example, the two-dimensional (2D) crystallization of a Cl^-/H^+ antiporter in a lipid bilayer would not restrict conformational changes and would allow relevant structural information to be obtained by electron crystallography, while allowing for widely changed environmental parameters, such as variations in pH or Cl^- concentrations. This type of data could then be used to inform the rational design of drugs targeting Cl^-/H^+ antiporters. Indeed, the first structural information of CIC -ec1 came from electron crystallography [72]. It is likely that some combination of biochemical and biophysical approaches will be necessary to ultimately provide a clear picture of the structure and function of this unique family of proteins.

ACKNOWLEDGEMENTS

This work was in part supported by the Swiss National Science Foundation, grant 315230_146929. We thank Shirley A Muller for her critical reading of the manuscript at the beginning.

References:

- [1] T.J. Jentsch, K. Steinmeyer, G. Schwarz, Primary structure of Torpedo marmorata chloride channel isolated by expression cloning in *Xenopus* oocytes, *Nature*, 348 (1990) 510-514.
- [2] T.J. Jentsch, V. Stein, F. Weinreich, A.A. Zdebik, Molecular structure and physiological function of chloride channels, *Physiological reviews*, 82 (2002) 503-568.
- [3] A. Picollo, M. Pusch, Chloride/proton antiporter activity of mammalian CLC proteins CLC-4 and CLC-5, *Nature*, 436 (2005) 420-423.
- [4] O. Scheel, A.A. Zdebik, S. Lourdel, T.J. Jentsch, Voltage-dependent electrogenic chloride/proton exchange by endosomal CLC proteins, *Nature*, 436 (2005) 424-427.
- [5] A.R. Graves, P.K. Curran, C.L. Smith, J.A. Mindell, The Cl⁻/H⁺ antiporter CLC-7 is the primary chloride permeation pathway in lysosomes, *Nature*, 453 (2008) 788-792.
- [6] S.M. Stobrawa, T. Breiderhoff, S. Takamori, D. Engel, M. Schweizer, A.A. Zdebik, M.R. Bosl, K. Ruether, H. Jahn, A. Draguhn, R. Jahn, T.J. Jentsch, Disruption of CLC-3, a chloride channel expressed on synaptic vesicles, leads to a loss of the hippocampus, *Neuron*, 29 (2001) 185-196.
- [7] M. Yoshikawa, S. Uchida, J. Ezaki, T. Rai, A. Hayama, K. Kobayashi, Y. Kida, M. Noda, M. Koike, Y. Uchiyama, F. Marumo, E. Kominami, S. Sasaki, CLC-3 deficiency leads to phenotypes similar to human neuronal ceroid lipofuscinosis, *Genes to cells : devoted to molecular & cellular mechanisms*, 7 (2002) 597-605.
- [8] L.W. Dickerson, D.J. Bonthius, B.C. Schutte, B. Yang, T.J. Barna, M.C. Bailey, K. Nehrke, R.A. Williamson, F.S. Lamb, Altered GABAergic function accompanies hippocampal degeneration in mice lacking CLC-3 voltage-gated chloride channels, *Brain research*, 958 (2002) 227-250.
- [9] T.J. Jentsch, Chloride transport in the kidney: lessons from human disease and knockout mice, *Journal of the American Society of Nephrology : JASN*, 16 (2005) 1549-1561.
- [10] R. Mohammad-Panah, L. Wellhauser, B.E. Steinberg, Y. Wang, L.J. Huan, X.D. Liu, C.E. Bear, An essential role for CLC-4 in transferrin receptor function revealed in studies of fibroblasts derived from *Clcn4*-null mice, *Journal of cell science*, 122 (2009) 1229-1237.

- [11] W. Gunther, N. Piwon, T.J. Jentsch, The ClC-5 chloride channel knock-out mouse - an animal model for Dent's disease, *Pflügers Archiv : European journal of physiology*, 445 (2003) 456-462.
- [12] M. Hara-Chikuma, Y. Wang, S.E. Guggino, W.B. Guggino, A.S. Verkman, Impaired acidification in early endosomes of ClC-5 deficient proximal tubule, *Biochemical and biophysical research communications*, 329 (2005) 941-946.
- [13] O.A. Weisz, Organelle acidification and disease, *Traffic*, 4 (2003) 57-64.
- [14] A. Hurtado-Lorenzo, M. Skinner, J. El Annan, M. Futai, G.H. Sun-Wada, S. Bourgoin, J. Casanova, A. Wildeman, S. Bechoua, D.A. Ausiello, D. Brown, V. Marshansky, V-ATPase interacts with ARNO and Arf6 in early endosomes and regulates the protein degradative pathway, *Nature cell biology*, 8 (2006) 124-136.
- [15] E.I. Christensen, O. Devuyst, G. Dom, R. Nielsen, P. Van der Smissen, P. Verroust, M. Leruth, W.B. Guggino, P.J. Courtoy, Loss of chloride channel ClC-5 impairs endocytosis by defective trafficking of megalin and cubilin in kidney proximal tubules, *Proceedings of the National Academy of Sciences of the United States of America*, 100 (2003) 8472-8477.
- [16] M. Hara-Chikuma, B. Yang, N.D. Sonawane, S. Sasaki, S. Uchida, A.S. Verkman, ClC-3 chloride channels facilitate endosomal acidification and chloride accumulation, *The Journal of biological chemistry*, 280 (2005) 1241-1247.
- [17] L. Wartosch, J.C. Fuhrmann, M. Schweizer, T. Stauber, T.J. Jentsch, Lysosomal degradation of endocytosed proteins depends on the chloride transport protein ClC-7, *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 23 (2009) 4056-4068.
- [18] C.A. Hubner, T.J. Jentsch, Ion channel diseases, *Hum Mol Genet*, 11 (2002) 2435-2445.
- [19] M. Lucas, J.A. Encinar, E.A. Arribas, I. Oyenarte, I.G. Garcia, D. Kortazar, J.A. Fernandez, J.M. Mato, M.L. Martinez-Chantar, L.A. Martinez-Cruz, Binding of S-methyl-5'-thioadenosine and S-adenosyl-L-methionine to protein MJ0100 triggers an open-to-closed conformational change in its CBS motif pair, *Journal of molecular biology*, 396 (2010) 800-820.
- [20] S. Ignoul, J. Eggermont, CBS domains: structure, function, and pathology in human proteins, *American journal of physiology. Cell physiology*, 289 (2005) C1369-1378.
- [21] T. Schmidt-Rose, T.J. Jentsch, Reconstitution of functional voltage-gated chloride channels from complementary fragments of CLC-1, *The Journal of biological chemistry*, 272 (1997) 20515-20521.
- [22] M. Maduke, C. Williams, C. Miller, Formation of CLC-0 chloride channels from separated transmembrane and cytoplasmic domains, *Biochemistry*, 37 (1998) 1315-1321.
- [23] T.J. Jentsch, CLC chloride channels and transporters: from genes to protein structure, pathology and physiology, *Critical reviews in biochemistry and molecular biology*, 43 (2008) 3-36.
- [24] C. Miller, Open-state substructure of single chloride channels from Torpedo electroplax, *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 299 (1982) 401-411.

- [25] M. Pusch, U. Ludewig, A. Rehfeldt, T.J. Jentsch, Gating of the voltage-dependent chloride channel ClC-0 by the permeant anion, *Nature*, 373 (1995) 527-531.
- [26] S.B. Long, E.B. Campbell, R. Mackinnon, Voltage sensor of Kv1.2: structural basis of electromechanical coupling, *Science*, 309 (2005) 903-908.
- [27] M. Pusch, U. Ludewig, T.J. Jentsch, Temperature dependence of fast and slow gating relaxations of ClC-0 chloride channels, *The Journal of general physiology*, 109 (1997) 105-116.
- [28] B. Bennetts, M.L. Roberts, A.H. Bretag, G.Y. Rychkov, Temperature dependence of human muscle ClC-1 chloride channel, *The Journal of physiology*, 535 (2001) 83-93.
- [29] E.A. Bykova, X.D. Zhang, T.Y. Chen, J. Zheng, Large movement in the C terminus of CLC-0 chloride channel during slow gating, *Nat Struct Mol Biol*, 13 (2006) 1115-1119.
- [30] M. Duffield, G. Rychkov, A. Bretag, M. Roberts, Involvement of helices at the dimer interface in ClC-1 common gating, *The Journal of general physiology*, 121 (2003) 149-161.
- [31] P. Fong, A. Rehfeldt, T.J. Jentsch, Determinants of slow gating in ClC-0, the voltage-gated chloride channel of *Torpedo marmorata*, *The American journal of physiology*, 274 (1998) C966-973.
- [32] J.Z. Sheng, A. Weljie, L. Sy, S. Ling, H.J. Vogel, A.P. Braun, Homology modeling identifies C-terminal residues that contribute to the Ca²⁺ sensitivity of a BKCa channel, *Biophysical journal*, 89 (2005) 3079-3092.
- [33] A. Accardi, C. Miller, Secondary active transport mediated by a prokaryotic homologue of ClC Cl⁻ channels, *Nature*, 427 (2004) 803-807.
- [34] G. Zifarelli, M. Pusch, Intracellular regulation of human ClC-5 by adenine nucleotides, *EMBO reports*, 10 (2009) 1111-1116.
- [35] K. Matulef, M. Maduke, The CLC 'chloride channel' family: revelations from prokaryotes, *Molecular membrane biology*, 24 (2007) 342-350.
- [36] J.W. Foster, *Escherichia coli* acid resistance: tales of an amateur acidophile, *Nature reviews. Microbiology*, 2 (2004) 898-907.
- [37] J. Gordon, P.L. Small, Acid resistance in enteric bacteria, *Infection and immunity*, 61 (1993) 364-367.
- [38] P. Small, D. Blankenhorn, D. Welty, E. Zinser, J.L. Slonczewski, Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: role of rpoS and growth pH, *Journal of bacteriology*, 176 (1994) 1729-1737.
- [39] J.P. Audia, C.C. Webb, J.W. Foster, Breaking through the acid barrier: an orchestrated response to proton stress by enteric bacteria, *International journal of medical microbiology : IJMM*, 291 (2001) 97-106.
- [40] H.T. Richard, J.W. Foster, Acid resistance in *Escherichia coli*, *Advances in applied microbiology*, 52 (2003) 167-186.
- [41] J. Lin, M.P. Smith, K.C. Chapin, H.S. Baik, G.N. Bennett, J.W. Foster, Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*, *Applied and environmental microbiology*, 62 (1996) 3094-3100.
- [42] E.F. Gale, H.M. Epps, The effect of the pH of the medium during growth on the enzymic activities of bacteria (*Escherichia coli* and *Micrococcus*

lysodeikticus) and the biological significance of the changes produced, *The Biochemical journal*, 36 (1942) 600-618.

[43] M.P. Castanie-Cornet, T.A. Penfound, D. Smith, J.F. Elliott, J.W. Foster, Control of acid resistance in *Escherichia coli*, *Journal of bacteriology*, 181 (1999) 3525-3535.

[44] R. Iyer, T.M. Iverson, A. Accardi, C. Miller, A biological role for prokaryotic CIC chloride channels, *Nature*, 419 (2002) 715-718.

[45] C. Miller, M.M. White, Dimeric structure of single chloride channels from *Torpedo electrophorus*, *Proceedings of the National Academy of Sciences of the United States of America*, 81 (1984) 2772-2775.

[46] R. Dutzler, E.B. Campbell, M. Cadene, B.T. Chait, R. MacKinnon, X-ray structure of a CIC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity, *Nature*, 415 (2002) 287-294.

[47] A. Accardi, M. Walden, W. Nguiragool, H. Jayaram, C. Williams, C. Miller, Separate ion pathways in a Cl⁻/H⁺ exchanger, *J Gen Physiol*, 126 (2005) 563-570.

[48] J. Yin, Z. Kuang, U. Mahankali, T.L. Beck, Ion transit pathways and gating in CIC chloride channels, *Proteins*, 57 (2004) 414-421.

[49] R. Dutzler, E.B. Campbell, R. MacKinnon, Gating the selectivity filter in CIC chloride channels, *Science*, 300 (2003) 108-112.

[50] M. Pusch, K. Steinmeyer, T.J. Jentsch, Low single channel conductance of the major skeletal muscle chloride channel, CIC-1, *Biophysical journal*, 66 (1994) 149-152.

[51] A.A. Zdebik, G. Zifarelli, E.Y. Bergsdorf, P. Soliani, O. Scheel, T.J. Jentsch, M. Pusch, Determinants of anion-proton coupling in mammalian endosomal CLC proteins, *The Journal of biological chemistry*, 283 (2008) 4219-4227.

[52] H. Jayaram, A. Accardi, F. Wu, C. Williams, C. Miller, Ion permeation through a Cl⁻-selective channel designed from a CLC Cl⁻/H⁺ exchanger, *Proceedings of the National Academy of Sciences of the United States of America*, 105 (2008) 11194-11199.

[53] M. Walden, A. Accardi, F. Wu, C. Xu, C. Williams, C. Miller, Uncoupling and turnover in a Cl⁻/H⁺ exchange transporter, *The Journal of general physiology*, 129 (2007) 317-329.

[54] S.P. Bell, P.K. Curran, S. Choi, J.A. Mindell, Site-directed fluorescence studies of a prokaryotic CIC antiporter, *Biochemistry*, 45 (2006) 6773-6782.

[55] D. Basilio, K. Noack, A. Picollo, A. Accardi, Conformational changes required for H⁺/Cl⁻ exchange mediated by a CLC transporter, *Nat Struct Mol Biol*, 21 (2014) 456-463.

[56] S.M. Elvington, C.W. Liu, M.C. Maduke, Substrate-driven conformational changes in CIC-ec1 observed by fluorine NMR, *EMBO J*, 28 (2009) 3090-3102.

[57] S. Lobet, R. Dutzler, Ion-binding properties of the CIC chloride selectivity filter, *The EMBO journal*, 25 (2006) 24-33.

[58] S.H. White, The progress of membrane protein structure determination, *Protein science : a publication of the Protein Society*, 13 (2004) 1948-1949.

- [59] J.D. Faraldo-Gomez, B. Roux, Electrostatics of ion stabilization in a CIC chloride channel homologue from *Escherichia coli*, *J Mol Biol*, 339 (2004) 981-1000.
- [60] T.Y. Chen, C. Miller, Nonequilibrium gating and voltage dependence of the CIC-0 Cl⁻ channel, *The Journal of general physiology*, 108 (1996) 237-250.
- [61] D. Wang, G.A. Voth, Proton transport pathway in the CIC Cl⁻/H⁺ antiporter, *Biophysical journal*, 97 (2009) 121-131.
- [62] W. Han, R.C. Cheng, M.C. Maduke, E. Tajkhorshid, Water access points and hydration pathways in CLC H⁺/Cl⁻ transporters, *Proc Natl Acad Sci U S A*, 111 (2014) 1819-1824.
- [63] A. Koglin, C. Klammt, N. Trbovic, D. Schwarz, B. Schneider, B. Schafer, F. Lohr, F. Bernhard, V. Dotsch, Combination of cell-free expression and NMR spectroscopy as a new approach for structural investigation of membrane proteins, *Magnetic resonance in chemistry : MRC*, 44 Spec No (2006) S17-23.
- [64] P.M. Hwang, L.E. Kay, Solution structure and dynamics of integral membrane proteins by NMR: a case study involving the enzyme PagP, *Methods in enzymology*, 394 (2005) 335-350.
- [65] S.F. Poget, S.M. Cahill, M.E. Girvin, Isotropic bicelles stabilize the functional form of a small multidrug-resistance pump for NMR structural studies, *Journal of the American Chemical Society*, 129 (2007) 2432-2433.
- [66] G. Wang, NMR of membrane-associated peptides and proteins, *Current protein & peptide science*, 9 (2008) 50-69.
- [67] K. Henzler-Wildman, D. Kern, Dynamic personalities of proteins, *Nature*, 450 (2007) 964-972.
- [68] M.A. Danielson, J.J. Falke, Use of ¹⁹F NMR to probe protein structure and conformational changes, *Annual review of biophysics and biomolecular structure*, 25 (1996) 163-195.
- [69] L.A. Luck, J.J. Falke, Open conformation of a substrate-binding cleft: ¹⁹F NMR studies of cleft angle in the D-galactose chemosensory receptor, *Biochemistry*, 30 (1991) 6484-6490.
- [70] S. Rozovsky, G. Jogl, L. Tong, A.E. McDermott, Solution-state NMR investigations of triosephosphate isomerase active site loop motion: ligand release in relation to active site loop dynamics, *Journal of molecular biology*, 310 (2001) 271-280.
- [71] A. Matin, B. Wilson, E. Zychlinsky, M. Matin, Proton motive force and the physiological basis of delta pH maintenance in *thiobacillus acidophilus*, *Journal of bacteriology*, 150 (1982) 582-591.
- [72] J.A. Mindell, M. Maduke, C. Miller, N. Grigorieff, Projection structure of a CIC-type chloride channel at 6.5 Å resolution, *Nature*, 409 (2001) 219-223.
- [73] M.C. Koch, K. Steinmeyer, C. Lorenz, K. Ricker, F. Wolf, M. Otto, B. Zoll, F. Lehmann-Horn, K.H. Grzeschik, T.J. Jentsch, The skeletal muscle chloride channel in dominant and recessive human myotonia, *Science*, 257 (1992) 797-800.
- [74] K. Steinmeyer, C. Lorenz, M. Pusch, M.C. Koch, T.J. Jentsch, Multimeric structure of CIC-1 chloride channel revealed by mutations in dominant myotonia congenita (Thomsen), *The EMBO journal*, 13 (1994) 737-743.

- [75] T. Sander, H. Schulz, K. Saar, E. Gennaro, M.C. Riggio, A. Bianchi, F. Zara, D. Luna, C. Bulteau, A. Kaminska, D. Ville, C. Cieuta, F. Picard, J.F. Prud'homme, L. Bate, A. Sundquist, R.M. Gardiner, G.A. Janssen, G.J. de Haan, D.G. Kasteleijn-Nolst-Trenite, A. Bader, D. Lindhout, O. Riess, T.F. Wienker, D. Janz, A. Reis, Genome search for susceptibility loci of common idiopathic generalised epilepsies, *Human molecular genetics*, 9 (2000) 1465-1472.
- [76] N. Piwon, W. Gunther, M. Schwake, M.R. Bosl, T.J. Jentsch, CIC-5 Cl⁻ channel disruption impairs endocytosis in a mouse model for Dent's disease, *Nature*, 408 (2000) 369-373.
- [77] S.E. Lloyd, S.H. Pearce, S.E. Fisher, K. Steinmeyer, B. Schwappach, S.J. Scheinman, B. Harding, A. Bolino, M. Devoto, P. Goodyer, S.P. Rigden, O. Wrong, T.J. Jentsch, I.W. Craig, R.V. Thakker, A common molecular basis for three inherited kidney stone diseases, *Nature*, 379 (1996) 445-449.
- [78] M. Poet, U. Kornak, M. Schweizer, A.A. Zdebik, O. Scheel, S. Hoelter, W. Wurst, A. Schmitt, J.C. Fuhrmann, R. Planells-Cases, S.E. Mole, C.A. Hubner, T.J. Jentsch, Lysosomal storage disease upon disruption of the neuronal chloride transport protein CIC-6, *Proceedings of the National Academy of Sciences of the United States of America*, 103 (2006) 13854-13859.
- [79] U. Kornak, D. Kasper, M.R. Bosl, E. Kaiser, M. Schweizer, A. Schulz, W. Friedrich, G. Delling, T.J. Jentsch, Loss of the CIC-7 chloride channel leads to osteopetrosis in mice and man, *Cell*, 104 (2001) 205-215.
- [80] S. Uchida, F. Marumo, Severely impaired urine-concentrating ability in mice lacking the CLC-K1 chloride channel, *Experimental nephrology*, 8 (2000) 361-365.
- [81] www.blanco.biochemical.uci.edu

Tables

Table 1. Subcellular localizations, physiological roles and genetic diseases associated with human CIC proteins.

CIC Protein	Subcellular Localization	Predominant Tissue(s)	Physiological role	Human Disease
CIC-1	Plasma membrane	Skeletal muscles	Muscle relaxation after contraction	Many myopathies Becker or Thompson Myotonia [73, 74]
CIC-2	Plasma membrane	Ubiquitous	Ion homeostasis	Increased susceptibility to generalized Epilepsy [75]
CIC-3	Late endosomes	Brain	Acidification critical for neuronal health [6]	-

CIC-4	Early and recycling endosomes	Ubiquitous	Acidification modulates function of transferring receptor [10]	-
CIC-5	Early and recycling endosomes	Proximal tube	Support in renal-absorption from the primary urine [11, 76] Chronic kidney stones	Dent's Disease [77], proteinuria, kidney stones
CIC-6	Late endosomes	Brain	Allows degradation of lysosomal material [78]	Neuronal Ceroid Lipofuscinosis [78]
CIC-7	Lysosomes (osteoclast's plasma membrane)	Bone tissue and brain	Bone resorption and degradation of lysosomal material [17, 79]	Osteopetrosis [79]
CIC-Ka	Plasma membrane	Thin ascending loop of Henle (ALH) and inner ear	Renal fluid homeostasis [80]	-
CIC-Kb	Plasma membrane	Thick ALH and inner ear	Allows sodium re-absorption in the distal nephron [80]	Bartter Syndrome [80]

Table 2. Summary of published crystal structures for Cl^-/H^+ antiporters [81].

Organism	Protein	Year	Resolution (Å)	PDB Entry	Description
<i>Salmonella typhimurium</i>	Cl^-/H^+ antiporter	2002	3.0	1KPL	<i>E. coli</i> protein, 3.5 (Å):1KPK
<i>E. coli</i>	Cl^-/H^+ antiporter	2003	2.51	1OTS	E148A mutant, 3.0 (Å):1OTT E148Q mutant, 3.30 (Å):1OTU
<i>E. coli</i>	Cl^-/H^+ antiporter	2006	3.20	2EXW	E148Q mutant without bound ions, 3.1 (Å): 2EXY S107A/E148Q/Y445A mutant with bound Br^- , 3.45 (Å): 2EZO

<i>E. coli</i>	Cl ⁻ /H ⁺ antiporter	2010	3.10	3NMO	CIC transporter was engineered to place tryptophan residues (I201W; I422W) at the monomer-monomer interface to prevent dimerization. E148 mutant
<i>E. coli</i>	Cl ⁻ /H ⁺ antiporter	2012	3.02	4FG6	
<i>E. coli</i>	Cl ⁻ /H ⁺ antiporter	2012	2.50	4ENE	
<i>Cynidioschyzon merolae</i>	eukaryotic Cl ⁻ /H ⁺ antiporter	2010	3.50	3ORG	Truncation: Residues 2-16 at N-terminal and 461-464 at C-terminal. E202Y mutant, 3.20 Å: 4FTP
<i>Synechocystis sp. Pcc 6803</i>	eukaryotic Cl ⁻ /H ⁺ antiporter	2011	3.20	3ND0	In the presence of Br ⁻ , 3.60 Å: 3Q17

1

2

3

4

5

6

7

8 **Figure Legends**

9 **Figure 1. Human CIC channels and antiporters.** The nine members of the CIC
10 protein family are divided into three subfamilies based on their sequence
11 homology (1). The first subfamily consists Cl⁻ channels while the second and the
12 third consist of Cl⁻/H⁺ antiporters. Protein shown in red have been associated
13 with human genetic diseases (Table 1).

14

Figure 2. Structure of the CIC-ec1 chloride ion permeation pathway [46, 59].

The α -helices are shown as cylinders in the Cl^- permeation path as viewed from side of the membrane. **A.** For clarity, residues 81-95/285-299 are not shown. Cl^- ions are shown as green spheres. Three Cl^- -binding sites at the ion conduction pathway are labeled as S_{ext} (external Cl^- -binding site), S_{cen} (central Cl^- -binding site), and S_{int} (internal Cl^- -binding site). Key residues involved in the Cl^- permeation pathway are highlighted: E148, S107, and Y445. Positively charged amino acids located at N-terminal of helices D, F, and N are highlighted in pink. **B.** Structure showing the location of the proposed fourth Cl^- binding site (magenta sphere, S_{ext}^*). Two Cl^- ions (green spheres) are shown as seen in the crystal structure. Two conserved motifs are illustrated: K131-GSGIP, which mediates ion binding at the intracellular site, and R340-GGGFN, which presumably mediates binding at the extracellular site are illustrated. Water molecules are highlighted in pale cyan. **C.** An alternative view of the Cl^- binding sites. Antiparallel arrangement of helices C, D, and E on the intracellular side and helices K, L and M on the extracellular side. This motif occurs within each of the CIC-ec1 subunits. The Lys131-GSGIP and Arg340-GGGFN motifs are highlighted in blue. For clarity, residues 81-95/285-299 and helix K are not shown. **D.** Structural details from panels A-C are combined to illustrate a possible Cl^- permeation pathway.

Figure 1.

1

2

Figure 2.

1

2