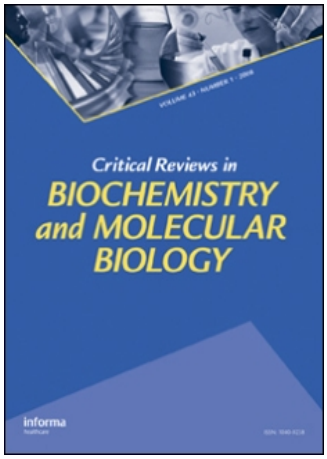

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CLC Chloride Channels and Transporters: From Genes to Protein Structure, Pathology and Physiology

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CLC Chloride Channels and Transporters: From Genes to Protein Structure, Pathology and Physiology

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ABSTRACT CLC genes are expressed in species from bacteria to human and encode Cl^--channels or Cl^-/H^+-exchangers. CLC proteins assemble to dimers, with each monomer containing an ion translocation pathway. Some mammalian isoforms need essential β-subunits (barttin and Ostm1). Crystal structures of bacterial CLC Cl^-/H^+-exchangers, combined with transport analysis of mammalian and bacterial CLCs, yielded surprising insights into their structure and function. The large cytosolic carboxy-termini of eukaryotic CLCs contain CBS domains, which may modulate transport activity. Some of these have been crystallized.

Mammals express nine CLC isoforms that differ in tissue distribution and subcellular localization. Some of these are plasma membrane Cl^- channels, which play important roles in transepithelial transport and in dampening muscle excitability. Other CLC proteins localize mainly to the endosomal-lysosomal system where they may facilitate luminal acidification or regulate luminal chloride concentration. All vesicular CLCs may be Cl^-/H^+-exchangers, as shown for the endosomal ClC-4 and -5 proteins. Human diseases and knockout mouse models have yielded important insights into their physiology and pathology. Phenotypes and diseases include myotonia, renal salt wasting, kidney stones, deafness, blindness, male infertility, leukodystrophy, osteopetrosis, lysosomal storage disease and defective endocytosis, demonstrating the broad physiological role of CLC-mediated anion transport.

KEYWORDS Bartter syndrome, Dent's disease, antiport, anion transport, ostm1, NCL, pH

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INTRODUCTION

The CLC gene family of chloride channels was first identified by the expression cloning of a voltage-gated Cl^- channel, ClC-0, from the electric organ of the marine ray *Torpedo marmorata* (Jentsch *et al.*, 1990). Homology screening and data base searches soon revealed that it was the founding member of a highly conserved gene family with orthologs being expressed in all phyla, from bacteria

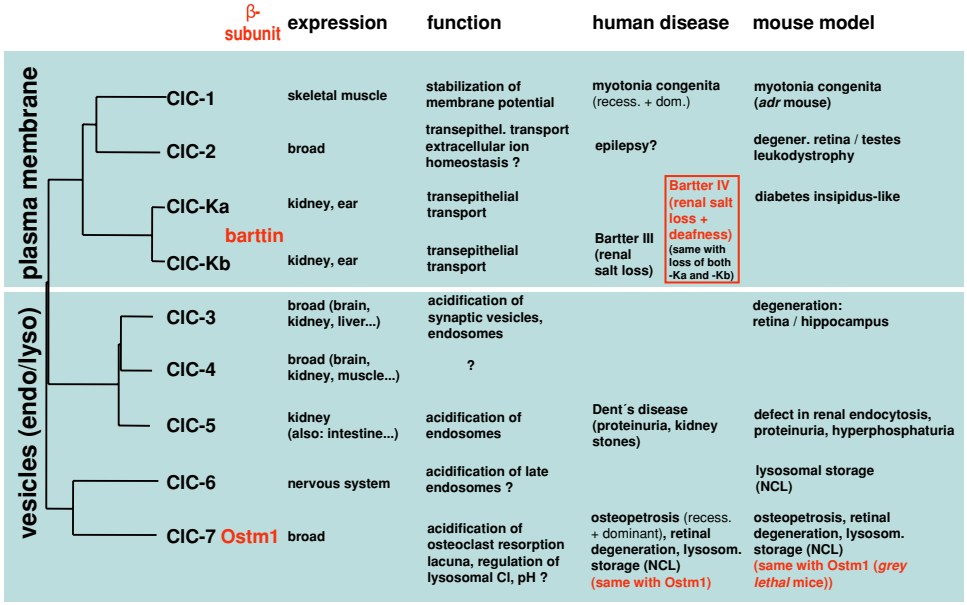

FIGURE 1 **Overview of the mammalian CLC family of chloride channels, indicating known β-subunits, tissue distribution, and human and mouse pathologies observed upon the disruption of the respective gene. Diseases observed upon the disruption of β-subunits are shown in red. The combined disruption of both ClC-Ka and ClC-Kb causes Bartter syndrome type IV, a disorder which is more frequently observed with loss-of-function mutations of the common β-subunit barttin. Please note that the gene nomenclature differs from the protein names given in the table: *CLCN1*, *CLCN2*, ..., and *BSND* for barttin. Human genes are given in upper case, and murine genes in lower case.**

to man. There are for instance two CLC isoforms in the bacterium *Escherichia coli* and one in the yeast *Saccharomyces cerevisiae*. Plants and animals have more than three CLC isoforms, with nine CLC genes being present in mammals (Figure 1). All CLC proteins were originally believed to function as Cl^- channels, but it became clear recently that many of them rather function as electrogenic Cl^-/H^+-exchangers (Accardi and Miller, 2004; Picollo and Pusch, 2005; Scheel *et al.*, 2005).

CLC proteins function as dimers. Both homo- and heterodimers have been observed upon heterologous expression (Lorenz *et al.*, 1996; Weinreich and Jentsch, 2001; Scholl *et al.*, 2006). Although ClC-4 and ClC-5 were reported to form heteromers *in vivo* (Mohammad-Panah *et al.*, 2003), the biological importance of CLC heteromers remains unclear. Some mammalian CLC proteins are also known to require smaller β-subunits (Barttin and Ostm1) for proper function (Estévez *et al.*, 2001; Lange *et al.*, 2006). Dimeric CLC proteins have two ion translocation pathways (Ludewig *et al.*, 1996; Middleton *et al.*, 1996), each of which is entirely contained within a single subunit (Weinreich and Jentsch, 2001; Dutzler *et al.*, 2002). The crystal structure of bacterial CLC proteins from *E. coli* and *Salmonella typhimurium* showed that each subunit has 17 intramembrane helices that often do not cross the width of the lipid bilayer (Dutzler *et al.*, 2002). These crystals also revealed several anion binding sites (Dutzler *et al.*, 2002; Dutzler *et al.*, 2003). A glutamate side chain that apparently blocks the access of extracellular anions to a central anion binding site is crucial for the voltage-dependent gating of CLC chloride channels (Dutzler *et al.*, 2003) and for the exchange-coupling of H^+ to Cl^- (Accardi and Miller, 2004). It appears that another glutamate, apparently found specifically in Cl^-/H^+-exchangers, is necessary for the transport of protons, the path of which diverges from that for Cl^- (Accardi *et al.*, 2005; Zdebik *et al.*, 2008). CLC proteins from eukaryotes and from some *archae* have a large, carboxyterminal portion that contains two CBS domains. These may have important roles in regulating transport activity.

The physiological functions of CLC anion transport proteins were largely deduced from the phenotypes of knockout mice and human diseases. Mutations in five CLC genes underlie human inherited disease, with symptoms as different as myotonia (muscle stiffness), renal salt loss, deafness, urinary protein loss, kidney stones, osteopetrosis, blindness, and lysosomal storage disease (Jentsch *et al.*, 2005). Mutations in either of the two known β-subunits (barttin and Ostm1) also underlie human disease (Estévez *et al.*, 2001; Lange *et al.*, 2006). The disruption of CLC genes in mice has

revealed other important pathologies (*e.g.*, male infertility and leukodystrophy). These findings indicate diverse roles for CLC-mediated ion transport. Thus, ClC-1 stabilizes the plasma membrane voltage of skeletal muscle (Steinmeyer *et al.*, 1991a). ClC-2 might be involved in the homeostasis of extracellular ion concentrations as well as in transepithelial transport (Bösl *et al.*, 2001; Blanz *et al.*, 2007), a task which is carried out by ClC-K/barttin channels in certain renal and cochlear epithelia (Simon *et al.*, 1997; Matsumura *et al.*, 1999; Estévez *et al.*, 2001). Whereas these proteins, which all belong to the first homology branch, function as plasma membrane Cl^- channels, members of the two other branches (ClC-3 to -5, and ClC-6 and -7, respectively) reside mainly in membranes of the endosomal/lysosomal system and in synaptic vesicles (ClC-3) (Jentsch, 2007). Several of these intracellular CLCs could not yet be studied biophysically. However, it emerged recently that the endosomal ClC-4 and ClC-5 proteins are electrogenic Cl^-/H^+-exchangers (Picollo and Pusch, 2005; Scheel *et al.*, 2005), resembling in that respect the *E. coli* EcClC-1 (Accardi and Miller, 2004). It seems likely that all vesicular CLC proteins function as exchangers rather than channels. Vesicular CLCs are believed to facilitate of luminal acidification by providing an anion conductance that neutralizes the currents of the electrogenic vesicular H^+-ATPase, a notion confirmed experimentally for ClC-3 and ClC-5. It therefore came as a surprise that they also allow for H^+-transport. However, even though H^+-transport will lead to a partial efflux of H^+ from vesicles, CLC Cl^-/H^+-exchangers will still facilitate vesicular acidification. The most important difference compared to a similar, hypothetical role of Cl^- channels might be a larger accumulation of Cl^- in the vesicular lumen (Jentsch, 2007). This notion is supported by the observation that a plant CLC, AtClC-a from *Arabidospis thaliana*, serves to accumulate nitrate in plant vacuoles (De Angeli *et al.*, 2006).

In this review, I will first summarize the most important findings concerning the structure-function analysis of CLCs, an area that was considerably boosted by the recent crystallization of bacterial CLCs and of cytosolic fragments of mammalian CLC proteins. This will be followed by a short overview over the individual mammalian CLCs, their biophysical properties, physiological functions, and pathologies resulting from their absence. Studies of CLC proteins from other species, like from the nematode *C. elegans* (see Petalcorin *et al.*, 1999; Schriever *et al.*, 1999; Rutledge *et al.*, 2001; Denton *et al.*, 2004; Denton *et al.*, 2006; He *et al.*, 2006) and the yeast *Saccharomyces cerevisiae* (Greene *et al.*, 1993; Davis-Kaplan *et al.*, 1998; Gaxiola *et al.*, 1998; Schwappach *et al.*, 1998; Li *et al.*, 1999; Wächter and Schwappach, 2005; Metz *et al.*, 2006) have also yielded highly interesting results. Unfortunately, they cannot be discussed here due to length constraints. Readers interested in a more thorough discussion of CLC biophysics, structure and function are referred to several excellent recent reviews (Chen, 2005; Miller, 2006; Dutzler, 2007; Zifarelli and Pusch, 2007).

STRUCTURE AND FUNCTION OF CLC PROTEINS

Homomeric CLC transporters have two identical ion translocation pathways which operate largely independently from each other. This was first deduced from single channel analysis of the reconstituted *Torpedo* channels by Miller (1982), who observed two equidistant conductance steps which could be interpreted as the opening and closing of two identical pores that opened and closed independently with time constants in the 10 ms range. While such recordings might also be interpreted as the gating of two independent channels, the observation of long periods in which there was no channel activity pointed to a single channel with two pores ('double-barrelled channel'). While every pore has its own gating process (the 'protopore gate,' or 'fast gate' in ClC-0), the long periods of time in which no channel activity could be detected indicated a common channel in which an additional, slow (or common) gate closed both pores simultaneously. The alternative explanation–a channel with two subconductance levels–seemed less likely as (1) the conductance levels were exactly equally spaced (entirely compatible with the opening of one or two pores) and because (2) the statistical analysis of the frequency of conductance states could be explained perfectly by voltage-dependent opening of two independent pores. The same single-channel pattern was observed when the cloned *Torpedo* ClC-0 cDNA was expressed in *Xenopus* oocytes, suggesting that a functional channel is composed of one or several copies of the same protein (Bauer *et al.*, 1991). The concept of a homodimeric channel with two pores was later proven by experiments in which wild-type (WT) and mutant ClC-0 subunits were hooked together in single, concatemeric constructs (Ludewig *et al.*, 1996; Middleton *et al.*, 1996). In single channel recordings, the smaller

conductance level of the mutant, which in some cases also had slightly changed ion selectivity, was observed together with WT conductance levels. When *Torpedo* ClC-0 (10 pS conductance) and mammalian ClC-2 (~3 pS) monomers were forced into an artificial dimer, independent ~10 pS and ~3 pS conductance levels were observed (Weinreich and Jentsch, 2001), an almost irrefutable evidence for each channel pore being entirely contained within a single subunit–a situation that starkly contrasts with cation channels in which pores are formed at the interface of four subunits.

This picture was beautifully confirmed by the crystal structures of bacterial CLCs (Dutzler *et al.*, 2002; Dutzler *et al.*, 2003). These revealed a rhombus-like dimer in which the two subunits contact each other at a broad interface. Chloride ions were found approximately in the centre of each subunit (Dutzler *et al.*, 2002). Each subunit has 17 intramembrane helices, which are tilted with respect to the plain of the membrane and have variable lengths, often turning back before spanning the width of the bilayer. This complicated arrangement explains why a previous biochemical analysis of the transmembrane topology of CLC channels had met with insurmountable difficulties in some parts of the protein (Schmidt-Rose and Jentsch, 1997b). The crystal structure also revealed an internal, antiparallell repeat pattern of either subunit. A centrally located chloride ion is coordinated by main-chain amide nitrogen atoms from isoleucine and phenylalanine residues, and by side chains from a serine and a tyrosine residues, all from different parts of the protein (Dutzler *et al.*, 2002). The coordinating tyrosine is located at the N-terminus of the last intramembrane helix R that connects the transmembrane part with the large cytoplasmic carboxy-terminus, suggesting the intriguing possibility that it may influence transport activity *via* that helix. There are no fully positive charges like those of arginines or lysines to keep the anion in place. As the Cl^- co-ordinating residues are located at the ends of α-helices, the helix dipole may create a favorable environment for anion binding (Dutzler *et al.*, 2002). Model calculations, however, favoured the view that this 'broken helix' architecture is not a salient feature of the energy profile of the CLC permeation pathway (Cohen and Schulten, 2004; Faraldo-Gómez and Roux, 2004). Such calculations showed that a strictly conserved lysine in helix E (K131 in EcClC-1, K149 in ClC-0), which is completely buried within the protein, stabilizes chloride binding by a relatively long range electrostatic interaction (Corry *et al.*, 2004; Faraldo-Gómez and Roux, 2004). This notion is strongly supported by structure-function studies of ClC-0 mutants in K149 (Zhang *et al.*, 2006; Engh *et al.*, 2007b; Engh *et al.*, 2007a). These revealed changes in anion selectivity and in fast gating, which could be interpreted as being caused by changes in affinity of the chloride binding sites or energy profiles in the pore. A second (internal) chloride binding site was detected in the channel vestibule that opens to the cytoplasm when higher resolution crystal structures were obtained (Dutzler *et al.*, 2003).

Remarkably, a glutamate side chain blocks the access of anions from the extracellular side, suggesting that the crystal represents a nontransporting, 'closed' state of the protein. With the exception of epithelial ClC-K channels, where it is replaced by valine, this glutamate is present in almost all CLC proteins. In previous mutagenesis studies, neutralizing this particular glutamate in ClC-1 and ClC-4 and -5 had led to drastic changes in voltage-dependence (Fahlke *et al.*, 1997; Friedrich *et al.*, 1999), and converting the equivalent valine to glutamate in ClC-K1 introduced time-dependent current activation by hyperpolarization (Waldegger and Jentsch, 2000). An additional, 'external' Cl^- binding site was created when this glutamate was mutated to glutamine in the *E. coli* EcClC-1, and the equivalent mutation in *Torpedo* ClC-0 abolished its voltage-dependent gating (Dutzler *et al.*, 2003). It was therefore suggested that this residue plays a role in the 'gating by the permeant anion.' This simple four-state model (Pusch *et al.*, 1995a) explained the voltage- and concentration-dependent gating of ClC-0 in terms of chloride, which binds to a site within the pore and by doing so shifts the equilibrium towards the open configuration. Extracellular Cl^- would be driven into the pore by an inside-positive voltage. This basic model explained the voltage- and Cl^--concentration-dependent open probability of the 'fast' gate of ClC-0 remarkably well over a range of Cl^--concentrations and voltages (Pusch *et al.*, 1995a). Chloride serves as an 'extrinsic' voltage-sensor, contrasting with the intrinsic voltage-sensor of positively charged amino acids of the S4 segment of voltage-gated cation channels. The effect of chloride on ClC-0 gating was then studied in more detail by Chen and Miller (Chen and Miller, 1996), who showed that an increase of external $[Cl^-]$ increased the opening rate, whereas the closing rate was more sensitive to internal chloride. These authors suggested that external chloride binding occurs in a voltage-independent manner (Chen and Miller,

1996) and proposed a five-state model for ClC-0 gating. However newer data from Maduke's laboratory, which benefit from the crystal structure of bacterial CLCs and previous model calculations, favor a four-state model with a voltage-dependent chloride binding step (Engh *et al.*, 2007a).

The crystal structure of EcClC-1 immediately suggested an attractive physical basis for the chloride-dependent gating of ClC-0: external chloride competes for an anion binding site with the negatively charged side chain of the 'gating glutamate,' which turns away and thereby opens the channel (Dutzler *et al.*, 2003). A protonation of this glutamate may also explain the apparent opening of several CLC channels by acidic pH (Rychkov *et al.*, 1996; Chen and Chen, 2001; Pusch, 2004; Traverso *et al.*, 2006). Indeed, the mechanism of channel opening may be a chloride-induced proton gating (Bostick and Berkowitz, 2004). Chloride-dependent gating is also observed with other CLC channels like ClC-1 or ClC-2 (Rychkov *et al.*, 1998; Pusch *et al.*, 1999; Niemeyer *et al.*, 2003) or CLCs from *C. elegans* (Schriever *et al.*, 1999).

Using crystallization in the presence of different anion concentrations of the 'closed' WT EcClC-1 and of its 'open' E148Q mutant (in which the external binding site is accessible for anion binding), the occupation of the three anion binding sites and even their binding affinities were determined (Lobet and Dutzler, 2006). All three sites can be occupied simultaneously, with binding constants for Cl^- in a physiologically meaningful range (4–40 mM). Both K^+ and Cl^- channels thus have multi-ion pores, in which mutual repulsion between neighbouring ions lower the energy barriers for ion diffusion. The internal binding site, which is already in contact with the aqueous environment of the channel vestibule, had the lowest affinity (Lobet and Dutzler, 2006). With the rather low (10 to 40 mM) cytoplasmic Cl^- concentrations of mammalian cytosol, the internal binding site might only be occupied when the channel is opened and this site faces the high ($\sim$ 100 mM) extracellular Cl^- concentration. This may have important implications for the sidedness of ion-dependent gating of CLC channels.

The functional reconstitution of EcClC-1 into lipid bilayers yielded a big surprise: In contrast to the well-studied ClC-0 Cl^- channel, it turned out to mediate Cl^-/H^+-exchange (Accardi and Miller, 2004). The shifts of reversal potentials by changing Cl^-- and H^+-concentrations strongly suggested that two chloride ions were exchanged for one proton. A Cl^- gradient could drive H^+ against its electrochemical gradient and *vice versa* (Accardi and Miller, 2004). Fluxes of these ions are obligatorily coupled without significant 'slippage' of one of the ions (Nguitragool and Miller, 2006). Neutralizing the 'gating glutamate' mentioned above by mutating it to alanine (E148A) abolished proton coupling and converted the exchanger into a pure Cl^- conductance (Accardi and Miller, 2004). Proton permeation requires another glutamate as a proton acceptor close to the intracellular surface of the protein (Accardi *et al.*, 2005). As this 'proton glutamate' is located at some distance from the chloride-conducting funnel that opens to the cell interior, the paths for Cl^- and H^+, which presumably meet at the 'gating glutamate' near the center of the CLC monomer, bifurcate towards the cytoplasm. When this 'proton glutamate' was neutralized, ecClC-1 was again converted into a pure Cl^- conductance. Whereas this is compatible with the idea that this glutamate is needed for proton transport, it is surprising that a blockade of the H^+ path does not block Cl^- transport as well (Accardi *et al.*, 2005). Such a situation would be expected with a centrally located obligatory Cl^-/H^+ exchange site when the supply of H^+ is abrogated. This situation was indeed found with the mammalian ClC-4 and ClC-5 proteins (Zdebik *et al.*, 2008). When their 'proton glutamates' were mutated to alanine, both currents and H^+-transport were suppressed. Currents, but not H^+-transport, could be rescued in these mutants by uncoupling Cl^-- from H^+-fluxes with a mutation in the 'gating glutamate.' Cl^-/H^+-exchange was still observed when the 'proton glutamate' was replaced by other protonatable residues like aspartate, histidine, and even tyrosine (Zdebik *et al.*, 2008).

Interestingly, polyatomic anions such as NO_3^- or SCN^- permeate ecClC-1 with partial or total uncoupling from H^+-countertransport (Nguitragool and Miller, 2006). Likewise, H^+-coupling is reduced with these anions in ClC-4 and ClC-5, but rather surprisingly these anions did not yield significant currents when the 'proton glutamate' was neutralized (Zdebik *et al.*, 2007). Crystallography revealed that only the internal, but not the external and central anion binding sites of EcClC-1 were occupied with $SeCN^-$ (which replaced SCN^- for technical reasons) (Nguitragool and Miller, 2006). Certain mutations in tyrosine 445, which contributes to the co-ordination of Cl^- in the central binding site, also uncoupled Cl^- fluxes from H^+, with a correlation

between proton coupling and the occupation of the central anion binding site (which was determined crystallographically) (Accardi *et al.*, 2006). Thus, anion occupation of that site is related to exchange coupling in some poorly understood way.

The observation that ecClC-1 is a $2Cl^-/H^+$ antiporter rather than a Cl^- channel raised the question whether some mammalian CLC proteins also function as exchangers. Attention focused on ClC-4 and ClC-5, because noise analysis had indicated very low single channel currents for ClC-4 (Hebeisen *et al.*, 2003) and because their extreme outward rectification had precluded measurements of reversal potentials that could have tested stringently for channel behavior (Friedrich *et al.*, 1999). Using different expression systems (*Xenopus* oocytes or transfected HEK cells) and either external or internal pH measurements, two groups showed that these endosomal proteins mediate electrogenic Cl^-/H^+ exchange (Picollo and Pusch, 2005; Scheel *et al.*, 2005). In contrast to EcClC-1, but in accord with the strong outward rectification of ClC-4 and -5 currents (Steinmeyer *et al.*, 1995; Friedrich *et al.*, 1999), H^+-transport could only be observed at positive voltages (Figure 2A). Like observed with EcClC-1, inward transport of Cl^- could drive H^+ efflux against its gradient. No proton transport could be detected when the *Torpedo* channel ClC-0 was studied under identical conditions (Figure 2B), confirming its identity as a chloride channel. Neutralization of their 'gating glutamates' also converted ClC-4 and -5 into Cl^- conductances (Picollo and Pusch, 2005; Scheel *et al.*, 2005). As described previously (Friedrich *et al.*, 1999), these mutations additionally abolished their voltage-dependence. Both ClC-4 and -5, as well as all other endosomal/lysosomal CLC proteins (ClC-3 through ClC-7) have a glutamate at the position of the EcClC-1 'proton glutamate,' whereas this position is occupied by valine residues in the known CLC channels (ClC-0, -1, -2, -K). Thus, the presence of a glutamate at this position may indicate that a CLC protein functions as antiporter.

The mapping of an inhibitor binding site in mammalian ClC-1 indicated a strong structural conservation between bacterial and mammalian CLC proteins (Estévez *et al.*, 2003). 9-anthracene carboxylic acid was found to bind in a hydrophobic pocket that overlaps with the internal anion binding site. The bacterial template has since then been used many times successfully in structure-function studies of mammalian CLC Cl^- channels (see Engh and Maduke, 2005; Zhang *et al.*, 2006).

In contrast to the bacterial CLC proteins that have been crystallized, all eukaryotic and even some prokaryotic CLC proteins have large cytoplasmic

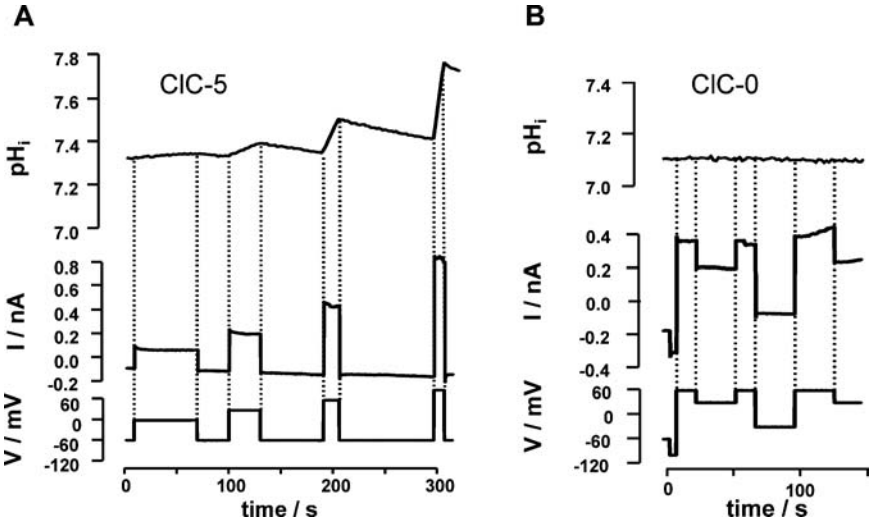

FIGURE 2 **ClC-5 is a Cl^-/H^+-exchanger, whereas ClC-0 is a Cl^--channel. HEK293 cells transfected with ClC-5 (*A*) or ClC-0 (*B*) were loaded with the fluorescent pH-indicator BCECF and were clamped to different voltages (lower panels). We used the gramicidin-perforated patch clamp technique to minimize the equilibration of the internal pH (pH_i) with the patch pipette. The centre panels shows clamp currents, while the upper panel shows pH_i. When ClC-5-transfected cells were clamped to voltages more positive than +30 mV, pH_i increased, indicating an exit of H^+ in exchange for Cl^- entry. Consistent with the steep outward rectification of ClC-5 currents (Steinmeyer *et al.*, 1995), the rate of intracellular alkalinization increased steeply with inside-positive voltage. By contrast, a similar voltage-clamp protocol did not change the pH_i of cells expressing ClC-0 from *Torpedo marmorata* (Jentsch *et al.*, 1990) (*B*), confirming that it functions as a chloride channel. Panels taken from (Scheel *et al.*, 2005).**

C-termini that contain two copies of CBS domains (Bateman, 1997; Ponting, 1997), which are named after cystathionine-β-synthase, an enzyme in which these domains are found as well. CBS domains, which are found in many different protein classes and all phyla, are usually present in tandem repeats and sometimes have regulatory roles (Ignoul and Eggermont, 2005). Truncations that delete the second CBS domain of either ClC-0 or ClC-1 led to a loss of function that could be rescued by coexpressing the missing CBS2 (Schmidt-Rose and Jentsch, 1997a; Maduke *et al.*, 1998; Estévez *et al.*, 2004). These effects may result from changed transport to the plasma membrane (Estévez *et al.*, 2004). Complementation studies in yeast with ScClC (gef1p) also indicated that intact CBS domains are important for proper intracellular targeting (Schwappach *et al.*, 1998). However, CBS1 or CBS2 could be deleted in frame in ClC-1 without abolishing its plasma membrane currents (Hryciw *et al.*, 1998; Estévez *et al.*, 2004; Hebeisen *et al.*, 2004), and truncated mutants of ClC-1 could be functionally rescued by short carboxy-terminal fragments lacking intact CBS2 (Wu *et al.*, 2006). Biochemical evidence suggested that CBS1 and CBS2 bind each other within a CLC subunit, and also to CBS domains of the associated, second monomer (Estévez *et al.*, 2004), findings that have now been confirmed by crystallography (Markovic and Dutzler, 2007).

Deletions and domain swaps in the cytoplasmic carboxyterminus altered the slow, common gate of ClC-0 (Fong *et al.*, 1998). Several mutations targeting predicted surface residues of CBS2 changed gating of ClC-1, again probably by affecting the common gate (Estévez *et al.*, 2004). Spectroscopical techniques were used to observe large movement of the carboxy-termini of ClC-0 during slow (common) gating (Bykova *et al.*, 2006). Such large movements are compatible with the large temperature-dependence of the 'slow' common gate of ClC-0 (Pusch *et al.*, 1997). This contrasts with the much smaller temperature dependence of 'fast' protopore gating (Pusch *et al.*, 1997), which may just involve a positional change of the 'gating glutamate' (Dutzler *et al.*, 2003), although this is probably not the whole story (Accardi and Pusch, 2003; Traverso *et al.*, 2003). The large differences in activation energy between common and protopore gating observed in ClC-0, however, was not found in ClC-1 (Bennetts *et al.*, 2001). Several point mutations in the transmembrane portion of CLC channels affect common gating (Lin *et al.*, 1999). Many of these cluster at the interface between the two subunits (Duffield *et al.*, 2003), as might be expected for a conformational change that affects both pores simultaneously. Common gating was also affected in concatemers between different CLC isoforms (Lorenz *et al.*, 1996) or between a WT ClC-0 subunit and a point mutant that lacked slow gating (Ludewig *et al.*, 1996), as would be expected from a common conformational change of both subunits. As common gating of ClC-0 is also influenced by pH and Cl^-, it might be somehow connected to the protopore gating, as also suggested for ClC-2 (Yusef *et al.*, 2006). Furthermore, a relation between slow and fast gating was also suggested by single channel studies of reconstituted *Torpedo* channels, which revealed that gating was driven by an ionic electrochemical gradient (Richard and Miller, 1990). An understanding of how structural changes in CBS domains affect gating of CLC channels may require the crystallization of CLC proteins containing these domains. The biophysical intricacies of CLC gating are discussed in detail in (Chen, 2005; Zifarelli and Pusch, 2007) and (Engh *et al.*, 2007a).

A new twist to the function of CBS domains came from the observation that tandem pairs of CBS domains from several proteins, including ClC-2, bound nucleotides like ATP *in vitro* (Scott *et al.*, 2004). This suggested that they may act as energy sensors. Indeed, the kinetics of ClC-2 gating may be slightly changed by intracellular ATP (Niemeyer *et al.*, 2004). More drastic effects of intracellular nucleotides were observed with ClC-1 (Bennetts *et al.*, 2005), where ATP, ADP and AMP, but not IMP, shifted the voltage-dependence of the common gate (but not of the protopore gate) to the right. Selected mutations in the CBS domains eliminated this effect, suggesting that these nucleotides exert their effect by binding to these domains (Bennetts *et al.*, 2005). Interestingly, the cytoplasmic ATP concentration influenced the pH-sensitivity of ClC-1 gating (Bennetts *et al.*, 2007; Tseng *et al.*, 2007).

More recently, the structures of the tandem CBS domains ClC-0, ClC-5 and ClC-Ka have been determined by X-ray crystallography of their isolated carboxyterminal, cytosolic domains (Meyer and Dutzler, 2006; Markovic and Dutzler, 2007; Meyer *et al.*, 2007). Surprisingly, the ClC-5 C-terminus (Meyer *et al.*, 2007) bound ATP and other nucleotides, but not those of ClC-0 (most closely related to ClC-1) or ClC-Ka. As determined by co-crystallization, ATP bound in a deep cleft of the protein in the interface of the two CBS domains. Mutating certain amino acids that contacted ATP in the crystal abolished nucleotide binding. However, current

properties were not affected when a full-length ClC-5 carrying such mutation was expressed (Meyer *et al.*, 2007). Surprisingly, changes in current rectification were observed when such mutations were studied in the background of the uncoupled ClC-5 gating glutamate mutant, a result of unclear significance. The binding affinities of ATP, ADP and AMP to the protein were not significantly different (close to 0.1 mM in all three cases), rendering a regulatory role of ATP on ClC-5 function rather unlikely (Meyer *et al.*, 2007). The lack of differentiation between these nucleotides, on the other hand, accords with the equally indiscriminate effects of ATP, ADP, and AMP on ClC-1 gating (Bennetts *et al.*, 2005). It will be interesting to determine crystallographically whether and how nucleotides bind to the CBS tandem of ClC-1. The inability to obtain crystals of the ClC-5 carboxy-terminus in the absence of nucleotides (Meyer *et al.*, 2007) may argue for a structural rather than regulatory role of ATP binding. However, the fact that mutations changing nucleotide binding evoked functional changes in the uncoupled mutant (Meyer *et al.*, 2007) may hint at a regulatory role of nucleotides under currently unknown circumstances.

The crystallization of the C-terminus of ClC-Ka provided the most detailed information on the oligomerization of CLC CBS domains (Markovic and Dutzler, 2007). Consistent with previous results for ClC-1 (Estévez *et al.*, 2004), CBS1 and CBS2 not only dimerized within the tandem, but formed a dimer with another CBS tandem (Markovic and Dutzler, 2007), mainly through CBS2-CBS2 interactions. The dimerization was confirmed biochemically and by mutagenesis, and additional experiments strongly suggested that it also occurs within the context of full-length ClC-0. So far, effects on ClC-0 currents of mutations impinging on the dimerization of CBS domains were not reported (Markovic and Dutzler, 2007).

Also other domains of CLC proteins may have important roles in channel gating. In ClC-2, an N-terminal cytoplasmic region is necessary for its opening by hyperpolarization, cell swelling, and mildly acidic pH (Gründer *et al.*, 1992; Jordt and Jentsch, 1997). Its deletion led to 'constitutively open' channels with an Ohmic behavior. They lacked hyperpolarization-activated gating and no longer responded to cell swelling or moderately acidic pH (6.5) when measured in *Xenopus* oocytes or in perforated patch recording of transfected cells. However, when measured in the excised patch or whole-cell configuration of the patch-clamp technique, also this 'open' mutant showed inward rectification, suggesting an effect of unknown factors present in the cytosol (Pusch *et al.*, 1999; Varela *et al.*, 2002). After transplantation of the N-terminal domain to the C-terminus between the two CBS domains, it retained its function (Gründer *et al.*, 1992), suggesting that it binds to an internal site of ClC-2. Additional mutagenesis identified a positively charged region between helices J and K that is accessible from the cytoplasm and that was suggested to serve as a receptor (Jordt and Jentsch, 1997). ClC-2 gating is influenced by the intracellular Cl^- concentration. When raised, it shifts its voltage-dependent to more positive potentials, resulting in an opening of the channel (Pusch *et al.*, 1999; Niemeyer *et al.*, 2003).

So far, only two auxiliary β-subunits are known for CLC proteins. Barttin is a 320 aa protein (Birkenhäger *et al.*, 2001) that associates specifically with ClC-Ka or ClC-Kb (Estévez *et al.*, 2001). It has two transmembrane spanning domains, with a small cytosolic amino-terminal part and a much larger cytosolic carboxyterminal part that contains a tyrosine-based internalization signal (Estévez *et al.*, 2001). Barttin boosts currents of ClC-K channels by dramatically increasing their surface expression. In addition, it alters some biophysical properties of the currents, e.g., their sensitivity to external Ca^{++} (Waldegger *et al.*, 2002) and gating (Scholl *et al.*, 2006). It has recently been proposed that barttin can bind separately to helices B and J in either subunit, a rather surprising result (Tajima *et al.*, 2007). Inactivating mutations in barttin cause renal salt loss and deafness in Bartter syndrome type IV (Birkenhäger *et al.*, 2001).

The other known β-subunit of CLC proteins is Ostm1, which associates specifically with ClC-7 (Lange *et al.*, 2006). It has a large, highly glycosylated luminal domain, a single transmembrane domain, and a short cytoplasmic tail. Its luminal domain is cleaved in the mature, lysosomal form (Lange *et al.*, 2006). The loss of Ostm1 leads to osteopetrosis and lysosomal storage disease because the stability of ClC-7 is compromised.

PATHOLOGY AND PHYSIOLOGY OF MAMMALIAN CLCs

The physiological roles of mammalian ClC chloride channels and transporters often became apparent from the discovery of human inherited diseases caused by mutations in CLC genes, or through the generation and analysis of KO mice (Figure 1). So far, five of

the nine human CLC genes are known to be mutated in human disease, as are the genes encoding either of the two known β-subunits. Mice with disrupted CLC genes are available for eight of the nine CLC genes. The mouse phenotypes often uncovered unexpected physiological roles of CLC proteins and in several cases also invalidated previous hypotheses. In the following, I will briefly summarize these findings.

ClC-1: A Voltage-Gated Chloride Channel Essential for the Control of Skeletal Muscle Excitability

Being almost exclusively expressed in skeletal muscle, ClC-1 shows the highest degree of tissue specificity within the CLC family (Steinmeyer *et al.*, 1991b). Its expression in skeletal muscle increases postnatally in mice (Steinmeyer *et al.*, 1991b) and is dependent on the electric activity of the muscle membrane (Klocke *et al.*, 1994). ClC-1 is a plasma membrane chloride channel with a small single channel conductance of about 1.5 pS (Pusch *et al.*, 1994; Saviane *et al.*, 1999). It is already open at the negative resting voltage of skeletal muscle and can be opened further by depolarization. In contrast to *Torpedo* ClC-0, both common and protopore gates open with depolarization. The common gate is much faster than that of the 'slow' common gate of ClC-0, requiring careful kinetic analysis to distinguish both gating modes (Saviane *et al.*, 1999; Bennetts *et al.*, 2005). Gating not only depends on voltage, chloride, and pH (Rychkov *et al.*, 1996), but also on the intracellular ATP concentration, a property that was suggested to possibly be important under severe energy depletion of muscle (Bennetts *et al.*, 2005). ClC-1 currents may be inhibited by the activation of protein kinase C (Rosenbohm *et al.*, 1999; Pierno *et al.*, 2007). The intricacies of ClC-1 gating are thoroughly discussed in the excellent review by Zifarelli and Pusch (2007).

ClC-1 provides the unusual high chloride conductance of skeletal muscle ($\sim$80% of resting conductance). It is thought that skeletal muscle uses predominantly Cl^- rather than K^+ to repolarize action potentials, because an efflux of potassium would significantly increase K^+-concentration in the small spaces of the t-tubules, thereby depolarizing their membranes. By contrast, when the same amount of charge is carried by Cl^-, $[Cl^-]_o$ will increase much less in relative terms (due to its $\sim$20-fold higher extracellular concentration compared to K^+). Hence it will cause almost no depolarization. This line of argumentation, however, hinges largely on the presence of ClC-1 in t-tubules. Such a localization was inferred from a decrease of Cl^--, but not K^+-conductance by tubular disruption with glycerol (Palade and Barchi, 1977). However, immunohistology failed to detect ClC-1 in t-tubules, rather showing expression in the sarcolemma (Gurnett *et al.*, 1995; Papponen *et al.*, 2005).

As ClC-1 is important for repolarizing action potentials and for stabilizing the membrane voltage in skeletal muscle, loss of ClC-1 currents leads to muscle hyperexcitability which manifests itself as myotonia, a form of 'muscle stiffness'. Patients and affected animals have difficulties in relaxing their muscle after voluntary contractions. Electrophysiological correlate are 'myotonic runs', series of action potentials that can be triggered by a single, short depolarization. Based on previous observations that skeletal muscle Cl^- conductance is reduced in some forms of myotonia (Lipicky and Bryant, 1966; Bryant and Morales-Aguilera, 1971; Lipicky *et al.*, 1971), ClC-1 was first shown to be mutated in myotonic mice (Steinmeyer *et al.*, 1991a), followed by humans (Koch *et al.*, 1992) and later by goats (Beck *et al.*, 1996) and dogs (Rhodes *et al.*, 1999). Human myotonia can be inherited as a recessive or as a clinically less severe dominant disease. If a dominant negative ClC-1 mutant assembles with WT subunits with unchanged affinity, and totally inactivates the function of the resulting dimer, ClC-1 currents in heterozygous patients are expected to decrease to 25% (because a quarter of the dimers will be composed entirely of WT subunits). This remnant current explains why dominant myotonia congenita (Thomsen's disease) is generally clinically less severe than recessive (Becker-type) myotonia, which can be associated with a complete loss of ClC-1. These considerations also apply for ClC-7 related osteopetrosis, where the dominant disease may be much more benign than the recessive one (Cleiren *et al.*, 2001; Kornak *et al.*, 2001). Interestingly, the ClC-1 mutation of Dr. Thomsen, who first described dominant myotonia (Thomsen, 1876) and who was himself affected by this disorder, has been identified (Steinmeyer *et al.*, 1994). The mutant exerted a dominant negative effect on coexpressed WT ClC-1, revealing for the first time a multimeric structure of CLC channels (Steinmeyer *et al.*, 1994).

A dominant negative effect of ClC-1 mutants may result from disturbed trafficking or early degradation of WT/mutant heteromers, or could result from changed biophysical properties of these heteromers. It

is important to realize that the architecture of CLC channels, in which each of the two pores is entirely contained within a monomer, leaves less room for dominant negative effects than in K^+-channels. In these latter tetrameric channels, one aberrant subunit can destroy the single pore that forms at the interface between all four subunits. Many dominant ClC-1 mutations change the gating of the dimer by shifting its voltage-dependence of opening to positive voltages where ClC-1 can no longer repolarize action potentials (Pusch *et al.*, 1995b). As expected, such mutations affect the common gate that closes both pores (Saviane *et al.*, 1999). When CLC crystal structures became available, it was realized that many dominant mutations affect residues close to the subunit interface (Duffield *et al.*, 2003).

Myotonic dystrophy is a multisystem disorder which is caused by nucleotide repeats in untranslated regions of two different genes. Myotonia, a cardinal symptom of that disease, may be caused by aberrant splicing of ClC-1, leading to a large decrease in ClC-1 protein levels (Charlet *et al.*, 2002; Mankodi *et al.*, 2002).

ClC-2—A Broadly Expressed Plasma Membrane Channel with Multiple Roles

ClC-2 is a very broadly expressed, inwardly rectifying plasma membrane Cl^- channel that is found for instance in many epithelia, neurons, glia, and heart (Thiemann *et al.*, 1992). ClC-2 has a single channel conductance of about 3 pS (Weinreich and Jentsch, 2001) and is activated by membrane hyperpolarization, cell swelling, and mildly acidic extracellular pH (Gründer *et al.*, 1992; Jordt and Jentsch, 1997). Mechanisms involved in ClC-2 gating have been mentioned above and were studied in detail by several groups (Gründer *et al.*, 1992; Jordt and Jentsch, 1997; Niemeyer *et al.*, 2003; Zuñiga *et al.*, 2004; de Santiago *et al.*, 2005; Yusef *et al.*, 2006), as thoroughly discussed by Zifarelli and Pusch (2007). ClC-2 gating is not only influenced by the patch-clamp configuration (whole-cell *vs*. perforated patch), but also depends on unknown factors of cells in which it is expressed (Park *et al.*, 1998). It is unclear whether this might be due to differences in membrane concentrations of cholesterol, which influences the gating properties of ClC-2 (Hinzpeter *et al.*, 2007).

Although ClC-2 certainly does not underlie the swelling-activated Cl^- current known as $I_{cl,swell}$ or VRAC (volume-regulated anion conductance) (Nilius *et al.*, 1997; Sardini *et al.*, 2003) that is observed in almost all cells and that starkly differs from ClC-2 by its outward-rectification and I>Cl selectivity, ClC-2 currents can be activated by cell swelling in heterologous expression systems (Gründer *et al.*, 1992; Furukawa *et al.*, 1998) and plays a role in volume regulation (Furukawa *et al.*, 1998). Swelling-activation of ClC-2 has also been observed in native cells (Clark *et al.*, 1998; Huber *et al.*, 2004; Comes *et al.*, 2006), but no effect on the volume regulation of mouse salivary acinar cells could be observed when comparing WT and KO mice (Nehrke *et al.*, 2002).

Unexpectedly, disruption of ClC-2 in mice led to retinal and testicular degeneration (Bösl *et al.*, 2001; Nehrke *et al.*, 2002), as well as to leukoencephalopathy, a widespread vacuolation of the white matter in the brain and spinal cord (Blanz *et al.*, 2007). Loss of hyperpolarization-activated Cl^- currents in *Clcn2*$^{-/-}$ mice was demonstrated in Sertoli cells (Bösl *et al.*, 2001), salivary acinar cells (Nehrke *et al.*, 2002), and erythrocytes (Huber *et al.*, 2004). Contrary to previous speculations, neither a defect in gastric acid secretion, lung development, nor epilepsy was observed (Bösl *et al.*, 2001). The degeneration of germ cells and photoreceptors was tentatively attributed to impaired transport across supporting epithelia (formed by Sertoli cells and retinal pigment epithelial cells, respectively). In support of this hypothesis, the short circuit currents across the retinal pigment epithelium were reduced in ClC-2 KO mice (Bösl *et al.*, 2001). Thus, a change in the extracellular ion concentration surrounding germ cells and photoreceptors might cause their degeneration.

Likewise, it was speculated that the vacuolation of the white matter of *Clcn2*$^{-/-}$ mice is secondary to changes in extracellular ion concentrations (Blanz *et al.*, 2007). A few weeks after birth, vacuoles began to appear within the myelin sheaths of central, but not of peripheral neurons. The size of these vacuoles increased over the following months. The axons, even those close to vacuolated myelin sheaths, appeared normal. No signs of neuronal cell loss were detected. Accordingly, except for the previously reported blindness (Bösl *et al.*, 2001), the neurological phenotype was mild and consisted in a slowed nerve conduction velocity (Blanz *et al.*, 2007). In the CNS, ClC-2 is expressed in neurons and in glia (Sik *et al.*, 2000; Blanz *et al.*, 2007). Immunoreactivity could be detected, *e.g.*, in principal neurons of the hippocampus and in Bergmann glia of the cerebellum.

Interestingly, ClC-2 immunoreactivity was detected in astrocytic endfeet that contact the endothelium of brain capillaries (Sik *et al.*, 2000; Blanz *et al.*, 2007), a localization shared by the inwardly rectifying K^+ channel Kir4.1 and aquaporin 4. ClC-2 immunoreactivity also surrounded cell bodies of oligodendrocytes, which similarly express Kir4.1 and connexin 47 (Cx47). These transport proteins are thought to be involved in 'potassium siphoning,' a process by which K^+ is removed from the small clefts between neurons and astrocytes and is equilibrated finally with the blood *via* astrocytic end-feet. Interestingly, disruption of Kir4.1, as well as the double KO of Cx32 and Cx42, led to myelin vacuolation as in *Clcn2*$^{-/-}$ mice (Neusch *et al.*, 2001; Menichella *et al.*, 2003). The analogy even goes further: In Cx32/47 double KO mice, vacuolation of the optic nerve could be prevented by inhibiting its activity (Menichella *et al.*, 2006), as could be expected if these proteins 'siphon' extracellular K^+ that leaves neurons when action potentials are repolarized. Likewise, no vacuolation was observed in the optic nerve of *Clcn2*$^{-/-}$ mice (Blanz *et al.*, 2007), which is inactive because *Clcn2*$^{-/-}$ retina degenerates rapidly after birth. It was therefore proposed that ClC-2 regulates extracellular ion concentration in brain.

ClC-2 was also proposed to regulate intraneuronal chloride concentration, which in turn is important for neuronal inhibition through $GABA_A$- and glycine receptor Cl^- channels (Staley *et al.*, 1996). Given the dependence of ClC-2 gating on $[Cl^-]_i$ (Pusch *et al.*, 1999; Niemeyer *et al.*, 2003), a rise in intraneuronal Cl^- concentration may open ClC-2 and lead to an efflux of Cl^-. Indeed, transfection of dorsal root ganglion neurons with ClC-2 converted their excitatory response to GABA to being inhibitory (Staley *et al.*, 1996). The expectation that ClC-2 KO mice would hence display epilepsy, however, was not met (Bösl *et al.*, 2001; Nehrke *et al.*, 2002). These mice neither displayed reduced seizure thresholds when challenged by different proconvulsive agents (Bösl *et al.*, 2001; Blanz *et al.*, 2007). This can be rationalized by the predominant role of the K-Cl cotransporter KCC2 in generating the low $[Cl^-]_i$ needed for GABAergic inhibition (Hübner *et al.*, 2001). On the other hand, three different *CLCN2* mutations were described in three families with clinically different forms of epilepsy (Haug *et al.*, 2003). The inheritance pattern suggested that these mutations, which were found in a heterozygous state, should act dominantly. Surprisingly, whereas two of these mutations (a frame shift and an intronic deletion) would result in a loss of function, another mutation (G715E) was described to increase Cl^- currents at physiological voltages and $[Cl^-]_i$ (Haug *et al.*, 2003). G715 is located between CBS1 and CBS2 and the G175E mutation may affect ATP binding to the CBS domains Scott *et al.*, 2004). The gain-of-function effect of G715E described by Haug et al. (2003), however, could not be reproduced (Niemeyer *et al.*, 2004). The latter work rather reported moderate changes in gating kinetics in the presence of 1 mM intracellular AMP, but not ATP, a finding of unclear physiological significance. These authors neither found an effect of the intronic deletion on ClC-2 splicing (Niemeyer *et al.*, 2004). In contrast to the purported dominant negative effect of the truncating mutation in the original report (Haug *et al.*, 2003), no such effect was seen in two other studies (Niemeyer *et al.*, 2004; Blanz *et al.*, 2007) and would neither be expected for a large truncation. Indeed, almost all truncating mutations in ClC-1 and ClC-7 were associated with recessive, and not dominant, forms of myotonia and osteopetrosis, respectively. ClC-2 amino acid exchanges described subsequently in other patients with epilepsy (D'Agostino *et al.*, 2004) turned out to be innocuous polymorphisms (Blanz *et al.*, 2007). Thus, the most convincing mutation described by Haug and coworkers (Haug *et al.*, 2003) results in a heterozygous ClC-2 truncation that is expected to lead to a 50% loss of function. The sum of these observations, together with the fact that no further convincing *CLCN2* mutations have been identified in patients with epilepsy (D'Agostino *et al.*, 2004; Coppola *et al.*, 2006; Stogmann *et al.*, 2006; Everett *et al.*, 2007), warrants skepticism towards the proposed causative role of ClC-2 in epilepsy.

ClC-2 was also proposed to provide a pathway for epithelial Cl^- secretion in parallel to the cystic fibrosis transmembrane conductance regulator CFTR (Schwiebert *et al.*, 1998), raising hopes that activation of ClC-2 might be useful in cystic fibrosis (CF). However, when mice carrying the CFTR mutation ΔF508 (the most common mutation found in CF) were crossed with ClC-2 KO mice, the double-deficient mice survived better than *Cftr*$^{\Delta F508/\Delta F508}$ mice (probably due to less intestinal obstipation) and failed to show any additional CF pathology like lung disease (Zdebik *et al.*, 2004). Using chamber experiments examining transport across colonic epithelia suggested that ClC-2 was present in basolateral membranes, a localization consistent with a role in Cl^- reabsorption rather than

secretion. This localization has now been confirmed by immunohistochemistry by several groups (Catalán *et al.*, 2002; Lipecka *et al.*, 2002; Catalán *et al.*, 2004), including our own (Zdebik and Jentsch, *unpublished data*) and was in part controlled by KO tissue. A dileucine motif in CBS2 of ClC-2 was implicated in its trafficking to the basolateral membrane in transfected epithelial cells (Peña-Münzenmayer *et al.*, 2005). The functional analysis of ClC-2 KO mice (Zdebik *et al.*, 2004) and the basolateral localization of ClC-2 is inconsistent with the proposed mechanism of the purported ClC-2 activator lubiprostone in alleviating obstipation (see, e.g. Camilleri *et al.*, 2006). Because a basolateral localization of ClC-2 is consistent with a role in Cl^- reabsorption, ClC-2 activation might rather be useful in diarrhea, but detrimental in obstipation. The contention that lubiprostone activates ClC-2 is questionable as well, as it is based on a single paper (Cuppoletti *et al.*, 2004) that reported drug effects on currents that differ drastically from typical ClC-2 currents.

Several proteins were reported to bind to ClC-2, including components of the dynein motor complex (Dhani *et al.*, 2003) and the heat shock proteins Hsp70 and Hsp90 (Hinzpeter *et al.*, 2005). The physiological relevance of these findings remains to be established.

ClC-K/barttin: Chloride Channels Involved in Transepithelial Transport

In mammals, there are two closely homologous ClC-K channels, named ClC-K1 and ClC-K2 in rodents (Uchida *et al.*, 1993; Adachi *et al.*, 1994; Kieferle *et al.*, 1994), and ClC-Ka and ClC-Kb in humans (Kieferle *et al.*, 1994). This terminology was chosen as sequence comparison did not allow to assign species orthologs, because the degree of amino-acid identity was higher within a species (*e.g.*, between ClC-Ka and –Kb) than between orthologs of different species. Subsequent localization studies have revealed that ClC-K1 corresponds to ClC-Ka, and ClC-K2 to ClC-Kb. Only rat ClC-K1 (rClC-K1) gave currents by itself (Uchida *et al.*, 1993; Waldegger and Jentsch, 2000). The currents reported for ClC-K2 and a variant carrying a deletion in the transmembrane block (Adachi *et al.*, 1994) are probably endogenous to the cells used for expression. Chimeras of ClC-Kb containing only a small part of ClC-K1 (roughly helices N to R) gave currents that differed in ion selectivity and Ca^{++}-sensitivity from those of ClC-K1 (Waldegger and Jentsch, 2000). ClC-K1 currents had a roughly linear I/V curve, with little gating relaxations. Mutating valine 166 to glutamate converted ClC-K1 to an inwardly rectifying channel with large gating relaxations, proving that the observed currents were indeed due to ClC-K1 (Waldegger and Jentsch, 2000). We now know that this mutation had introduced a 'gating glutamate,' the crucial role of which became apparent only after the crystallization of bacterial CLC proteins (Dutzler *et al.*, 2003). Indeed, ClC-K channels are the only mammalian CLC proteins in which this highly conserved glutamate is not present. It seems that evolution has selected a mutant abolishing the 'gating glutamate' to create non-gating, ohmic 'leak' channels that are suited for transepithelial transport over a large range of plasma membrane voltages.

As immunocytochemistry (Uchida *et al.*, 1995; Vandewalle *et al.*, 1997) and genetics (Simon *et al.*, 1997; Matsumura *et al.*, 1999) suggested that ClC-K channels are involved in salt transport across the plasma membrane, the inability to obtain currents with most ClC-K isoforms suggested that they may need an accessory β-subunit (Waldegger and Jentsch, 2000). Indeed barttin, a small membrane-spanning protein identified as the product of the *BSND* gene that is mutated in human Bartter syndrome type IV (Birkenhäger *et al.*, 2001), was identified as an auxiliary β-subunit of both ClC-K isoforms (Estévez *et al.*, 2001). Barttin has two predicted transmembrane domains, with both the amino- and the much longer carboxy-terminus being located in the cytosol (Estévez *et al.*, 2001). Coexpression of ClC-K1 with barttin led to a more than tenfold stimulation of current amplitudes, while coexpression with human ClC-Ka and –Kb revealed plasma membrane anion currents that could not be detected without barttin. The mechanism by which barttin boosted or enabled plasma membrane currents is primarily an increase in surface expression of ClC-K proteins, which otherwise mostly remain in the endoplasmic reticulum (Estévez *et al.*, 2001; Waldegger *et al.*, 2002; Hayama *et al.*, 2003; Scholl *et al.*, 2006). In addition to its effect on surface expression (which was not observed when coexpressing barttin with other CLC proteins) (Estévez *et al.*, 2001), barttin also changed the current properties of ClC-K currents (Waldegger *et al.*, 2002; Scholl *et al.*, 2006). Currents of ClC-Ka/barttin and ClC-Kb/barttin are stimulated by an increase of extracellular Ca^{++}-concentration from 0 to 1.8 and to 5 mM (Estévez *et al.*, 2001), an effect of unclear physiological significance. Similarly, rClC-K1 is stimulated by $[Ca^{++}]_o$ over the range between 0

to 11.8 mM. However, upon coexpression with barttin, the enhancement of currents by $[Ca^{++}]_o$ already saturated at 1.8 mM Ca^{++}, rendering it essentially Ca^{++}-insensitive at physiological concentrations (Waldegger *et al.*, 2002). In addition to this modulation of Ca^{++}-sensitivity, barttin also affects gating of ClC-K channels and may increase their single channel conductance (Scholl *et al.*, 2006). The effect on gating was most evident with the 'gating glutamate' mutant V166E, that induced a slow, hyperpolarization-activated gating in rClC-K1 (Waldegger and Jentsch, 2000). Coexpressing this mutant with barttin caused a drastic change to a depolarization-activated gating (Scholl *et al.*, 2006). The unitary conductance of the rClC-K1 mutant V166E was reported to increase from about 6.5 pS to nearly 20 pS upon coexpression with barttin (Scholl *et al.*, 2006). This mutant was chosen because its slow activation by hyperpolarization allowed non-stationary noise analysis. However, as the mutation changes a crucial residue in the permeation pathway, these values may not correspond to WT rClC-K1.

The carboxyterminus of barttin could be truncated considerably without losing its stimulatory effect on ClC-K currents, but the extent of tolerated deletions varies between reports that used either oocytes (Estévez *et al.*, 2001) or transfected mammalian cells (Scholl *et al.*, 2006). It was concluded that the transmembrane region of barttin acts as a chaperone for surface expression, whereas a proximal cytoplasmic stretch, which needs to be attached to the transmembrane part, influences gating and conductance (Scholl *et al.*, 2006). A direct binding of barttin to ClC-K was suggested by coimmunoprecipation experiments using heterologously expressed proteins (Waldegger *et al.*, 2002; Hayama *et al.*, 2003). These results were extended by a report suggesting that barttin can bind independently to helices B and J of a ClC-K subunit (Tajima *et al.*, 2007), which both lie at the periphery of CLC monomers.

The cytoplasmic carboxy-terminus of barttin contains an amino-acid sequence (PQPPYVRL) that conforms to the consensus of a tyrosine-based internalization motif (YxxL) (Bonifacino and Traub, 2003) and that bears weak resemblance to so-called PY moitifs that interact with WW-domains (Einbond and Sudol, 1996; Otte *et al.*, 2003). WW-domains are for instance present in certain classes of ubiquitin ligases. PY-motif dependent ubiquitylation leads, *e.g.*, to the internalization of the Na^+-channel ENaC (Staub *et al.*, 1997) and of ClC-5 (Schwake *et al.*, 2001; Hryciw *et al.*, 2004). Mutating this tyrosine of barttin to alanine indeed increased currents of ClC-Ka/barttin and ClC-Kb/barttin approximately two-fold (Estévez *et al.*, 2001). This observation is compatible with a role of this tyrosine in either mechanism of endocytosis. It was reported that the ubiquitin ligase Nedd4-2 mediates the downregulation of ClC-K/barttin currents in *Xenopus* oocytes (Embark *et al.*, 2004). However, our laboratory could not detect significant effects of dominant negative mutants of several WW-domain containing ubiquitin-ligases on ClC-K/barttin currents (unpublished results), suggesting that the YVRL sequence in barttin may rather function in AP-complex mediated endocytosis.

Currents mediated by ClC-K/barttin channels show little time-dependent activation or deactivation. Contrasting with ClC-Ka/barttin, ClC-Kb/barttin are moderately outwardly rectifying (Estévez *et al.*, 2001; Waldegger *et al.*, 2002; Picollo *et al.*, 2004). Both channels display a Cl>Br>I permeability sequence, are decreased by extracellular acidification and enhanced by raising the extracellular Ca^{++}-concentration (Estévez *et al.*, 2001). The pharmacological inhibition and activation of ClC-Ka/barttin and ClC-Kb/barttin has been studied in considerable detail, including the mapping of binding sites by mutagenesis (Liantonio *et al.*, 2003; Liantonio *et al.*, 2004; Picollo *et al.*, 2004; Liantonio *et al.*, 2006; Picollo *et al.*, 2007). Interestingly, in spite of the high degree of sequence similarity between ClC-Ka and –Kb, there are compounds which show a roughly five-fold higher selectivity for ClC-Ka (Picollo *et al.*, 2004). As these inhibitors bind from the extracellular side (in contrast to the binding of two inhibitors to ClC-1 [Estévez *et al.*, 2003], they may be useful as diuretics.

Immunocytochemistry was used to localize ClC-K channels in the kidney (Uchida *et al.*, 1995; Vandewalle *et al.*, 1997; Estévez *et al.*, 2001; Kobayashi *et al.*, 2001a; Kobayashi *et al.*, 2001b) and the inner ear (Estévez *et al.*, 2001; Sage and Marcus, 2001). As most ClC-K antibodies detect both highly homologous isoforms, these studies were complemented by studying mice expressing EGFP under the control of the human ClC-Kb promoter (Kobayashi *et al.*, 2002), as well as by a histochemical analysis of mice lacking ClC-K1 (Matsumura *et al.*, 1999; Kobayashi *et al.*, 2001b). These studies indicated that ClC-K1 (and by extension probably ClC-Ka) is present in the thin limb of Henle's loop, which lacks comparable expression levels of ClC-K2 (-Kb). ClC-K1 may be present both in the basolateral and apical

membrane of these cells (Uchida *et al.*, 1995), although another study only detected basolateral staining (Vandewalle *et al.*, 1997). ClC-K2 (-Kb) is prominently expressed in exclusively basolateral membranes of the thick ascending limb of Henle's loop (TAL), the distal convoluted tubule, and in intercalated cells of the collecting duct. One cannot exclude that these cells also express minor amounts of ClC-K1 (-Ka). In the inner ear, ClC-K channels (most likely both isoforms) are expressed in basolateral membranes of marginal cells of the stria vascularis as well as in dark cells of the vestibular organ (Estévez *et al.*, 2001; Sage and Marcus, 2001). These epithelial cells secrete K^+-ions into the scala media and vestibular canal, respectively. The presence of ClC-Kb in these cells was independently confirmed by the expression of EGFP that was driven by the human ClC-Kb promoter (Kobayashi *et al.*, 2002). The expression of ClC-K proteins in the kidney and the inner ear is faithfully mirrored by that of barttin (Estévez *et al.*, 2001), strongly supporting the notion that both form heteromers also *in vivo*.

The physiological functions of the two ClC-K isoforms and of barttin are evident from human and mouse pathologies that result from their inactivation: in humans, loss-of-function mutations of ClC-Kb (encoded by the *CLCNKB* gene) result in the massive salt loss of Bartter syndrome type III (Simon *et al.*, 1997). Mutations in *BSND* (encoding barttin) lead to Bartter syndrome type IV (Birkenhäger *et al.*, 2001) that combines congenital deafness with even more severe renal symptoms than those observed with a loss of ClC-Kb. Finally, the disruption in mice of ClC-K1 led to a defect in urinary concentration ability that resembled human nephrogenic diabetes insipidus (Matsumura *et al.*, 1999).

These results, together with the localization and biophysical properties of ClC-K/barttin channels, suggest that ClC-K1 is essential for the transepithelial transport of chloride in the thin limb of Henle. This segment plays an important role in establishing the high osmolarity in kidney medulla, which in turn is needed for the aquaporin-mediated resorption of water in the collecting duct (Fenton and Knepper, 2007). Indeed, medullary solute accumulation was drastically decreased in *Clcnk1*$^{-/-}$ kidneys (Akizuki *et al.*, 2001). So far, no human mutation affecting exclusively *CLCNKA* has been identified, but there are rare individuals who carry deletions in both *CLCNKA* and *CLCNKB* (Schlingmann *et al.*, 2004) (these genes are very close to each other on chromosome 1p36) (Brandt and Jentsch, 1995; Simon *et al.*, 1997). Their disease phenotype resembles Bartter syndrome IV, as discussed below.

The thick ascending limb of Henle's loop is a major site of NaCl reabsorption in the kidney. The main apical uptake of Na^+ and Cl^- occurs through the absorptive NaK2Cl-cotransporter NKCC2 (SLC12A1). The co-transported K^+-ions have to be recycled over the apical membrane through ROMK (Kir1.1; encoded by the *KCNJ1* gene). While Na^+ is secreted over the basolateral membrane by the Na,K-ATPase, Cl^- leaves the cell through ClC-Kb/barttin Cl^- channels. This model is very well supported by human genetics: mutations in NKCC2 underlie Bartter syndrome type I (Simon *et al.*, 1996a), those in ROMK Bartter II (Simon *et al.*, 1996b), in ClC-Kb Bartter III (Simon *et al.*, 1997), and finally those in barttin Bartter IV (Birkenhäger *et al.*, 2001).

In the inner ear, ClC-K/barttin channels are important for K^+-secretion into the scala media. The antibodies used to stain ClC-K in the epithelial marginal cells of the stria vascularis could not distinguish between ClC-K1 and ClC-K2 (Estévez *et al.*, 2001; Sage and Marcus, 2001). RT-PCR on cochlear RNA detected both isoforms (Estévez *et al.*, 2001). Moreover, the demonstration by EGFP-expressing transgenic mice that ClC-Kb is expressed in marginal cells (Kobayashi *et al.*, 2002), together with the observation that patients lacking functional ClC-Kb (in Bartter III) do *not* display deafness, strongly suggests the presence of both ClC-K isoforms in this epithelium. Only if both ClC-Ka/barttin and ClC-Kb/barttin are nonfunctional, deafness ensues. This occurs most often by loss-of-function mutations of the common subunit barttin (Birkenhäger *et al.*, 2001; Estévez *et al.*, 2001), but is also observed in rare patients having lost *both* ClC-K isoforms (Schlingmann *et al.*, 2004). As loss of barttin also affects transport in the thin limb, the nephron segment that seems to express exclusively ClC-Ka, their renal symptoms are generally more severe than those observed with a selective loss of ClC-Kb in Bartter III.

How does a loss of both ClC-Ka/barttin and ClC-Kb/barttin cause deafness? The fluid of the scala media has a highly unusual ion composition in which almost all sodium is replaced by potassium. The extraordinarily high K^+-concentration is needed to depolarize sensory hair cells, since their apical mechanosensitive channels function as K^+-channels. The high K^+- concentration of the scala media is established by the

epithelium of the stria vascularis. It uses the basolateral Na,K-ATPase together with the secretory NaK2Cl-cotransporter NKCC1 to transport K^+ into the cytoplasm. The chloride ions that were coaccumulated by NKCC1 have to be recycled via ClC-Ka/barttin and ClC-Kb/barttin over the basolateral membrane. This exit of chloride may also depolarize this membrane, which may be important for the generation of the positive potential (+90 mV) of the scala media. Potassium leaves marginal cells apically by diffusion through KCNQ1/KCNE1 potassium channels. In support of this model, loss-of-function mutations in either *KCNQ1* or *KCNE1* gene also can lead to deafness (Neyroud *et al.*, 1997; Schulze-Bahr *et al.*, 1997).

Intriguingly, a T481S polymorphism in ClC-Kb leads to a dramatic, ~20-fold increase in ClC-Kb/barttin currents, possibly by increasing its open probability (Jeck *et al.*, 2004a). These currents retain their typical dependence on external pH and Ca^{++}. It was hypothesized that this common sequence variant (found in 20% to 40% of the European population) might increase renal salt reabsorption and might thereby contribute to arterial hypertension. An initial study indeed found a weak correlation of this polymorphism with high blood pressure (Jeck *et al.*, 2004b). However, this finding could not be reproduced in several other cohorts (Kokubo *et al.*, 2005; Speirs *et al.*, 2005; Fava *et al.*, 2007). More recently, the presence of the T481S allele was suggested to correlate with more sensitive hearing (lower hearing threshold) in humans (Frey *et al.*, 2006), whereas a (very different) polymorphism in *CLCNKA* was associated with hypertension (Barlassina *et al.*, 2007). Moreover, a barttin variant (V431I) was identified in African Americans and other populations, but not in Caucasians (Sile *et al.*, 2007). Upon coexpression with ClC-Kb, this variant showed only about 30% current when compared to WT ClC-Kb/barttin. However, it was not associated with a protection against high blood pressure (Sile *et al.*, 2007). Clearly, more work is needed before definitive conclusions can be drawn.

ClC-3: A Putative Cl^-/H^+-Exchanger of Endosomes and Synaptic Vesicles

ClC-3 is very broadly expressed member of the second homology branch of the CLC family (Kawasaki *et al.*, 1994; Borsani *et al.*, 1995). Like its close homologs

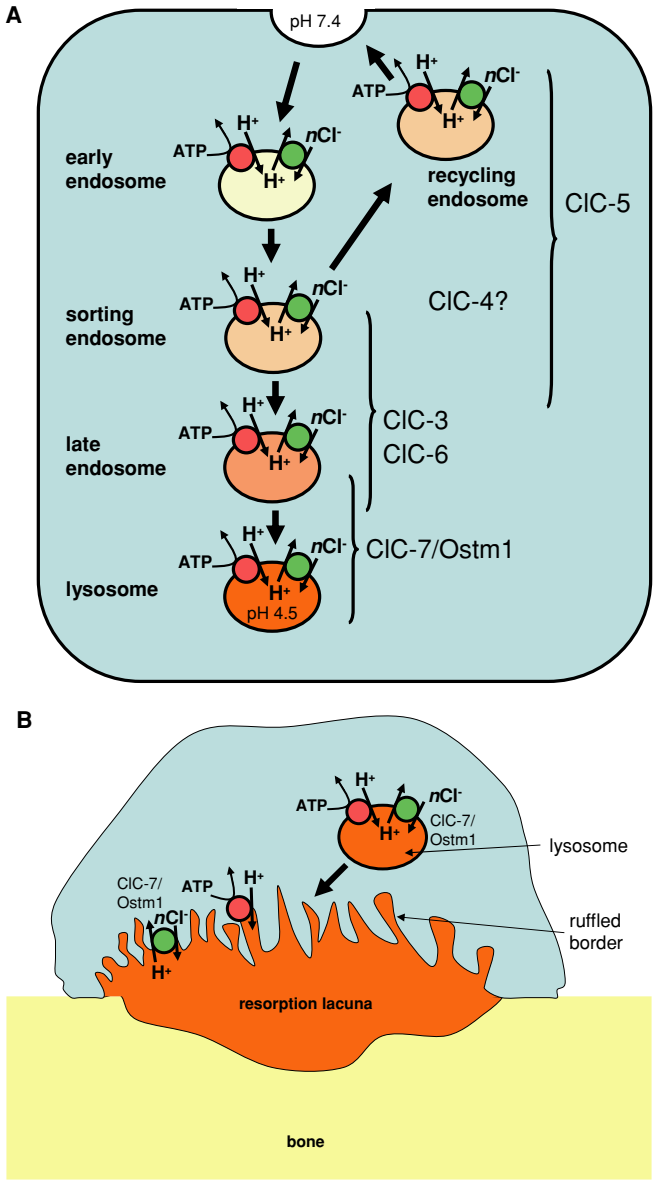

FIGURE 3 **Subcellular localization of vesicular CLC proteins. *(A)*, proposed localization of vesicular CLC isoforms along the endosomal-lysosomal pathway. Vesicles are progressively acidified from an extracellular pH of ~7.4 to the acidic pH (~4.5) of lysosomes. Acidification is carried out by a V-type H^+-ATPase that needs a net influx of negative charge for electroneutrality. This neutralizing current is thought to be mediated by various CLC isoforms. ClC-4 and -5 mediate nCl^-/H^+-exchange (with an imprecisely known stoichiometry *n*, which might be $n = 2$ as in ecClC-1) and this very likely applies for ClC-3 as well. ClC-6 and ClC-7/Ostm1 are also shown as antiporters, although this remains to be shown. With a stoichiometry of $2Cl^-/1H^+$, one out of three H^+-ions transported by the proton pump will leave the vesicle through the CLC antiporter. The localization of ClC-4 is quite uncertain, but may also be endosomal (Mohammad-Panah *et al.*, 2003; Suzuki*et al.*, 2006). *B*, ClC-7/Ostm1 is co-inserted with the proton pump from lysosomes into the highly infolded 'ruffled border' of bone-attached osteoclasts. Proton secretion over this specialized membrane acidifies the underlying resorption lacuna. The acidic pH is needed for the chemical dissolution of inorganic bone material and for the activity of lysosomal enzymes that are secreted into the lacuna. (modified from Jentsch, 2007).**

ClC-4 and ClC-5, ClC-3 is expressed in membranes of the endocytic system (Stobrawa *et al.*, 2001; Miller *et al.*, 2007) (Figure 3A), but additionally localizes to synaptic vesicles (Stobrawa *et al.*, 2001; Salazar *et al.*, 2004). Upon heterologous expression, ClC-3 was also observed mainly in endosomal/lysosomal compartments (Li *et al.*, 2002; Hara-Chikuma *et al.*, 2005b; Suzuki *et al.*, 2006; Weylandt *et al.*, 2007; Zhao *et al.*, 2007). ClC-3 may reach endosomes by transiently trafficking through the plasma membrane, from where it is rapidly endocytosed in a process that requires an interaction of an amino-terminal dileucine motif with actin (Zhao *et al.*, 2007). Several lines of evidence suggested that ClC-3 reaches synaptic vesicles (or synaptic-like microvesicles of neurosecretory PC12 cells) by a mechanism that involves the adaptor complex AP-3 (Salazar *et al.*, 2004; Seong *et al.*, 2005). Intriguingly, a carboxyterminal splice variant of ClC-3 replaces part of CBS2 (without destroying CBS consensus) and adds a PDZ-binding motif (Ogura *et al.*, 2002). The resulting protein was named ClC-3B and was shown to bind to PDZ domains of EBP50 (Ogura *et al.*, 2002), PDZK1 and the Golgi protein GOPC (Gentzsch *et al.*, 2003). The functional importance of this interesting finding is not yet clear.

The almost exclusive intracellular localization of ClC-3 rendered a biophysical characterization of ClC-3 currents notoriously difficult. Weinman's group reported that only 6% of the protein was present in the plasma membrane of their overexpressing COS cells (Zhao *et al.*, 2007) and described associated currents (Li *et al.*, 2000; Li *et al.*, 2002). Weylandt *et al.* (2001) observed some ClC-3 surface expression in overexpressing HEK cells, but did not detect any associated current. Several other groups, including our own (Friedrich *et al.*, 1999), were also unable to detect ClC-3 currents despite many efforts. On the other hand, there is a large body of literature on currents that were ascribed to ClC-3. ClC-3 was originally reported to yield time-independent, very slightly outwardly rectifying plasma membrane Cl^- currents that were completely abolished by activating protein kinase C (Kawasaki *et al.*, 1994). ClC-3 was also claimed to mediate the physiologically important swelling-activated chloride current that is present in most cells (Duan *et al.*, 1997). ClC-3 was also published to be a Ca^{++}-dependent, CamKII-activated Cl^--channel (Huang *et al.*, 2001) that may modulate excitatory synaptic transmission in the hippocampus (Wang *et al.*, 2006). These mutually incompatible results probably reflect the poor surface expression of ClC-3 and the presence of endogenous Cl^--currents in the cells that were investigated. As Nelson and coworkers reported that postsynaptic CamKII-activated anion currents were abolished in *Clcn3*$^{-/-}$ mice (Wang *et al.*, 2006), one might speculate that those currents were mediated by channels that are in some way activated by ClC-3.

The ClC-3 currents reported by Weinman and colleagues (Li *et al.*, 2000, 2002) seem to be most trustworthy. These authors reported very strongly outwardly rectifying currents that displayed a Cl>I conductance sequence (Li *et al.*, 2000). Both characteristics closely match those of ClC-4 and ClC-5 (Steinmeyer *et al.*, 1995; Friedrich *et al.*, 1999), which share about 80% sequence identity with ClC-3. Moreover, a mutation changing a critical glutamate residue in ClC-4 and -5 abolished their strong outward rectification (Friedrich *et al.*, 1999), and exactly the same effect was observed when Li *et al.* (2002) expressed the corresponding ClC-3 mutant. We now know that the mutated residue is the 'gating glutamate', and that ClC-4 and -5 are Cl^-/H^+-exchangers that lose their proton-coupling by that mutation (Picollo and Pusch, 2005; Scheel *et al.*, 2005). These results strongly suggests that ClC-3 is a vesicular Cl^-/H^+-exchanger as well. Unfortunately, this well-founded hypothesis could not yet be tested experimentally, as ClC-3 expression was too low (Picollo and Pusch, 2005).

Recently, Lamb and colleagues also reported strongly rectifying Cl^- currents upon overexpression of ClC-3 (Matsuda *et al.*, 2007), with a neutralization of the gating glutamate again leading to linear currents. However, those currents activated more slowly than ClC-3 currents described by Weinman and colleagues (Li *et al.*, 2000; Li *et al.*, 2002) and currents from ClC-4 and ClC-5 (Steinmeyer *et al.*, 1995; Friedrich *et al.*, 1999). They were >80% inhibited by 30 μM phloretin (Matsuda *et al.*, 2007), an inhibitor that has no effect on ClC-5 currents at those concentrations (Bergsdorf, Zdebik, and Jentsch, unpublished).

To gain insights into the physiological role of ClC-3, three groups disrupted its gene in mice (Stobrawa *et al.*, 2001; Dickerson *et al.*, 2002; Yoshikawa *et al.*, 2002). Stobrawa *et al.* were first to describe a dramatic postnatal degeneration of the hippocampus and retina in *Clcn3*$^{-/-}$ mice (Stobrawa *et al.*, 2001). The retina degenerated within a few weeks after birth, which resulted in complete blindness. The hippocampus already showed signs of incipient degeneration at P12. It progressed rapidly

over the following weeks, resulting in a virtual absence of the hippocampus after a couple of months. The degeneration, which was accompanied by an activation of microglia, was not restricted to the hippocampus. In spite of this massive degeneration, the mice survived for more than a year and showed rather moderate behavioral abnormalities (Stobrawa *et al.*, 2001). Yoshikawa *et al.* (2002) reported that their ClC-3 KO mice displayed some features of neuronal ceroid lipofuscinosis, a subtype of a lysosomal storage disease. This included an accumulation of subunit c of the mitochondrial ATP synthase, a hydrophobic protein that is normally degraded in lysosomes. On the other hand, a comparison of ClC-7 KO mice (which display severe lysosomal storage) with mice disrupted for ClC-3 (Stobrawa *et al.*, 2001) showed that the latter nearly lacked intraneuronal storage material and only minimally accumulated subunit c (Kasper *et al.*, 2005). While neuronal cell loss started in the hippocampal CA1 region in the mouse of Stobrawa *et al.* (2001), Dickerson, Lamb, and coworkers (2002) described that the degeneration progressed from the dentate gyrus to CA3 and CA1 in their mouse model. These authors also observed an initial upregulation of $GABA_A$-receptors and subsequent loss of GABA-synthesizing neurons in the dentate gyrus. Responses to drugs suggested an altered response to GABA as well. The reason for these changes, as well as for the neurodegeneration observed in all three mouse models, remains essentially unclear. It should be stressed that ClC-3 expression is by no means restricted to the nervous system. ClC-3 KO mice probably have abnormalities in many different organs, complicating the establishment of causal relationships leading to phenotypes. For instance, ClC-3 KO mice are smaller than WT littermates and have less body fat, pointing to metabolic abnormalities that may influence several organs (Stobrawa *et al.*, 2001; Dickerson *et al.*, 2002).

All three independent *Clcn3*$^{-/-}$ mouse models have been used to test the assertion by Hume, Duan, and coworkers (1997) that ClC-3 may be the long-sought swelling-activated Cl^--channel. Invalidating this hypothesis, swelling activated anion currents were unaffected in pancreatic acinar cells and hepatocytes (Stobrawa *et al.*, 2001), salivary acinar cells (Arreola *et al.*, 2002), as well as cardiomyoctes (Gong *et al.*, 2004). Hume and coworkers confirmed that cardiac myocytes retained typical swelling-activated chloride currents, observing only some changes in their regulation (Yamamoto-Mizuma *et al.*, 2004). This constitutes overwhelming evidence that ClC-3 does not mediate $I_{Cl,swell}$. Additionally, Ca^{++}-activated Cl^- currents of salivary acinar cells were unchanged by ClC-3 disruption (Arreola *et al.*, 2002).

The vesicular localization of ClC-3 rather suggested that it may affect the luminal acidification of these compartments by neutralizing proton pump currents, a role suggested previously for ClC-5 (Günther *et al.*, 1998; Piwon *et al.*, 2000). Indeed, suspensions of synaptic vesicles acidified less efficiently when isolated from *Clcn3*$^{-/-}$ mice (Stobrawa *et al.*, 2001). Steady-state pH in FITC-dextran loaded liver endosome fractions was reported to be slightly more alkaline in the KO (Yoshikawa *et al.*, 2002). Endosomal pH and Cl^--concentrations were followed in cultured WT and *Clcn3*$^{-/-}$ hepatocytes (Hara-Chikuma *et al.*, 2005b). As predicted by the hypothesis, KO vesicles showed less rapid alkalinization and chloride accumulation.

If ClC-3 is as drastically outwardly-rectifying as ClC-4 and ClC-5 (Friedrich *et al.*, 1999; Picollo and Pusch, 2005; Scheel *et al.*, 2005), as strongly suggested by ClC-3 current measurements (Li *et al.*, 2000, 2002; Picollo and Pusch, 2005), it should however be nearly shut-off at lumen-positive voltages that might be created by the vesicular V-type H^+-ATPase (inside-positive vesicular voltages correspond to the inside-negative voltages over the plasma membrane). This potential problem would not be encountered if ClC-3 were to provide an electric shunt for the currents generated by the NADPH oxidase. This enzyme, which is involved in the generation of reactive oxygen species (ROS), rather transports negative charge into the vesicular lumen (or the extracellular space), predicting a voltage that would activate ClC-3. Indeed, interesting and provocative data by Lamb and coworkers suggest that ClC-3 may influence the activity of NADPH oxidase in both neutrophils (Moreland *et al.*, 2006, 2007) and vascular smooth muscle cells (Miller *et al.*, 2007), using ClC-3 KO cells as control. It is obvious that much more work is needed to understand the multiple roles of this important vesicular anion transport protein.

ClC-4: A Cl^-/H^+-Exchanger with Poorly Understood Biological Significance

ClC-4 belongs to the same homology branch as ClC-3 and ClC-5 and is broadly expressed in many

tissues (van Slegtenhorst *et al.*, 1994; Jentsch *et al.*, 1995), prominently including brain, skeletal muscle, liver, and kidney. Although mainly localized to intracellular membranes, it reaches the plasma membrane to some degree upon heterologous expression in *Xenopus* oocytes or in transfected cells. ClC-4 currents are strongly outwardly rectifying and closely resemble those of ClC-5 (Friedrich *et al.*, 1999). Whereas ClC-4 was previously thought to be a chloride channel, it is now clear that it mediates voltage-dependent electrogenic Cl^-/H^+-exchange (Picollo and Pusch, 2005; Scheel *et al.*, 2005) just like ClC-5 or the bacterial EcClC-1 (Accardi and Miller, 2004). The stoichiometry of Cl^-/H^+-exchange could not yet be determined, but may be identical to the 2:1 stioiometry of EcClC-1 (Accardi and Miller, 2004). Like observed also with ClC-5, current amplitudes are largest with SCN^-, followed by NO_3^-, Cl^-, and I^- (Friedrich *et al.*, 1999; Hebeisen *et al.*, 2003). Whereas one report suggested that ClC-4 channels have a single channel conductance of 3 pS (Vanoye and George, 2002), noise analysis indicated a very small conductance (Hebeisen *et al.*, 2003). The subcellular localization of ClC-4 is controversial, which probably reflects in part problems of antibody specificity. For instance, Bear and colleagues colocalized ClC-4 with CFTR in apical membranes of intestinal epithelial cells (Mohammad-Panah *et al.*, 2002), but also to subapical regions of proximal tubules that are enriched in endosomes (Mohammad-Panah *et al.*, 2003). In transfected cells, one group found ClC-4 in endosomal compartments were it partially overlapped with ClC-3 and ClC-5 (Suzuki *et al.*, 2006). Another group, however, reported a localization to the endoplasmic reticulum and suggested a role of the ClC-4 N-terminus in this localization (Okkenhaug *et al.*, 2006). Overexpression of ClC-4 provided circumstantial evidence that it may play a role in copper incorporation into ceruloplasmin (Wang and Weinman, 2004), while others suggested a role in endosomal acidification and trafficking (Mohammad-Panah *et al.*, 2003). Unpublished results from my laboratory show that the disruption of ClC-4 does not impair proximal tubular endocytosis. ClC-4 may form heteromers with ClC-3 and ClC-5 (Mohammad-Panah *et al.*, 2003; Suzuki *et al.*, 2006), but whether this occurs *in vivo* remains to be unambiguously shown. Much more work seems necessary to elucidate possible roles of ClC-4.

ClC-5: A Cl^-/H^+-Exchanger Involved for Renal Endocytosis

ClC-5 displays a more restricted tissue distribution than ClC-3 and ClC-4. It is mostly, but not exclusively, expressed in epithelia, most prominently in kidney and intestine (Steinmeyer *et al.*, 1995; Vandewalle *et al.*, 2001). In the kidney, it is most prominently expressed in acid-transporting intercalating cells of the distal nephron and in proximal tubules (Günther *et al.*, 1998; Sakamoto *et al.*, 1999). Lower amounts could also be detected in other nephron segments like the thick ascending limb of Henle's loop (Devuyst *et al.*, 1999). ClC-5 is expressed on endosomes, where it colocalizes with the V-type H^+-ATPase and endocytosed protein (Günther *et al.*, 1998; Sakamoto *et al.*, 1999). In transfected cells, it accumulated in endosomes that were artificially enlarged by the constitutively active Q79L mutant of rab5 (Günther *et al.*, 1998). In proximal tubular kidney cells and intestinal epithelial cells, it was apparently only present in apical endosomes (Günther *et al.*, 1998; Vandewalle *et al.*, 2001). The endosomal localization of ClC-5 has been confirmed by many groups (Sakamoto *et al.*, 1999; Mohammad-Panah *et al.*, 2003; Wang *et al.*, 2005; Suzuki *et al.*, 2006). A portion of ClC-5 may also be transiently present in the plasma membrane of native cells, for instance in the brush border membrane of proximal tubular epithelia (Sakamoto *et al.*, 1999). Significant surface expression is achieved upon heterologous expression, conveniently allowing a biophysical analysis of associated ion transport. Like its close relative ClC-4, ClC-5 mediates sharply outwardly rectifying currents (Steinmeyer *et al.*, 1995; Friedrich *et al.*, 1999) that reflect an electrogenic Cl^-/H^+-exchange (Picollo and Pusch, 2005; Scheel *et al.*, 2005). The $SCN^- > NO_3^- > Cl^- > I^-$ conductance sequence is also identical to that of ClC-4 (Friedrich *et al.*, 1999). Neutralization of its 'gating glutamate' uncoupled its anion transport from the countertransport of H^+ (Picollo and Pusch, 2005; Scheel *et al.*, 2005). Concatemers between WT and mutant CLC-5 subunits showed that each monomer performs CL^-/H^+-exchange independent from the other subunit (Zdebik *et al.*, 2008). Noise analysis indicated that this exchange occurs in bursts that resemble gating (Zdebik *et al.*, 2008).

ClC-5 was independently identified by homology cloning (Steinmeyer *et al.*, 1995) and by linkage analysis

of patients with Dent's disease (Fisher *et al.*, 1994; Lloyd *et al.*, 1996). This inherited disorder affects almost exclusively males as the *CLCN5* gene is present on the X-chromosome. Since the identification of *CLCN5* as the gene underlying Dent's disease, more than 40 mutations changing the ClC-5 amino-acid sequence were described. About half of these mutations severely truncate the protein and hence predict a complete loss of function. Almost all of the human missense mutations that were tested in heterologous expression resulted in reduced or undetectable plasma membrane currents (Lloyd *et al.*, 1996; Lloyd *et al.*, 1997; Morimoto *et al.*, 1998). Some mutants that were examined for subcellular localization upon heterologous expression did not to reach the plasma membrane (Ludwig *et al.*, 2005). Many human ClC-5 mutations affect residues that are located close to the dimer interface of ClC-5 (Wu *et al.*, 2003), an intriguing observation that still awaits an enlightening interpretation.

The clinically important symptoms of Dent's disease are recurrent kidney stones and nephrocalcinosis (Wrong *et al.*, 1994). When leading to renal insufficiency, these symptoms may eventually require kidney transplantation. The incidence of these pathologies is quite variable, in contrast to the low molecular weight proteinuria that is observed in almost all patients with *CLCN5* mutations. The selective urinary loss of small proteins indicated that the glomerular filter is intact and hinted at a problem in proximal tubular endocytosis. Indeed, two ClC-5 KO mouse models reproduced the human proteinuria and revealed defective endocytosis by the proximal tubule (Piwon *et al.*, 2000; Wang *et al.*, 2000). Exploiting the random inactivation of X-chromosomes in females, Piwon et al. (2000) analyzed chimeric tubules in which only some cells expressed ClC-5. *In vivo* endocytosis of fluorescently labeled proteins or FITC-dextrane showed that both receptor-mediated as well as fluid phase endocytosis were drastically reduced in a cell-autonomous manner in cells lacking ClC-5. These authors also showed that megalin, an apical receptor for proteins and other ligands that is highly expressed in proximal tubules, was significantly reduced in KO cells–again a cell-autonomous effect (Piwon *et al.*, 2000). This finding implied that receptor-mediated endocytosis is even more drastically reduced than fluid-phase endocytosis. We speculated that ClC-5 might be necessary for the recycling of endocytosed megalin back to the brush border (Günther *et al.*, 2003). Indeed, subsequent electron-microscopical studies by Christensen and coworkers revealed a drastically reduced brush border expression of megalin and its coreceptor cubulin (Christensen *et al.*, 2003). It should be stressed, however, that the loss of megalin only exacerbates the broad defect in endocytosis of KO proximal tubules lacking functional ClC-5. Indeed, in addition to the strongly reduced fluid phase endocytosis, the loss of ClC-5 also slowed the PTH-induced endocytosis of apical transport proteins of proximal tubular cells like the Na^+-phosphate cotransporter NaPi-2a or the Na^+/H^+-exchanger NHE3 (Piwon *et al.*, 2000). Hence, the loss of the endosomal ClC-5 protein leads to a broad defect in proximal tubular endocytosis.

The defect in endocytosis was ascribed to a defective acidification of renal endosomes. This hypothesis was ascertained experimentally by measuring ATP-induced acidification of renal cortical membrane preparations (Piwon *et al.*, 2000; Günther *et al.*, 2003). Moreover, endosomal pH and Cl^- concentration were measured in WT and KO cells as a function of time after endocytosis of dyes coupled to either transferrin or α_2-macroglobulin (Hara-Chikuma *et al.*, 2005a). These coupled indicators should report early and recycling endosomes, or late endosomes, respectively. Alkalinization and chloride accumulation were reduced in early, but not late endosomes of *Clcn5*$^{y/-}$ mice (Hara-Chikuma *et al.*, 2005a), consistent with our current understanding of the subcellular localization of ClC-5.

It was suggested that ClC-5 provides an electric shunt for the electrogenic V-type H^+-ATPase (Günther *et al.*, 1998; Piwon *et al.*, 2000). Indeed, it has been known since long that chloride is necessary for the acidification of endosomes, lysosomes, synaptic vesicles, as well as secretory vesicles (Mellman *et al.*, 1986). This observation led to the hypothesis that vesicular chloride channels prevent the generation of a large voltage across vesicular membranes that would inhibit further proton pumping on energetic grounds. The more recent finding that ClC-5 is not a Cl^- channel, but rather an electrogenic Cl^-/H^+-exchanger, does not invalidate the assumption that it enables vesicular acidification. It implies, however, that more energy is needed to achieve the same pH value and suggests that the direct coupling of H^+-gradients to Cl^--gradients plays a previously unrecognized role (Jentsch, 2007).

The impairment of proximal tubular endocytosis in ClC-5 KO mice might thus be a direct effect of an impaired endosomal acidification. Indeed, the binding to the endosomes of ARNO and Arf6, two important

regulators of endosomal trafficking, was shown to be dependent on an acidic luminal pH (Maranda *et al.*, 2001). The luminal pH has to be 'measured' and communicated to the cytoplasmic side where those regulatory proteins bind. This task is probably carried out by a subunit of the V-type ATPase (Hurtado-Lorenzo *et al.*, 2006). Several groups have also investigated effects of alkalinizing endosomes by weak bases like chloroquin or by applying the V-type H^+-ATPase inhibitor bafilomycin (Chapman and Munro, 1994; Gekle *et al.*, 1995; Presley *et al.*, 1997; Wang *et al.*, 2005). Such treatments led to disturbed trafficking in the endosomal pathway and an overall reduction of endocytosis in cell culture. A defect in endocytosis was also shown in primary cell cultures derived from proximal tubules of ClC-5 KO animals (Wang *et al.*, 2005). Treatment with bafilomycin inhibited endocytosis in cells derived from WT, but not in those derived from ClC-5 KO mice, supporting the hypothesis that ClC-5 modulates endocytosis by facilitating endosomal acidification (Wang *et al.*, 2005).

Whereas a defective luminal acidification might be sufficient to explain the strongly reduced endocytosis in ClC-5 KO proximal tubules, one should not discard prematurely alternative or additional roles of ClC-5. In addition to well documented binding of the ClC-5 C-terminus to certain ubiquitin ligases, the cytoplasmic amino- and carboxy-termini of the Cl^-/H^+-exchanger are likely to bind other proteins. Yeast two hybrid screens revealed that cofilin, an enzyme involved in actin depolymerization, binds to ClC-5 (Hryciw *et al.*, 2003), and ClC-5 was also found to associate with the PDZ-domain protein NHERF2 (Hryciw *et al.*, 2006). Silencing NHERF2 in opossum kidney (OK) cells slightly reduced endocytosis. Although this procedure also decreased the surface expression of ClC-5 (Hryciw *et al.*, 2006), it remains unclear whether the effect on endocytosis occurred through changes in ClC-5 trafficking.

The experiments and considerations discussed above provide a reasonably well founded explanation for the proteinuria of patients with Dent's disease, but what might be the mechanism leading to kidney stones? Patients (Wrong *et al.*, 1994) and the two ClC-5 KO mouse models (Piwon *et al.*, 2000; Wang *et al.*, 2000) lose inorganic phosphate into the urine. To a large extent phosphate is reabsorbed in the proximal tubule. The main transporter responsible for that uptake is NaPi-2a, a sodium-coupled phosphate cotransporter that is highly regulated by parathyroid hormone (PTH). An increase in PTH leads to a rapid internalization of NaPi-2a from the plasma membrane and to its lysosomal degradation. Consistent with the observed hyperphosphaturia, Piwon et al. (2000) found that the presence in the brush-border of NaPi-2a and its overall expression were decreased in ClC-5 KO proximal tubules. As lacking endocytosis of NaPi-2a itself would lead to an increase, rather than a decrease, of NaPi-2a in the plasma membrane and hence to less phosphate in the urine, these results suggested a change in PTH signaling. Plasma membrane levels of this hormone, however, were normal (Piwon *et al.*, 2000). However, PTH receptors are also expressed in the apical membranes of proximal tubular cells, and PTH–being a small peptide hormone–is filtered into the primary urine. A large portion of PTH is normally removed from the urine by megalin-dependent endocytosis (Hilpert *et al.*, 1999), a process that is impaired in ClC-5 KO mice. Indeed, levels of PTH were found to be increased in the urine of ClC-5 KO mice (Piwon *et al.*, 2000) and patients with Dent's disease (Norden *et al.*, 2001). We therefore proposed (Piwon *et al.*, 2000) that the abnormal increase in late proximal tubular PTH concentration, a consequence of a primary defect in endocytosis, excessively stimulates apical PTH receptors in late proximal tubular cells. The resulting endocytosis of NaPi-2a then leads to a higher urinary phosphate concentration, a factor contributing to the formation of kidney stones.

The proximal tubule plays a major role in the metabolism of vitamin D, a hormone crucially involved in the calcium homeostasis of the body. In these cells the inactive precursor 25(OH)-VitD_3 is converted to the active hormone 1,25$(OH)_2$-VitD_3 by the mitochondrial 25(OH)-Vit$D_3$1α-hydroxylase. Additionally, both the precursor as well as 1,25$(OH)_2$-VitD_3 can be hydroxylated at position 24 to create inactive metabolites, a reaction carried out by VitD_3 24-hydroxylase. 1,25$(OH)_2$-Vit$D_3$is transcriptionally inhibited, and 24-hydroxylase transcriptionally activated by cellular levels of the active hormone 1,25$(OH)_2$-VitD_3 (Murayama *et al.*, 1999). Additionally, PTH activates the transcription of 1α-hydroxylase (Brenza *et al.*, 1998; Murayama *et al.*, 1999). In contrast to most other cells of the body, the major uptake pathway of vitamin D and its metabolites into proximal tubular cells is through apical, megalin-dependent endocytosis in a complex with its binding protein (Nykjaer *et al.*, 1999). Vitamin D binding protein can pass the glomerular filter and binds to megalin. Thus, the partial blockade of 1,25$(OH)_2$-VitD_3 uptake

into PT cells that lack ClC-5 predicts both an upregulation of the activating enzyme 1α-hydroxylase, as well as a decrease of the inactivating 24 hydroxylase. We additionally predict a contribution of the stimulation of apical PTH receptors by abnormally high levels of PTH to an increase in 1α-hydroxylase activity. We indeed found these changes in $VitD_3$ hydroxylase levels in ClC-5 KO mice (Piwon *et al.*, 2000; Günther *et al.*, 2003; Maritzen *et al.*, 2006b). These changes in metabolizing enzymes would predict an increase in circulating levels of the active hormone $1,25(OH)_2$-$VitD_3$, which would lead to an increased intestinal reabsorption of calcium and phosphate and ultimately result in hypercalciuria as a compensatory mechanism. Indeed, in patients with Dent's disease levels of $1,25(OH)_2$-$VitD_3$ are generally moderately elevated (Scheinman, 1998). However, there is a complication: due to the defect in PT endocytosis, the uptake of the precursor into PT cells is drastically reduced, and both the precursor and the active hormone are lost into the urine. Hence there are two opposing mechanisms that may lead, depending on genetic factors and nutrition, either to an increase or a decrease in plasma levels of $1,25(OH)_2$-$VitD_3$. This model may well explain the clinical variability of patients with ClC-5 mutations, who do not always show hypercalciuria and kidney stones. Interestingly, this variability is also reflected in the phenotypes of the two ClC-5 KO mouse models (Piwon *et al.*, 2000; Wang *et al.*, 2000): the mouse from our laboratory has reduced levels of $1,25(OH)_2$-$VitD_3$ and lacks hypercalciuria (Piwon *et al.*, 2000), whereas the mouse model of Guggino and colleagues has hypercalciuria and signs of renal calcification in the presence of slightly elevated $1,25(OH)_2$-$VitD_3$ levels (Wang *et al.*, 2000; Guggino, 2007).

The effects of impaired endocytosis of hormones in the ClC-5 KO mouse not only leads to transcriptional changes in the proximal tubule (like the decrease of $VitD_3$-induced $VitD_3$ 24-hydroxylase), but also in more distal nephron segments. In spite of reduced plasma levels of $1,25(OH)_2$-$VitD_3$, Maritzen *et al.* (2006b) found an upregulation of renal $VitD_3$ regulated genes like the epithelial Ca^{++} channels TRPV5 and TRPV6 or the Ca^{++}-binding proteins calbindin D 28k and D 9k. These proteins are expressed in nephron segments distal to the proximal tubule. Whereas the defective endocytosis in the ClC-5 KO decreases $1,25(OH)_2$-$VitD_3$ levels in proximal tubular cells, it increases the hormone exposure of cells located downstream in the tubule. Hence $VitD_3$-induced genes are downregulated in the proximal tubule, but upregulated in more distal segments. Interestingly, also the RNA of a lipocalin-encoding gene, which is known to be transcriptionally activated by retinoic acid, was strongly increased. This fits to the drastic increase of retinol binding protein in the urine of ClC-5 KO mice (Piwon *et al.*, 2000). These results suggest that distal, luminal effects of hormones should be considered with any pathology leading to defective proximal tubular endocytosis.

Summarizing this section, the pathogenesis of kidney stones in Dent's disease could be a indirect consequence of defective proximal tubular endocytosis, leading to an abnormal metabolism of PTH and vitamin D and eventually to an internalization of NaPi-2a and an increased intestinal reabsorption of calcium and phosphate (Piwon *et al.*, 2000; Günther *et al.*, 2003; Maritzen *et al.*, 2006b). However, another study failed to detect a stimulation of intestinal Ca^{++}-reabsorption and rather described an increased bone turnover (Silva *et al.*, 2003; Guggino, 2007).

Only a few other organs were investigated for a possible role of ClC-5 in endocytosis. There was no gross difference of asialoglycoprotein uptake into the liver between WT and ClC-5 KO mice (Piwon *et al.*, 2000). Two reports investigated the role of ClC-5 in the thyroid (Maritzen *et al.*, 2006a; van den Hove *et al.*, 2006), where endocytosis and subsequent intracellular processing of thyroglobulin is central to organ function. Both studies coincided in that ClC-5 protein expression is rather modest in this organ (10% to 20% of the kidney level), that KO mice were euthyroid, and that megalin levels were unaffected. Whereas no defects were observed in the 'Jentsch mouse' (Maritzen *et al.*, 2006a), the 'Guggino mouse' displayed goiter and was reported to have reduced levels of the apical I^-/Cl^- exchanger pendrin (van den Hove *et al.*, 2006). The reason for these differences and the mechanism leading to a downregulation of pendrin are not known.

Also other transport proteins may be changed in ClC-5 KO mice. Whereas the subcellular localization of the V-type H^+-ATPase was unchanged in ClC-5 KO kidneys, both in proximal tubules and in intercalated cells (Piwon *et al.*, 2000), one report describes an inverted localization of the proton pump. It was found at the basolateral side of proximal tubules in biopsies from patients with Dent's disease (Moulin *et al.*, 2003). Furthermore, expression of the basolateral glucose transporter GLUT2 was reportedly reduced at both the RNA and protein levels in mice lacking ClC-5

(Souza-Menezes *et al.*, 2007). It is unclear how these observations can be linked to the absence of ClC-5.

Several mechanisms have been proposed to regulate ClC-5 localization and activity. ClC-5 carries in its cytoplasmic tail between its two CBS domains a PY-motif that is a potential binding site for WW-domains (Pirozzi *et al.*, 1997; Schwake *et al.*, 2001). Recent crystallographic and NMR data from CLC carboxy-termini (including ClC-5) suggest that the region encompassing the PY-motif is unstructured, flexible, and accessible for binding other proteins (Meyer and Dutzler, 2006; Alioth *et al.*, 2007; Meyer *et al.*, 2007). A peptide containing this sequence bound several WW-domain proteins *in vitro*, most strongly interacting with the fourth WW-domain of the E3 ubiquitin ligase WWPII (Pirozzi *et al.*, 1997). This ubiquitin ligase is broadly expressed, including the kidney (Schwake *et al.*, 2001). When ClC-5 point mutants that destroyed this PY motif were expressed in *Xenopus* oocytes, ClC-5 expression in the plasma membrane and currents were approximately doubled (Schwake *et al.*, 2001). Coexpression with several dominant negative forms of WWPII with WT ClC-5, but not with its PY-mutants, also increased currents about two-fold. These experiments strongly suggested that ClC-5 can be ubiquitinated in a PY-motif dependent manner and that ubiquitylation induces an endocytotic removal of the transporter from the plasma membrane. It was shown later that ClC-5 can also be modified by the WW-domain containing ubiquitin ligase Nedd4 and Nedd4-2 (Hryciw *et al.*, 2004). An attachment of tagged ubiquitin to overexpressed ClC-5 was demonstrated biochemically. Surprisingly, ubiquitylation could only be observed when cells were incubated with a proteasome inhibitor together with albumin, a procedure thought to stimulate endocytosis. No controls with ClC-5 PY mutants were reported, however. Knocking down Nedd4-2 with RNAi in opossum kidney cells (a cell culture model for the proximal tubule) reduced albumin endocytosis by about 25% (Hryciw *et al.*, 2004), but it is unclear whether this effect was mediated by reduced ubiquitylation of ClC-5. A role of ClC-5 ubiquitylation for endocytosis remains to be shown *in vivo*.

The CBS domains of ClC-5 were shown to bind nucleotides (Meyer *et al.*, 2007). As they bound ATP, ADP, and AMP with similar affinities, and because a mutation inhibiting nucleotide did not change currents when introduced into WT ClC-5 (Meyer *et al.*, 2007), it is currently unclear how nucleotides could regulate ClC-5 activity.

ClC-6: A Late Endosomal Neuronal Chloride Transporter Important for Lysosomal Function

Together with ClC-7, ClC-6 forms the third branch of the mammalian CLC gene family (Brandt and Jentsch, 1995). ClC-6 did not give any new currents when expressed in oocytes or other cells (Brandt and Jentsch, 1995; Buyse *et al.*, 1997; Buyse *et al.*, 1998). Functional complementation of the gef1 yeast mutant that carries a deletion in the single yeast CLC gene (Greene *et al.*, 1993) revealed that ClC-6, but not ClC-7, could substitute for ScClC (Kida *et al.*, 2001). As the transport properties of ClC-6 could not yet been determined, it remains unclear whether it is a Cl^- channel or a Cl^-/H^+-exchanger. The latter possibility, however, is suggested by the presence of a 'proton glutamate' that has so far only been found in CLC Cl^-/H^+-exchangers, but not in Cl^- channels (Accardi *et al.*, 2005).

The functional significance of ClC-6 splice variants (Eggermont *et al.*, 1997) is unclear, in particular since they often truncate the protein. On the RNA level, ClC-6 is very broadly expressed, including the brain, kidney and testes (Brandt and Jentsch, 1995; Kida *et al.*, 2001). Message levels in whole mouse embryos increased from E7 to E11 (Brandt and Jentsch, 1995). *In situ* hybridization revealed expression in epithelial cells of the lung, intestine, pancreas, and Sertoli cells (Kida *et al.*, 2001). Surprisingly, however, the ClC-6 protein is almost exclusively expressed in the nervous system (Poët *et al.*, 2006).

ClC-6 was at first reported to reside in the endoplasmic reticulum in a study that used tagged ClC-6 overexpressed in COS cells (Buyse *et al.*, 1998). In contrast, KO-controlled immunocytochemistry and Western blotting of membrane fractions revealed that native ClC-6 is an endosomal protein (Poët *et al.*, 2006). The partial colocalization in immunocytochemistry with lamp1, together with the presence of small amounts of ClC-6 in lysosomal membrane fractions and its partial shift to these fractions in ClC-7 KO mice, suggested that ClC-6 is mainly expressed in late endosomes (Poët *et al.*, 2006). This localization of native ClC-6 was confirmed in a neuroblastoma cell line (Ignoul *et al.*, 2007). In transfected cells, ClC-6 was found in a detergent-resistant membrane fraction ('lipid rafts'), a localization that depended on an stretch of positively charged amino-acids in its amino-terminus (Ignoul *et al.*, 2007). The biological significance of the latter finding,

which has been obtained with COS cells overexpressing ClC-6, remains to be determined.

The physiological function of ClC-6 was addressed by generating a knockout mouse model (Poët *et al.*, 2006). These mice had no immediately apparent phenotype, were fertile and had an approximately normal life span. Closer examination, however, revealed a lysosomal storage disease which became apparent at 4 weeks of age, was strong after 3 months, and was associated with lipofuscin accumulation. Storage material stained positively for lysosomal proteins like lamp1, cathepsin D, and ClC-7. This material was also positive for subunit c of mitochondrial ATP synthase, a hydrophobic protein that is typical component of storage material in human neuronal ceroid lipofuscinosis (NCL). In contrast to the lysosomal storage of *Clcn7*$^{-/-}$ mice (see below), storage material accumulated specifically in initial axon segments of *Clcn6*$^{-/-}$ neurons and no obvious neuronal cell loss was noted (Poët *et al.*, 2006). ClC-6 is highly expressed in dorsal root ganglia. In the KO, these sensory neurons displayed particular high levels of storage material, which was correlated with a decrease in pain sensitivity as assayed by tail-flick analysis. In view of the role of other intracellular CLCs in acidifying vesicular lumina, Poët *et al.* (2006) tested the hypothesis that a more alkaline lysosomal pH could underlie the lysosomal storage of *Clcn6*$^{-/-}$ neurons. However, their lysosomal pH turned out to be normal under steady-state conditions (Poët *et al.*, 2006).

As the pathology of *Clcn6*$^{-/-}$ mice resembled clinically mild forms of human neuronal ceroid lipofuscinosis (NCL), 75 patients with predominantly late onset NCL were screened for mutations in *CLCN6* (Poët *et al.*, 2006). Two missense mutations that were not present on 200 control chromosomes were identified. However, these sequence variants were present in a heterozygous state, falling short of proving that *CLCN6* is a human NCL gene. Nonetheless, *CLCN6* remains a candidate gene for rare forms of human NCL.

ClC-7/Ostm1: A Broadly Expressed Lysosomal Cl^- Transporter with Important Roles in Lysosomes and Osteoclasts

ClC-7 has been cloned by homology to ClC-6, with which it shares approximately 45% amino acid identity (Brandt and Jentsch, 1995). Despite many attempts, no currents could be detected in *Xenopus* oocytes or mammalian cells overexpressing ClC-7 (Brandt and Jentsch, 1995). Acid-activated currents that were observed with *Xenopus* oocytes expressing ClC-7 (Diewald *et al.*, 2002) are probably endogenous to the expression system. ClC-7 neither complemented the function of the single yeast CLC gene (Kida *et al.*, 2001). As with ClC-6, the presence of a 'proton glutamate' that is important for the H^+-transport of the bacterial Cl^-/H^+-exchanger EcClC-1 (Accardi *et al.*, 2005) and of CIC-4 and CLC-5 (Zdebik *et al.*, 2008) suggests that ClC-6 might be an exchanger as well. However, this point remains to be proven.

ClC-7 mRNA was found in every tissue examined (Brandt and Jentsch, 1995). *In situ* hybridization of mouse embryos showed particularly high labeling of dorsal root and trigeminal ganglia and of the brain (Kornak *et al.*, 2001). Hybridization of adult mouse sections resulted in labeling of, *e.g.*, cerebellar Purkinje cells, tracheal and pancreatic epithelia, renal proximal tubules and Sertoli cells (Kida *et al.*, 2001). Immunocytochemistry using KO-controlled antibodies revealed broad neuronal staining (Kasper *et al.*, 2005). The subcellular localization of ClC-7 was addressed by immunocytochemistry of native cells (in both tissues and cell culture) and by probing subcellular fractions by Western blotting (Kornak *et al.*, 2001; Kasper *et al.*, 2005). Both techniques indicated a presence in late endosomes and lysosomes. The latter localization was additionally confirmed by immunogold labeling of neuronal lysosomes *in situ* (Kasper *et al.*, 2005). ClC-7 is the only mammalian CLC protein that is predominantly expressed in the lysosomal membrane. This localization, which is also observed in transfected mammalian cells (Lange *et al.*, 2006), most likely explains the lack of measurable ClC-7 plasma membrane currents.

Physiological roles of ClC-7 were elucidated by generating KO mice (Kornak *et al.*, 2005). *Clcn7*$^{-/-}$ mice were born at approximately Mendelian ratio, but were smaller and died after about 6 weeks. They displayed a severe osteopetrosis that resulted in secondary effects like a lack of teeth eruption. Calcification of bone marrow cavities led to extramedullary blood production. Investigation of KO mice expressing X-Gal under the endogenous ClC-7 promoter and immunocytochemistry of bone sections revealed that ClC-7 is highly expressed in osteoclasts (Kornak *et al.*, 2005). In these cells, the protein was concentrated in the acid-secreting 'ruffled border,' a specialized membrane created by the exocytotic insertion of lysosomal membranes. ClC-7 colocalizes

in the ruffled border with its β-subunit Ostm1 (see below) and with the a3 subunit of the V-type H^+-ATPase (Kornak *et al.*, 2005; Lange *et al.*, 2006). Osteoclasts were present in approximately normal numbers in *Clcn7*$^{-/-}$ mice, but electron microscopy showed that their ruffled border was underdeveloped (Kornak *et al.*, 2001). When cultured on ivory slices, *Clcn7*$^{-/-}$ osteoclasts were unable to acidify their resorption lacuna and to significantly degrade bone material. In a few osteoclasts, however, the transcytotic transit of biotinylated bone material could be observed (Kornak *et al.*, 2001). These experiments suggested that ClC-7 is necessary for the neutralization of electrical currents generated by the H^+-ATPase, similar to the roles proposed for ClC-5 and ClC-3 in the acidification of endosomal and synaptic vesicles (Figure 3B). Indeed, staining ivory-attached osteoclasts with acridine orange showed that WT, but not *Clcn7*$^{-/-}$ osteoclasts acidify their resorption lacuna (Kornak *et al.*, 2001).

ClC-7 mice are also blind due to a rapid postnatal degeneration of the retina (Kornak *et al.*, 2001). In human osteopetrosis, blindness often results from a compression of the optic nerve by the thickened bone. Whereas the optic nerve was compressed in ClC-7 KO mice (Kornak *et al.*, 2001), also KO mice whose osteopetrosis was rescued by TRAP-promoter driven expression of ClC-7 in osteoclasts displayed retinal degeneration. Hence retinal degeneration is caused by a tissue-intrinsic mechanism (Kasper *et al.*, 2005).

The severe osteopetrosis of ClC-7 KO mice suggested that mutations in the human *CLCN7* gene might underlie infantile malignant osteopetrosis. Indeed, in a cohort of 10 patients with this severe form of osteopetrosis, we found one patient who was a compound heterozygote for two mutations in the *CLCN7* gene (Kornak *et al.*, 2001). In the same cohort, we identified seven patients who carried mutations in the a3 subunit of the vacuolar H^+-ATPase (Kornak *et al.*, 2000). The fact that loss-of-function mutations in either the proton pump or the ClC-7 Cl^-transporter cause very similar osteopetrotic phenotypes provides additional, albeit indirect, genetic support for the hypothesis that both proteins jointly acidify the resorption lacuna.

Until now, more than 30 *CLCN7* mutations have been identified in human osteopetrosis (Frattini *et al.*, 2003; Waguespack *et al.*, 2003; Waguespack *et al.*, 2007). This includes dominant osteopetrosis (Albers-Schönberg disease) (Cleiren *et al.*, 2001), in which the clinical phenotype is more benign and becomes evident only later in life when compared to infantile malignant osteopetrosis. Dominant mutations in *CLCN7* are always missense mutations. Even if these mutations totally inactivate the function of the CLC dimer when being in associated with a WT subunit, the homo-dimeric structure of CLC proteins predicts that heterozygous Albers-Schönberg patients would still display 25% of normal ClC-7 activity, which agrees with the comparatively mild osteopetrosis. Osteoclasts isolated from patients with dominant osteopetrosis showed defects in degrading both the calcified and organic matrix of bone in culture (Chu *et al.*, 2006; Henriksen *et al.*, 2006). So far, no patient with two truncating *CLCN7* mutations has been described in malignant osteopetrosis–if a stop codon disrupted the ClC-7 reading frame on one allele, the other allele carried a missense mutation. This might suggest that even in those patients some residual ClC-7 function remains. Possibly, a total loss of ClC-7 function leads to early lethality in humans, but not in mice. An even more general role of ClC-7 in regulating bone density is suggested by the finding that *CLCN7* polymorphisms are associated with variations in bone mineral density (Kornak *et al.*, 2005; Pettersson *et al.*, 2005). These findings provide additional support for the hypothesis (Kornak *et al.*, 2001) that specific inhibitors of ClC-7 might be useful in treating osteoporosis, a very common disorder of mostly elderly women. Attempts are ongoing to develop such inhibitors (Schaller *et al.*, 2004; Karsdal *et al.*, 2005; Schaller *et al.*, 2005).

ClC-7 is very broadly expressed, with high expression levels in neurons (Kornak *et al.*, 2001; Kasper *et al.*, 2005). In addition to osteopetrosis and retinal degeneration, ClC-7 KO mice also display a severe lysosomal storage disease that leads to neuronal cell loss (Kasper *et al.*, 2005). Typical biochemical and morphological features of neuronal ceroid lipofuscinosis (NCL), a subtype of lysosomal storage disease, were observed. Electron-dense deposits were found in neuronal cell bodies, but not in initial axon segments like with *Clcn6*$^{-/-}$ mice. Some osteopetrotic patients with *CLCN7* mutations on both alleles also display severe neurological symptoms (Frattini *et al.*, 2003).

Attempts were made to demonstrate a lysosomal defect in *Clcn7*$^{-/-}$ cells in culture. However, both the activity of a lysosomal enzyme (PPT1) and the steady-state pH of lysosomes were normal in fibroblasts and neurons derived from KO mice (Kornak *et al.*, 2001).

ClC-7 needs a small integral membrane protein, Ostm1, as auxiliary β-subunit (Lange *et al.*, 2006).

Ostm1 was identified as the gene underlying the severe osteopetrotic phenotype of the spontaneous *grey lethal* mouse (Chalhoub *et al.*, 2003). Mutations in *OSTM1* also underlie rare cases of human recessive, malignant infantile osteopetrosis (Chalhoub *et al.*, 2003; Quarello *et al.*, 2004; Ramírez *et al.*, 2004; Maranda *et al.*, 2007). Ostm1 was independently cloned by a yeast-2-hybrid approach using RGS-GAIP, a protein interacting with a G-protein, as a bait (Fischer *et al.*, 2003). It was proposed to have an E3 ubiquitin ligase activity. However, it was shown that a putative RING finger domain (Fischer *et al.*, 2003), which must be cytoplasmic for mediating ubiquitylation, is highly glycosylated and therefore located on the luminal side of the ER and subsequent intracellular compartments (Lange *et al.*, 2006). The amino-terminal hydrophobic stretch of Ostm1 serves as a cleavable signal peptide and the resulting type I transmembrane protein is anchored in the membrane through a single transmembrane domain close to the Ostm1 C-terminus. The luminal part of Ostm1 is proteolytically cleaved on the way to or in lysosomes, where mature Ostm1 is located in native cells (Lange *et al.*, 2006). Transfection experiments revealed that Ostm1 needs ClC-7 to reach lysosomes, whereas ClC-7 does not require Ostm1 to reach that compartment. The closest homologue of ClC-7, ClC-6, could not replace ClC-7 in directing Ostm1 to lysosomes, indicating a specific ClC-7/Ostm1 interaction. Indeed, Ostm1 could be coprecipitated with ClC-7 and *vice versa*, suggesting that Ostm1 is a β-subunit of ClC-7. Protein levels of Ostm1 were severely reduced in ClC-7 KO mice, and ClC-7 proteins levels were reduced to about 5% of WT in all tissues of *grey lethal* mice that were examined. Hence, the osteopetrotic phenotype of *grey lethal* mice and of patients with *OSTM1* mutations is most likely due to a loss of ClC-7 activity (Lange *et al.*, 2006). In addition to osteopetrosis, *grey lethal* mice also displayed lysosomal storage and neurodegeneration similar to *Clcn7*$^{-/-}$ mice. In the meantime, signs of CNS degeneration have also been found in osteopetrotic patients carrying mutations in *OSTM1* (Pangrazio *et al.*, 2006; Maranda *et al.*, 2007). Electron microscopical studies of skin biopsies from such patients revealed swollen unmyelinated axons containing spheorids and lipofuscin-containing secondary lysosomes in Schwann cells (Alroy *et al.*, 2007).

The exact mechanism by which ClC-7 becomes unstable in the absence of Ostm1 is not yet known. However, ClC-7 is the only mammalian CLC protein that is not glycosylated. This is particularly surprising for a lysosomal membrane protein, which are normally highly glycosylated in order to protect them from an attack by lysosomal enzymes. The highly glycosylated Ostm1 β-subunit was therefore proposed to shield ClC-7 from lysosomal degradation (Lange *et al.*, 2006).

Lysosomal steady-state pH was determined in *grey lethal* fibroblasts and neurons (Lange *et al.*, 2006). Just like in ClC-7 KO cells, it was found to be unchanged. These findings do not necessarily rule out a role of ClC-7/Ostm1 in luminal acidification. Lysosomal pH was measured after an overnight chase of an endocytosed pH indicator into lysosomes. A small remaining conductive pathway mediated by other proteins may suffice to neutralize the charge transferred by the proton ATPase, resulting in a normal steady-state pH after several hours. Nonetheless, these experiments raise the question of the pathogenesis of the lysosomal storage disease observed in ClC-7 or Ostm1 KO mice. The lysosomal pathology might be a consequence of a slower acidification rate during the transition from late endosomes to lysosomes. An alternative explanation implicates a role of lysosomal chloride concentration (Jentsch, 2007). If ClC-6 and ClC-7 were Cl^-/H^+-exchangers, as suggested by the presence of a 'proton glutamate' (Accardi *et al.*, 2005), the luminal [Cl^-] might be changed even in the presence of a normal lysosomal pH. If ClC-7/Ostm1 were the only pathway for Cl^- transport across lysosomal membranes (which is unlikely), a high luminal lysosomal [Cl^-] is predicted. Assuming a $2Cl^-/1H^+$ exchange like in EcClC-1, a lysosomal voltage of 0 mV, cytosolic pH of 7.2 and lysosomal pH of 4.5, lysosomal [Cl^-] is calculated to 224 mM when cytosolic [Cl^-] is as low as 10 mM (as found in neurons). However, the lysosomal voltage is not exactly known (but lumen-positive voltages would lead to even more chloride accumulation), and the calculated [Cl^-] would pose osmotic problems for lysosomes. Despite these caveats, this calculation demonstrates the potential of endosomal/lysosomal CLCs to significantly accumulate chloride in these compartments. In the same vein, AtClC-a (Hechenberger *et al.*, 1996), a NO_3^-/H^+-exchanger from the plant *Arabidopsis thaliana*, was shown to accumulate NO_3^- in vacuoles (De Angeli *et al.*, 2006). Thus, the pathologies observed with the loss of endosomal/lysosomal CLC transporters may be due in part to a changed chloride concentration in those compartments. Little information is available on a possible role of luminal chloride. For instance, the activity of

cathepsin C was shown to depend on the [Cl^-] (Cigic and Pain, 1999), and an endosomal calcium channel was reported to be inhibited by high luminal [Cl^-] (Saito *et al.*, 2007).

CONCLUSION AND OUTLOOK

Seventeen years after their molecular identification (Jentsch *et al.*, 1990), CLC chloride channels and transporters continue to be an exciting and ever more rapidly expanding field of research. Recent progress in crystallization of CLC proteins from bacteria, the finding that bacterial CLCs as well as vesicular CLCs represent Cl^-/H^+-exchangers rather than Cl^--channels, the elucidation of CLC pathologies and the identification of associated subunits represent major advances during the past few years. These important new insights will certainly trigger new exciting research into several directions. In the structure-function field, these include attempts to crystallize mammalian CLCs, to understand the structural differences between exchangers and channels, and to elucidate the mechanisms of common and single pore gating and the roles of CBS domains. On the functional level, elucidation of the regulation of CLC function and trafficking by protein networks and signal transduction cascades will be a major challenge. Last, but certainly not least, understanding the role of Cl^-/H^+-exchange by vesicular CLCs will be a fascinating area of research that may profoundly change our understanding of vesicular ion homeostasis and its role in many processes that are central to cell biology.

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