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Discovery of CLC transport proteins:

Cloning, structure, function and pathophysiology

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Abstract

After a personal description of the convoluted path leading 25 years ago to the molecular identification of the *Torpedo* Cl^- channel ClC-0 and the discovery of the CLC gene family, I succinctly describe general structural and functional features of these ion transporters before giving a short overview of mammalian CLCs. These can be categorized into plasma membrane Cl^- channels and vesicular Cl^-/H^+-exchangers. They are involved in the regulation of membrane excitability, transepithelial transport, extracellular ion homeostasis, endocytosis, and lysosomal function. Diseases caused by CLC dysfunction include myotonia, neurodegeneration, deafness, blindness, leukodystrophy, male infertility, renal salt loss, kidney stones and osteopetrosis, revealing a surprisingly broad spectrum of biological roles for chloride transport that was unsuspected when I set out to clone the first voltage-gated chloride channel.

Cloning of ClC-0 and identification of the CLC gene family

Exciting times broke in 1980s, when the first primary structures of ion channels and transporters were obtained by molecular cloning (Kopito & Lodish, 1985; Noda et al, 1986; Noda et al, 1982). We finally could 'see' proteins underlying ion transport, precisely manipulate their function, investigate their localization and structure, and determine their role in biology and disease. I joined Harvey Lodish's laboratory at the Whitehead Institute as postdoc in 1986 to clone the $NaHCO_3^-$ cotransporter, which I had been studying previously (Jentsch et al, 1984), by homology to the erythrocyte Cl^-/HCO_3^- exchanger that had just been cloned in his lab (Kopito & Lodish, 1985). We now know that both proteins are indeed distantly related, but there was no chance to clone 'my' cotransporter using low-stringency hybridization of phage libraries. I tried to broaden the search for anion transporters using DIDS and SITS, negatively charged stilbene derivates that inhibit and covalently bind many anion transporters. I reacted several cell lines with SITS and used antibodies against SITS to identify binding proteins in Western blots. As there were far too many bands to be followed up, I thought of model systems expressing anion transporters to very high levels – like band 3 in erythrocytes or the acetylcholine receptor (AChR) in the electric organ of the electric fish *Torpedo*, from which it had been cloned a few years earlier (Noda et al, 1982). That organ also expressed an exotic, DIDS-sensitive Cl^- channel (Miller & White, 1984) which had been found by Chris Miller while trying to reconstitute the AChR (White & Miller, 1979). Although having a weird 'double-barreled' single channel behaviour (Miller & White, 1984), this channel seemed a more worthwhile target than another anion exchanger. Neither K^+ nor Cl^- channels had yet been cloned by then (1986). Few physiologic studies had addressed Cl^- channels, which rather annoyed many electrophysiologists by obscuring cation

currents. However, a lack of Cl^- currents had been implicated in two genetic diseases, cystic fibrosis and myotonia. Hence, this uncharted territory promised to hold many novel biological insights and surprises.

In a first sample of *Torpedo* membranes obtained from Chris' nitrogen tank at nearby Brandeis University I detected a major broad SITS-labelled band. It resolved into two bands in fresher samples obtained from *Torpedo* shipped alive from California. One of these bands could be discarded (it was the α-subunit of the Na,K-ATPase, a warning sign!), but excitingly the other SITS-binding band was a disulphide-linked dimer – and the *Torpedo* channel appeared to have two pores! After purifying the protein and obtaining both N-terminal amino-acid sequence and specific antibodies, I pulled out overlapping clones from a cDNA library. Disappointingly, sequencing revealed only one strong candidate for a transmembrane domain. Although even total RNA from electric organ generated large Cl^- currents in *Xenopus* oocytes, nothing happened when I injected the cRNA encoding this SITS-binding protein (Jentsch et al, 1989). It might still have been a subunit of the channel, but antisense experiments in oocytes gave conflicting, and in the end negative, results. No homology was found to other proteins in the small DNA database available at that time, but now it appears to be a membrane-anchored glucosidase.

In the meantime, I had accepted an invitation to lead an independent research group at Hamburg University. I had been offered this 5-year position before it became clear that the SITS-binding protein was no Cl^- channel after all. Quite a stressful start into an independent career. I now really had to get that channel! I swore not to touch SITS again and started from scratch using expression cloning. Expression of precisely fractionated RNA from *Torpedo* electric organ in *Xenopus* oocytes indicated that the channel was encoded by a ~10 kb mRNA (Jentsch et al, 1990) that might encode a very large protein. Complete conversion of the mRNA into cDNA for 'positive' expression cloning appeared exceedingly difficult. Hence I turned to a cumbersome hybrid depletion approach in which we searched for cDNA clones that *delete* Cl^- channel-forming activity from electric organ RNA. In contrast to positive expression cloning, but similar to siRNA screens that we recently used to clone the volume-regulated anion channel VRAC (Voss et al, 2014), this approach allows for the identification of heteromultimeric channels if at least one subunit is not redundant. Since hybrid depletion requires a large molar excess of depleting DNA over RNA we used very small pools of clones instead of pool sizes of many hundreds that are possible with positive expression cloning. Single stranded DNAs derived from groups of just twelve individual clones picked from a highly size-selected cDNA library were hybridized to electric organ RNA and DNA-RNA hybrids removed by CsCl density ultracentrifugation. Reduction of Cl^- currents relative to those induced by acetylcholine (the *Torpedo* AChR served as internal control) was examined in the oocyte expression system (Fig. 1). After about two years, Klaus Steinmeyer and I finally isolated a *bona fide* full-length cDNA with a partial clone identified by this painstaking procedure. When injected into oocytes, cRNA derived

from that clone produced large Cl^- currents that showed the right kinetics and ion selectivity. Together with the hydropathy analysis of the predicted ~100 kDa protein, these results demonstrated that we had finally cloned the first voltage-gated Cl^- channel (Jentsch et al, 1990). Its primary structure did not resemble any other known protein. We later named it ClC-0 (Steinmeyer et al, 1991b) to highlight it as the founder of a ***Cl*** ***C***hannel gene family (Footnote 1). The ClC-0 cDNA was sufficient to reproduce the typical double-barreled single-channel appearance (Bauer et al, 1991), suggesting that we did not lack an important ancillary subunit.

Cloning of ClC-0 opened the way to identification of CLC proteins from mammals (Table I) and many other species and phylae. The past 25 years saw many exciting discoveries concerning associated β-subunits, structure and function, and physiology, pathology and human genetic disease. I will give at first a short introduction into general structural and functional features of CLC proteins, which will be addressed in more detail by other reviews in this issue. This is followed by a concise overview of mammalian CLC proteins and their roles in physiology and disease.

General features of CLC channels and ion exchangers

CLC proteins function as dimers with one ion translocation pathway per subunit. This arrangement explains the 'double-barreled' appearance of ClC-0 (Bauer et al, 1991; Miller, 1982). In single channel recordings of ClC-0 two conductances of equal magnitude are observed. They are gated independently by 'protopore gates' (also termed 'fast gate' in ClC-0), but can also be together closed by a common gate ('slow gate' in ClC-0). The dimeric nature of CLC proteins was substantiated by protein biochemistry (Middleton et al, 1994) and biophysical analysis of mutants (Ludewig et al, 1996; Middleton et al, 1996; Weinreich & Jentsch, 2001) and proven by X-ray structures of bacterial CLC Cl^-/H^+-exchangers (Dutzler et al, 2002). Since the translocation pathway is contained entirely within each subunit (Dutzler et al, 2002; Ludewig et al, 1996; Weinreich & Jentsch, 2001), a dimeric structure is, in principle, not required for ion transport. Indeed, a 'monomerized' ecClC-1 mutant retained ion transport activity (Robertson et al, 2010). It is not clear what advantage a dimeric structure confers to CLC transporters. It might bestow an increased spectrum of regulatory mechanisms, like the common gating that affects both subunits in CLC channels (Accardi & Pusch, 2000; Bauer et al, 1991; Miller, 1982) and transporters (Ludwig et al, 2013), or enable formation of heteromers with novel functions formation as observed *in vitro* with several mammalian CLC isoforms (Lorenz et al, 1996; Suzuki et al, 2006). From the perspective of pathology, the dimeric CLC structure allows for dominant negative effects with certain mutations of

CLCN1 in dominant myotonia (Steinmeyer et al, 1994) and of *CLCN7* in dominantly inherited osteopetrosis (Cleiren et al, 2001).

Each CLC monomer has 18 intramembrane helices, several of which do not span the membrane entirely (Dutzler et al, 2002). In eukaryotes the transmembrane block is followed by a large, intracellular carboxyterminus that contains two CBS (cystathionine-β-synthase) domains that bind each other and are in close contact with the intracellular face of the transmembrane block of the same subunit (Feng et al, 2010; Markovic & Dutzler, 2007; Meyer & Dutzler, 2006; Meyer et al, 2007; Schmidt-Rose & Jentsch, 1997). They also interact with the CBS domains from the partner monomer (Feng et al, 2010). Depending on the CLC isoform, these CBS domains can bind nucleotides like ATP (Bennetts et al, 2005; Markovic & Dutzler, 2007; Meyer & Dutzler, 2006; Meyer et al, 2007) and may regulate the common gating (Bennetts et al, 2005; Fong et al, 1998), a process that seems to be associated with a movement of these domains (Bykova et al, 2006; Ma et al, 2011). The carboxytermini of some CLC proteins can interact with other proteins like (in the case of ClC-5) ubiquitin ligases (Schwake et al, 2001), cofilin (Hryciw et al, 2003) or KIF3B (Reed et al, 2010), but the physiological relevance of these interactions remains unclear (Rickheit et al, 2010). Moreover, several mammalian CLCs interact with transmembrane proteins that are either obligatory β-subunits (Barttin for ClC-K channels (Estévez et al, 2001), Ostm1 for ClC-7 (Lange et al, 2006)) or may modulate their localization and function in only some tissues (GlialCAM for ClC-2 (Jeworutzki et al, 2012)).

Mutagenesis and crystal structures have identified the anion permeation pathway of CLC proteins. The first CLC crystal structure revealed the presence of a negatively charged glutamate side chain that apparently blocked the permeation pathway (Dutzler et al, 2002). Its position was occupied by a Cl^- ion upon mutation to glutamine, suggesting that this 'gating glutamate' is the structural basis of the 'protopore' gate observed in electrophysiology (Dutzler et al, 2003). Already before crystal structures were available, site-directed mutagenesis revealed the importance of this residue for rectification and gating (Friedrich et al, 1999; Waldegger & Jentsch, 2000) and of a Cl^- coordinating serine (Dutzler et al, 2002) for ion selectivity and single channel conductance (Ludewig et al, 1996). Subsequent mutations of the 'gating glutamate' in ClC-0 (Dutzler et al, 2003) and CLC exchangers (Leisle et al, 2011; Neagoe et al, 2010) likewise largely abolished the voltage-dependence of currents. However, channels lacking this glutamate still gate at the single channel level (Dutzler et al, 2003; L'Hoste et al, 2013), indicating that CLC gating cannot be explained entirely by the movement of its side chain. Voltage-gating of CLC channels depends on the Cl^- concentration. We proposed a reductionist model in which the gating charge is not provided by a charged intramembrane amino acid as in many cation channels, but by the permeant anion that feels the electrical field in the pore (Pusch et al, 1995a). Chloride may compete with the negative side chain of the 'gating glutamate' and thereby open the channel (Chen, 2003). Although channel

opening by Cl^- was invoked to explain the non-equilibrium gating of ClC-0 (Chen & Miller, 1996; Richard & Miller, 1990), the current model proposes that protonation of the 'gating glutamate' leads to channel opening which is accompanied by a (so far unmeasurable) proton flux (Lísal & Maduke, 2008). This protonation-dependent gating fits to the role of this glutamate residue in tightly coupled Cl^-/H^+-exchange by other CLC members.

It came as a shock that the bacterial EcClC-1 protein is not a well-behaved Cl^- channel, but rather a tightly coupled Cl^-/H^+-exchanger (Accardi & Miller, 2004). This seminal finding was quickly followed by the demonstration that mammalian ClC-4 and ClC-5 are also Cl^-/H^+-exchangers (Picollo & Pusch, 2005; Scheel et al, 2005) and that plant atClC-a is a NO_3^-/Cl^- antiporter (Bergsdorf et al, 2009; De Angeli et al, 2006). CLC anion/proton exchange is believed to rely on protonation of the 'gating glutamate', mutations in which convert the antiporter into a mere Cl^- conductance (Accardi & Miller, 2004; Bergsdorf et al, 2009; Leisle et al, 2011; Picollo & Pusch, 2005; Scheel et al, 2005). Protons reach the 'gating glutamate' from the cell interior through a path that diverges from that for chloride (Accardi et al, 2005). Proton transport of most, but not all (Feng et al, 2010), CLC exchangers depends on a 'proton glutamate' on the cytoplasmic side (Accardi et al, 2005; Zdebik et al, 2008).

Previously the main function of vesicular CLC 'channels' was seen in facilitating endosomal/lysosomal acidification by neutralizing H^+-ATPase currents (Günther et al, 1998; Günther et al, 2003). This notion seemed in doubt when mammalian vesicular CLCs were discovered to be Cl^-/H^+-antiporters rather than Cl^- channels. Naively, one thinks that these antiporters should rather shunt vesicular H^+-gradients. However, our reductionist model calculations (Fig. 2) revealed that they may rather acidify vesicles better than channels because $2Cl^-/H^+$ exchange shifts luminal potentials to more negative values (Weinert et al, 2010). Furthermore, H^+-driven, secondary active accumulation of Cl^- into vesicles may serve important, though unknown, physiological roles (Novarino et al, 2010; Weinert et al, 2010).

ClC-1, a Cl^- channel electrically stabilizing the skeletal muscle membrane

As the fish electric organ has developed from muscle, known to have a high Cl^- conductance since at least the 1950's (Hodgkin & Horowicz, 1959), we chose to isolate the first mammalian Cl^- channel from skeletal muscle. The Cl^- conductance of muscle, which exceeds K^+ conductance, stabilizes the muscle membrane voltage and aids in the repolarization of action potentials. Early work by Shirley Bryant and colleagues had indicated that muscle Cl^- conductance was reduced in goats (Lipicky & Bryant, 1966) and humans (Lipicky et al, 1971) with myotonia, a muscle stiffness caused by membrane hyperexcitability. Hence, a muscle Cl^- channel promised to be biologically and medically important. Indeed, shortly after identifying ClC-1 by homology cloning (Steinmeyer et al, 1991b), we found that a transposon had

destroyed the *Clcn1* gene in myotonic *adr* mice (Steinmeyer et al, 1991a) and identified a *CLCN1* mutation in human myotonia (Koch et al, 1992). This established myotonia congenita as one of the first known 'channelopathies'. We identified a *CLCN1* mutation in the family of Dr. Thomsen (Steinmeyer et al, 1994), who suffered from, and first described (Thomsen, 1876), the less severe dominantly inherited form of the disease (Thomsen disease). Heterozygous loss of *CLCN1* is not associated with myotonic symptoms. Therefore, mutant ClC-1 proteins of Thomsen disease patients must affect the function of the WT protein encoded by the non-mutated allele. We found that many ClC-1 mutants of patients with dominant myotonia shift the voltage-dependence of channel opening to non-physiological positive potentials and impose a similar, but variable, shift also on mutant/WT heteromers (Pusch et al, 1995b). This shift is owed to altered voltage-dependence of the common gate (Saviane et al, 1999). It results in a loss of function because ClC-1 will be now be largely closed at physiological voltages and can therefore neither repolarize action potentials, nor stabilize the resting voltage. Interestingly, abnormal splicing of *CLCN1* contributes to myotonia in myotonic dystrophy (Charlet et al, 2002; Mankodi et al, 2002) and in Huntington's disease (Waters et al, 2013), which are caused by trinucleotide repeats in different genes. A recent report that *CLCN1* polymorphisms may contribute to epilepsy (Chen et al, 2013) has met skepticism because ClC-1 shows very low expression in brain (Steinmeyer et al, 1991b) and since loss of ClC-1 function leads to myotonia, but not epilepsy.

The biophysics, structure/function relationship, regulation and pharmacology of ClC-1 has been studied extensively by ourselves and many other groups and will only be shortly mentioned here. ClC-1 is gated open by depolarization (Steinmeyer et al, 1991b) and displays the typical $Cl^- > I^-$ selectivity of CLC channels. It has a low single channel conductance of about 1 pS (Pusch et al, 1994; Saviane et al, 1999; Weinreich & Jentsch, 2001). Like ClC-0, it is 'double-barreled' and displays fast and slow gating relaxations (Accardi & Pusch, 2000). These correspond to 'protopore' and common gates like in ClC-0, although both ClC-1 gates are activated by depolarization (Saviane et al, 1999). Gating of ClC-1 is modulated by anions and pH (Rychkov et al, 1996; Rychkov et al, 1998). Interestingly, it is also modulated in a redox-dependent manner by intracellular ATP (Bennetts et al, 2007; Bennetts et al, 2005; Zhang et al, 2008) and β-nicotinamide adenine dinucleotide (Bennetts et al, 2012) through binding to the CBS domains in its cytoplasmic tail. This may provide a physiologically important coupling to muscle metabolism.

ClC-2, a widely expressed Cl^- channel with multiple roles

Shortly after ClC-1, we cloned ClC-2, an inwardly rectifying Cl^- channel present in almost all tissues (Thiemann et al, 1992). ClC-2 opens very slowly upon hyperpolarization (beyond ~-60 mV). Hypoosmotic cell swelling or moderately acidic extracellular pH decreased or abolished the inward rectification and thereby opened the channel (Gründer et al, 1992; Jordt & Jentsch, 1997). Residues involved in the slow voltage activation were identified in the amino-terminus (Gründer et al, 1992) and a positively charged intracellular loop (Jordt & Jentsch, 1997). Similar to ClC-0 and ClC-1, gating of ClC-2 is affected by extracellular (Pusch et al, 1999), and prominently also intracellular Cl^- (Niemeyer et al, 2004). ClC-2 has a single channel conductance of 2-3 pS (Weinreich & Jentsch, 2001). Although ClC-2 can be activated by cell swelling, its biophysical properties (e.g. the $Cl^->I^-$ selectivity (Thiemann et al, 1992)) clearly differ from the volume-regulated anion channel VRAC that displays a I>Cl selectivity sequence and that we recently found to be composed of LRRC8 heteromers (Voss et al, 2014). Whereas ablation (Voss et al, 2014) or partial knock-down (Qiu et al, 2014) of *LRRC8A* blocked or diminished, respectively, cell volume regulation, salivary gland cells from *Clcn2*$^{-/-}$ mice regulated their cell volume normally (Nehrke et al, 2002).

Clues for the physiological roles of ClC-2 were gleaned from *Clcn2*$^{-/-}$ mice that display early postnatal retinal and testicular degeneration (Bösl et al, 2001). We suggested that these pathologies result from disturbed extracellular ion homeostasis in the narrow spaces surrounding photoreceptors and germ cells. *Clcn2*$^{-/-}$ mice also develop leukodystrophy, with vacuoles slowly appearing in myelin sheaths of central axons (Blanz et al, 2007). Accordingly, nerve conduction velocity in the central auditory pathway was reduced. After the retraction of a widely cited publication (Haug et al, 2009) there is no convincing evidence for a role of ClC-2 in epilepsy (Blanz et al, 2007; Depienne et al, 2013; Niemeyer et al, 2010; Niemeyer et al, 2004). Recent results show that, like in mice, human *CLCN2* mutations rather result in leukodystrophy (Depienne et al, 2013) that can be associated with azoospermia (Di Bella et al, 2014).

ClC-2 was recently shown to bind to the cell adhesion molecule GlialCAM (Jeworutzki et al, 2012), which in turn binds a multiple membrane-spanning protein, Mlc1 (López-Hernández et al, 2011). Mutations in *GLIALCAM* or *MLC1* cause megalencephalic leukoencephalopathy with subcortical cysts (Leegwater et al, 2001; López-Hernández et al, 2011), suggesting a common pathophysiology with all three genes. GlialCAM anchors ClC-2 and Mlc1 to cell-cell junctions of transfected cells (Hoegg-Beiler et al, 2014; Jeworutzki et al, 2012; López-Hernández et al, 2011). Excitingly, co-expression of GlialCAM increased ClC-2 current amplitudes and almost abolished its rectification (Jeworutzki et al, 2012). The expression pattern of GlialCAM implies that it may affect ClC-2 only in glia. GlialCAM overexpression also changes the common gates of ClC-0, ClC-1, and ClC-K channels, but not of CLC Cl^-/H^+ exchangers (Jeworutzki et al, 2014). This observation is of biophysical interest

but lacks physiological consequences as glial expression of those channels is insignificant.

In line with *in vitro* data, we found that *Glialcam* disruption in mice affected the abundance and localization of both ClC-2 and Mlc1 in glial cells (Hoegg-Beiler et al, 2014). Unexpectedly, also *Mlc1* disruption also changed both GlialCAM and ClC-2 expression (Dubey et al, 2014; Hoegg-Beiler et al, 2014). Consistent with *in vitro* data, deletion of GlialCAM (or of Mlc1) reduced ClC-2 currents and introduced inward rectification in oligodendrocytes, but surprisingly not in cerebellar Bergmann glia that prominently co-express all three proteins (Hoegg-Beiler et al, 2014). The pathology resulting from GlialCAM and Mlc1 disruption cannot be explained only by reduced ClC-2 function beause mice lacking both ClC-2 and GlialCAM show more severe leukodystrophy than *Clcn2*$^{-/-}$ mice (Hoegg-Beiler et al, 2014).

ClC-2, GlialCAM and MLC1 are co-expressed at connections between oligodendrocytes and astrocytes, and at astrocytic endfeet that contact blood vessels (Blanz et al, 2007; Hoegg-Beiler et al, 2014; Jeworutzki et al, 2012). This localization resembles that of the K^+ channel Kir4.1 and the Cx47 gap junction protein (Blanz et al, 2007; Hoegg-Beiler et al, 2014), lack of which also cause leukodystrophy. These proteins are believed to have a role in K^+ siphoning (Wallraff et al, 2006), a process in which K^+ ions released from neurons are taken up by the glial syncytium and are equilibrated with serum at astrocytic endfeet. We postulated a similar role for ClC-2 in Cl^- siphoning that may be needed to electrically compensate the movement of K^+ (Blanz et al, 2007; Hoegg-Beiler et al, 2014). The linear voltage-dependence of ClC-2/GlialCAM channels allows Cl^- entry into glia when they are depolarized by the rise in $[K^+]_o$ during K^+ siphoning.

ClC-2 is also expressed in neurons where it might lower the cytoplasmic Cl^- concentration under certain circumstances (Földy et al, 2010; Rinke et al, 2010; Staley et al, 1996), although this notion has been questioned (Ratté & Prescott, 2011). A rise in $[Cl^-]_i$ above its electrochemical equilibrium may open ClC-2 by shifting its voltage-dependence to more positive potentials (Catalán et al, 2004; Pusch et al, 1999) and allow Cl^- to passively approach equilibrium values. When $[Cl^-]_i$ is lowered below its equilibrium by the K^+Cl^- cotransporter KCC2, closure of ClC-2 may prevent it from counteracting the effect of KCC2 on $[Cl^-]_i$.

A role of ClC-2 in transepithelial transport is not restricted to Sertoli cells and retinal pigment epithelial cells where it was invoked to explain the testicular and retinal degeneration of *Clcn2*$^{-/-}$ mice (Bösl et al, 2001), but has also been found in the intestine. ClC-2 is expressed in the basolateral membrane of colonic enterocytes (Catalán et al, 2002; Catalán et al, 2004) where it plays a role in Cl^- reabsorption (Catalán et al, 2012; Zdebik et al, 2004). This contrasts with the role of the apical Cl^- channel CFTR in Cl^- secretion. Indeed, we found that mice homozygous for the deleterious ΔF508 CFTR mutation survive *better* when ClC-2 is additionally disrupted (Zdebik et al, 2004).

ClC-K/barttin channels in renal and inner ear transepithelial ion transport

ClC-Ka and ClC-Kb (-K1 and –K2 in rodents) are highly homologous Cl^- channels (Adachi et al, 1994; Kieferle et al, 1994) that need barttin, a small β-subunit with two transmembrane domains, for full functionality (Estévez et al, 2001). Barttin (encoded by the *BSND* gene) is needed for channel activity, the transport of ClC-K to the plasma membrane (Estévez et al, 2001; Scholl et al, 2006; Waldegger et al, 2002), and for ClC-K protein stability *in vivo* (Nomura et al, 2011; Rickheit et al, 2008). Without barttin, only rat ClC-K1 gave currents that could be confirmed by rectification-changing mutagenesis (Waldegger & Jentsch, 2000). Exceptional within the CLC family, ClC-K channels lack a 'gating' glutamate and show little voltage-dependent gating. Insertion of such a glutamate introduces hyperpolarization-activated gating both in the absence and presence of barttin (L'Hoste et al, 2013; Waldegger & Jentsch, 2000). Mouse ClC-K1 has a 'double-barreled' appearance with a rather large single channel conductance of ~40 pS that is not changed by barttin co-expression (L'Hoste et al, 2013).

ClC-K proteins and barttin are almost exclusively expressed in the kidney and in the stria vascularis of the inner ear. In both renal (Kobayashi et al, 2001; Uchida et al, 1995; Vandewalle et al, 1997) and strial epithelia (Estévez et al, 2001; Rickheit et al, 2008) they reside in basolateral membranes, but ClC-K1 may additionally be apical in the thin limb of Henle's loop (Uchida et al, 1995). Mutations inactivating ClC-Kb cause the severe salt-losing nephropathy Bartter syndrome type III in humans (Simon et al, 1997), whereas disruption of mouse ClC-K1 caused a diabetes insipidus-like phenotype (Matsumura et al, 1999). Loss-of-function mutations in *BSND* underlie Bartter syndrome type IV that combines severe renal salt loss with congenital deafness (Birkenhäger et al, 2001). A rather benign missense mutation in BSND may underlie nonsyndromic hearing loss without renal symptoms (DFNB73; Riazuddin et al, 2009).

In the thick ascending limb (TAL), its main renal expression site, ClC-Kb/barttin provides the basolateral exit for Cl^- that is taken up from urine through the NaK2Cl-cotransporter NKCC2 (SLC12A1; mutated in Bartter I) (Fig. 3*A*). Na^+, which drives apical Cl^- uptake, is extruded basolaterally by the Na,K-ATPase, while K^+ is recycled apically through the ROMK (Kir1.1, KCNJ1) K^+-channel (mutated in Bartter II). As the TAL reabsorbs the bulk of filtered NaCl, *CLCNKB* mutations lead to severe congenital salt and fluid loss.

Clcnk1$^{-/-}$ mice show overt nephrogenic diabetes insipidus (Matsumura et al, 1999) owed to impaired solute accumulation in the inner medulla (Akizuki et al, 2001). Hence ClC-K1 (-Ka) appears crucial for the countercurrent system that creates the strongly hypertonic environment of the renal medulla required for urine concentration. Whereas humans with mutations in only *CLCNKA* have not yet been

described, there are a few patients with mutations in both *CLCNKA* and *CLCNKB* (Nozu et al, 2008; Schlingmann et al, 2004). Like patients with non-functional barttin, they display Bartter syndrome type IV that combines deafness with a particularly severe renal phenotype.

The stria vascularis is a multi-layered epithelium in the lateral wall of the cochlea. It generates a positive potential and a high K^+ concentration in the scala media, both of which are crucial for mechanotransduction currents in hair cells. Transport of ions across the most apical cell layer of the stria, the marginal cells, involves basolateral uptake of K^+ by the Na,K-ATPase and the NKCC1 NaK2Cl cotransporter (Fig. 3*B*). K^+ is secreted through apical KCNQ1/KCNE1 K^+ channels (loss of which cause deafness with cardiac arrhythmia), while Cl^- ions accumulated though NKCC1 are recycled basolaterally through ClC-Ka/barttin and ClC-Kb/barttin Cl^- channels (Rickheit et al, 2008). Disruption of only one of the ClC-K isoforms is compatible with hearing, but loss of both, or of their essential β-subunit barttin, leads to deafness (Birkenhäger et al, 2001; Schlingmann et al, 2004). Whereas constitutive barttin disruption entails early postnatal lethality due to renal salt and fluid loss (Rickheit et al, 2008), inner ear-specific *Bsnd* disruption revealed a breakdown of the endocochlear potential (Rickheit et al, 2008). Sensory outer hair cells showed anatomic degeneration over the first few postnatal weeks, but already showed functional impairment before these cells died, resulting in a hearing loss that was present from birth on, just like in humans with Bartter IV.

ClC-3 – a highly controversial endosomal Cl^-/H^+-exchanger

ClC-3 is the most controversial member of the CLC family. Several different plasma membrane Cl^- currents with mutually incompatible characteristics have been assigned to it. This prominently includes a purported role as volume-regulated anion channel VRAC (Duan et al, 1997), a claim sometimes repeated even today. However, VRAC currents were unaffected in our *Clcn3*$^{-/-}$ mice (Stobrawa et al, 2001), a finding confirmed in two other *Clcn3*$^{-/-}$ mouse models (Arreola et al, 2002; Gong et al, 2004). One might hope that the identification of LRRC8 proteins as essential VRAC components (Qiu et al, 2014; Voss et al, 2014) may finally convince the last proponents of the VRAC=ClC-3 hypothesis.

ClC-3 is expressed in almost all tissues. It is mainly found on endosomes (Hara-Chikuma et al, 2005b; Stobrawa et al, 2001; Suzuki et al, 2006) where it co-localizes partially with the late endosomal/lysosomal protein lamp1 (Stobrawa et al, 2001). It was also found on synaptic vesicles (SVs) (Salazar et al, 2004; Stobrawa et al, 2001) and synaptic-like microvesicles (Maritzen et al, 2008), but significant presence of ClC-3 on synaptic vesicles has been questioned recently (Schenck et al, 2009). The ClC-3B splice variant displaying a C-terminal PDZ-binding motif (Ogura et al, 2002) might localize to the Golgi (Gentzsch et al, 2003).

Most plasma membrane currents observed upon ClC-3 overexpression are probably carried by channels endogenous to the host cells. However, some groups (Guzman et al, 2013; Li et al, 2002; Matsuda et al, 2008; Picollo & Pusch, 2005) reported small strongly outwardly-rectifying Cl^- currents upon ClC-3 overexpression. They strongly resembled those of ClC-4 and ClC-5 (Friedrich et al, 1999; Steinmeyer et al, 1995) and were similarly affected by mutations of the gating glutamate (Li et al, 2002; Matsuda et al, 2008). Hence, they probably represent genuine ClC-3 currents. ClC-3 is most likely an intracellular voltage-dependent, electrogenic $2Cl^-/H^+$-exchanger (Guzman et al, 2013; Jentsch, 2008). However, because of its low transport rates at the plasma membrane, this could not yet been shown as convincingly as for ClC-4 through ClC-7 (Leisle et al, 2011; Neagoe et al, 2010; Picollo & Pusch, 2005; Scheel et al, 2005).

Similar to ClC-5 in renal proximal tubules (Günther et al, 2003; Hara-Chikuma et al, 2005a; Novarino et al, 2010; Piwon et al, 2000), ClC-3 may be important for endosomal acidification and chloride accumulation (Hara-Chikuma et al, 2005b). Unlike ClC-5, however, ClC-3 deletion did not impair renal endocytosis (Rickheit et al, 2010). Synaptic vesicles of *Clcn3*$^{-/-}$ mice showed impaired acidification *in vitro* (Stobrawa et al, 2001), but their reduced uptake of glutamate could be explained by reduced expression of the vesicular glutamate transporter vGlut1 (Stobrawa et al, 2001). It was suggested recently that SVs express only low amounts of ClC-3 (Schenck et al, 2009) and that vGlut1 itself provides a Cl^- conductance (Preobraschenski et al, 2014; Schenck et al, 2009). The impaired acidification of SVs from *Clcn3*$^{-/-}$ mice was therefore attributed to the reduction of vGlut1 in *Clcn3*$^{-/-}$ mice (Schenck et al, 2009) which may result from their severe neurodegeneration (Stobrawa et al, 2001). Amplitudes of miniature postsynaptic currents (mPSCs), which reflect the neurotransmitter contents of SVs, may reveal changes in vesicular neurotransmitter uptake which depends on the electrochemical potential of SVs as driving force. However, there is disagreement on whether mPSC amplitudes are changed in *Clcn3*$^{-/-}$ neurons (Guzman et al, 2014; Riazanski et al, 2011; Stobrawa et al, 2001). More work is needed to sort out these contradictory results.

Disruption of ClC-3 in mice leads to neuronal degeneration in the retina and brain that eventually leads to a dramatic loss of the hippocampus (Stobrawa et al, 2001) and degeneration of other brain areas (Dickerson et al, 2002; Stobrawa et al, 2001; Yoshikawa et al, 2002). Signs of pathological lysosomal storage were found in one study (Yoshikawa et al, 2002), but it was much milder than in *Clcn6*$^{-/-}$ (Poët et al, 2006) or *Clcn7*$^{-/-}$ mice (Kasper et al, 2005). The mechanism by which ClC-3 disruption leads to neurodegeneration remains unclear. It should also be noted that *Clcn3*$^{-/-}$ mice are systemically sick and display e.g. reduced body weight. Possible effects on e.g. insulin secretion might therefore not be a β-cell intrinsic consequence of ClC-3 disruption and require cautious interpretation (Jentsch et al, 2010; Maritzen et al, 2008).

ClC-4 – an endosomal Cl^-/H^+ exchanger with a possible role in brain development

ClC-4 is widely expressed in many tissues (Jentsch et al, 1995; van Slegtenhorst et al, 1994). ClC-4 is a strongly voltage-dependent $2Cl^-/H^+$-exchanger (Picollo & Pusch, 2005; Scheel et al, 2005) that is present on endosomes and, in heterologous expression, to a small degree on the plasma membrane (Mohammad-Panah et al, 2003; Suzuki et al, 2006). However, others reported a localization in the endoplasmic reticulum (Okkenhaug et al, 2006). Unfortunately no knock-out controlled immunohistochemistry is available for ClC-4. ClC-4 was suggested to function in endosomal acidification and trafficking (Mohammad-Panah et al, 2003). However, whereas *Clcn5*$^{y/-}$ mice display impaired proximal tubular endocytosis (Piwon et al, 2000) (see below), no such defect is seen in *Clcn4*$^{-/-}$ mice which also lack other obvious phenotypes (Rickheit et al, 2010).

Human genetics suggests that loss of ClC-4 impacts on brain function. Whereas *Clcn4* resides on chromosome 7 in inbred house mice, human *CLCN4* is located on the X chromosome (Rugarli et al, 1995). A patient carrying a deletion of the X-chromosome encompassing *CLCN4* and many other genes displayed severe psychomotor delay (Meindl et al, 1993) and a *de novo* loss-of-function mutation (G544R) was identified in a patient displaying severe early-onset epilepsy and delayed development (Veeramah et al, 2013). More convincingly, five families with different *CLCN4* mutations were identified in a screen for genes underlying X-linked intellectual disability (Hu et al, 2015). These mutations reduced ClC-4 currents in heterologous expression. Cultured neurons derived from *Clcn4*$^{-/-}$ mice, or primary neurons subjected to *Clcn4* shRNA knock-down, displayed moderately reduced neurite outgrowth and branching (Hu et al, 2015). Defective intracellular trafficking may underlie these disturbances.

ClC-5 – an endosomal Cl^-/H^+ exchanger crucial for renal endocytosis

CLCN5 was discovered in a search for genes underlying Dent's disease (Fisher et al, 1994), an X-linked renal disorder associated with low molecular weight proteinuria and variable presence of kidney stones, nephrocalcinosis, and renal failure (Wrong et al, 1994). ClC-5 is a $2Cl^-/H^+$-exchanger (Picollo & Pusch, 2005; Scheel et al, 2005) that is most highly expressed in renal and other epithelia (Steinmeyer et al, 1995; Vandewalle et al, 2001). Like ClC-4, upon heterologous expression ClC-5 partially reaches the plasma membrane where it can be studied electrophysiologically (Friedrich et al, 1999; Steinmeyer et al, 1995). Both transporters activate almost instantaneously upon strong depolarization (~+30 mV) and lack measurable tail currents. Although a small proportion of ClC-5 can be detected in the apical brush-border membrane of renal proximal tubular cells, the majority of the protein is located in apical endosomes in renal (Günther et al, 1998; Wartosch et al, 2009) and intestinal epithelia (Vandewalle et al, 2001). The plasma membrane expression of ClC-5 can be increased by mutating a PY motif located between both CBS domains

and that can interact with ubiquitin ligases (Hryciw et al, 2004; Schwake et al, 2001). However, disrupting this motif had no effect *in vivo* (Rickheit et al, 2010).

The pathogenesis of Dent's disease has been elucidated by knock-out (Günther et al, 2003; Piwon et al, 2000; Wang et al, 2000) and knock-in (Novarino et al, 2010; Rickheit et al, 2010) mouse models. These mice display proteinuria resulting from impaired proximal tubular endocytosis (Piwon et al, 2000; Wang et al, 2000) that is a cell-autonomous consequence of *Clcn5* disruption (Novarino et al, 2010; Piwon et al, 2000). Both receptor-mediated and fluid-phase endocytosis is affected, and the endocytic retrieval of apical membrane proteins like NaPi-IIa is slowed (Novarino et al, 2010; Piwon et al, 2000). The decreased expression of the apical scavenger receptor megalin (Piwon et al, 2000), which may result from impaired recycling, exacerbates the urinary loss of ligands like vitamin D binding protein or parathyroid hormone (PTH). The ensuing increased *tubular* PTH concentration may stimulate apical PTH receptors and thereby reduce the apical expression of the resorptive phosphate transporter NaPi-IIa (SLC34A1) (Piwon et al, 2000). The resultant phosphaturia contributes to the formation of kidney stones in Dent's disease. Many patients with *CLCN5* mutations display proteinuria, but lack hypercalciuria and kidney stones (Sekine et al, 2014). Likewise, the *Clcn5*$^{y/-}$ mice of (Wang et al, 2000), but not those generated in our laboratory (Piwon et al, 2000), displayed hypercalciuria. We attributed these different outcomes to opposing effects of *Clcn5* disruption on vitamin D levels. In *Clcn5*$^{y/-}$ proximal tubules, increased luminal PTH stimulates the conversion of the precursor 1(OH)-D_3 into the active form 1,25$(OH)_2$-D_3, but the levels of both the precursor and the active form are reduced by urinary loss. This delicate balance can result in a decrease or increase of serum 1,25$(OH)_2$-D_3, which may decrease or increase, respectively, intestinal absorption of Ca^{2+} and its subsequent renal excretion (Piwon et al, 2000). Hence hyperphosphaturia, hypercalciuria and kidney stones are secondary to a primary defect in renal endocytosis.

We hypothesized that impaired endocytosis is caused by impaired acidification of endosomes (Günther et al, 1998). Endosomal acidification was indeed reduced in vesicle preparations (Günther et al, 2003; Novarino et al, 2010) or cell cultures (Hara-Chikuma et al, 2005a) from *Clcn5*$^{y/-}$ kidneys. However, *Clcn5*$^{y/unc}$ mice (Novarino et al, 2010), in which we *unc*oupled Cl^- from H^+-countertransport by the E211A 'gating glutamate' mutation (Scheel et al, 2005), displayed normal proximal tubular endosomal acidification, but impaired tubular endocytosis that was as severe as in *Clcn5*$^{y/-}$ mice (Novarino et al, 2010). A similar uncoupling mutation (E211Q) was recently identified in a patient with Dent's disease (Sekine et al, 2014). Hence impaired endocytosis in *Clcn5*$^{y/-}$ and *Clcn5*$^{y/unc}$ mice cannot be attributed only to reduced endosomal acidification, but to changes of additional parameters like luminal Cl^- concentration or transmembrane voltage of endosomes (Weinert et al, 2010). Furthermore, the normal expression and localization of the mutant ClC-5 protein in *Clcn5*$^{y/unc}$ mice (Novarino et al, 2010) suggests that in *Clcn5*$^{y/-}$ mice a lack of interactions of ClC-5 with other proteins is not a major pathogenic factor.

ClC-6 – a mainly neuronal late endosomal Cl^-/H^+-exchanger

Together with ClC-7, ClC-6 forms the third branch of the CLC family (Brandt & Jentsch, 1995). Although the *Clcn6* mRNA is found in many tissues (Brandt & Jentsch, 1995), the ClC-6 protein is predominantly expressed in the nervous system (Poët et al, 2006). ClC-6 resides in endosomes of cultured cells (Ignoul et al, 2007; Suzuki et al, 2006) and of neurons *in situ* (Poët et al, 2006) and partially co-localizes with the late endosomal/lysosomal protein lamp1. Subcellular fractionation of brain membranes revealed that ClC-6, like ClC-3, is present in endosomal rather than lysosomal fractions (Poët et al, 2006).

The late endosomal localization of ClC-6 precluded its biophysical characterization for many years. Only recently we found that a GFP-ClC-6 fusion protein reaches the plasma membrane. It mediates electrogenic Cl^-/H^+-exchange as confirmed by point mutations in the 'gating glutamate' and an ion-selectivity changing mutation in the Cl-coordinating serine (Neagoe et al, 2010).

Clcn6$^{-/-}$ mice display a peculiar form of lysosomal storage disease in which intracellular deposits localize mainly at axon hillocks (Poët et al, 2006). Unlike *Clcn7*$^{-/-}$ mice, this storage disease progresses slowly, is not associated with significant neuronal cell loss, and causes little microglial activation (Poët et al, 2006; Pressey et al, 2010). The loss of ClC-6 did not change lysosomal pH. *Clcn6*$^{-/-}$ mice show mild behavioural abnormalities (Poët et al, 2006). This includes a reduction in pain sensitivity that correlates with strong lysosomal storage disease in dorsal root ganglion neurons. We considered *CLCN6* as a candidate gene for mild forms of human neuronal ceroid lipofuscinosis, but found only two heterozygous missense mutations in 2 out of 75 NCL patients (Poët et al, 2006). It may just be a matter of time until convincing *CLCN6* mutations are found in human NCL.

ClC-7/Ostm1 – a lysosomal Cl^-/H^+-antiporter crucial for brain and bone integrity

ClC-7 needs Ostm1, a highly glycosylated type I transmembrane protein, for ion transport activity (Leisle et al, 2011) and protein stability *in vivo* (Lange et al, 2006). ClC-7 is the only lysosomal CLC protein, as shown by subcellular fractionation (Poët et al, 2006) and immunohistochemistry of transfected and native cells (Kasper et al, 2005; Kornak et al, 2001; Lange et al, 2006; Suzuki et al, 2006). In bone-degrading osteoclasts, ClC-7/Ostm1 and the H^+-ATPase are inserted by lysosomal exocytosis into the ruffled border membrane that faces the acidic resorption lacuna (Kornak et al, 2001; Lange et al, 2006). Whereas ClC-7 traffics to lysosomes also without Ostm1, Ostm1 needs ClC-7 for lysosomal targeting, processing, and stability (Lange et al, 2006).

The lysosomal localization of ClC-7/Ostm1 complicated the characterization of its transport properties. Isolated lysosomes display Cl^-/H^+-exchange activity (Graves et al, 2008; Weinert et al, 2010) that was reduced in the absence of ClC-7 (Weinert et al, 2010). The identification of sorting signals in the ClC-7 N-terminus allowed the engineering of point mutants which partially localize to the plasma membrane (Stauber & Jentsch, 2010) where they can be analysed biophysically (Leisle et al,

2011). The mutant reaches the plasma membrane also without Ostm1, but needs the β-subunit Ostm1 for ion transport activity (Leisle et al, 2011). ClC-7/Ostm1 rectifies as strongly in the outward direction as ClC-4 through ClC-6, but in contrast to those transporters it activates very slowly (within seconds) upon depolarization. Deactivation of ClC-7/Ostm1 is also slow, permitting measurements of tail currents. These revealed that macroscopic current rectification is caused by voltage gating of an electrogenic exchange process with an almost linear intrinsic voltage-dependence (Leisle et al, 2011). Reversal potentials of tail currents established a $2Cl^-/1H^+$ exchange stoichiometry for ClC-7. Gating of ClC-7/Ostm1 involves a common gate (Ludwig et al, 2013). Intriguingly, several *CLCN7* mutations found in human osteopetrosis accelerate ClC-7/Ostm1 gating, suggesting that the slow gating of ClC-7 is physiologically important (Leisle et al, 2011).

The physiological roles of ClC-7 became apparent from KO mice (Kornak et al, 2001) which display severe osteopetrosis, retinal degeneration and neurodegeneration associated with lysosomal storage (Kasper et al, 2005). *Grey-lethal* mice that carry a mutation in the β-subunit Ostm1 display an almost indistinguishable phenotype (Chalhoub et al, 2003; Lange et al, 2006; Pressey et al, 2010). Likewise, total loss of either ClC-7 or Ostm1 function leads to severe infantile osteopetrosis in humans (Chalhoub et al, 2003; Kornak et al, 2001) which is probably associated with neurodegeneration as well (Frattini et al, 2003). Certain *CLCN7* missense mutations cause autosomal dominant osteopetrosis that is clinically more benign and lacks CNS involvement (Cleiren et al, 2001; Frattini et al, 2003).

Clcn7$^{-/-}$ mice die at around 6 weeks of age and show neuronal cell loss predominantly in the hippocampus (Kornak et al, 2001). Mice with forebrain-specific *Clcn7* disruption lived much longer and almost completely lost neurons in areas lacking ClC-7 (Wartosch et al, 2009). *In vivo* pulse-chase experiments revealed that protein degradation in proximal tubular cells lacking ClC-7 is impaired in a cell-intrinsic manner (Wartosch et al, 2009). The enlargement of lamp1-positive compartments in tubular cells lacking ClC-7, however, is not owed to protein accumulation as evident from cells in which protein uptake was impaired by ClC-5 disruption (Wartosch et al, 2009). This observation suggests a role of ClC-7 in vesicular trafficking or fusion processes. It was tempting to speculate that impaired protein degradation and lysosomal storage resulted from a less acidic lysosomal pH. However, careful ratiometric measurements showed normal lysosomal pH of mice lacking ClC-7/Ostm1 repeatedly (Kasper et al, 2005; Lange et al, 2006; Weinert et al, 2010). This can be rationalized by the presence of a lysosomal cation conductance that obviates the need for the ClC-7/Ostm1 conductance in neutralizing H^+-ATPase currents (Steinberg et al, 2010; Weinert et al, 2010). As expected for a pH-gradient driven transport of Cl^- by ClC-7, lysosomal Cl^- concentration was decreased in KO mice (Weinert et al, 2010).

Unlike the normal pH of *Clcn7*$^{-/-}$ lysosomes (Kasper et al, 2005; Lange et al, 2006; Weinert et al, 2010), the resorption lacuna of cultured *Clcn7*$^{-/-}$ osteoclasts was less acidic (Kornak et al, 2001). This fits to osteopetrosis because an acidic pH is required both for the dissolution of inorganic bone material and the activity of secreted proteases. Indeed, mutations in the a3 subunit of the lysosomal H^+-ATPase also cause osteopetrosis (Kornak et al, 2000; Scimeca et al, 2000). The failure of *Clcn7*$^{-/-}$ osteoclasts to acidify the resorption lacuna may be attributed to the lack of neutralizing currents, or to the underdevelopment of the ruffled border observed in electron microscopy (Kornak et al, 2001; Weinert et al, 2014). This underdevelopment may be a consequence of reduced lysosomal exocytosis.

Two other *Clcn7* mouse models were generated to elucidate the respective biological roles of ClC-7 ion transport and protein-protein interactions. Like *Clcn5*$^{y/unc}$ mice (Novarino et al, 2010), *Clcn7*$^{unc/unc}$ mice (Weinert et al, 2010) carry a 'gating glutamate' mutation that converts ClC-7 into a mere anion conductance, whereas in *Clcn7*$^{td/td}$ mice (Weinert et al, 2014) its ion transport was abolished by a mutation in the 'proton glutamate' (Leisle et al, 2011) (td: transport-deficient). Like *Clcn7*$^{-/-}$ mice, both mouse models had unchanged lysosomal pH but increased lysosomal Cl^- concentration. Both new mouse models were osteopetrotic and displayed lysosomal storage (Weinert et al, 2014; Weinert et al, 2010), although with different severities. Compared to the total KO, osteopetrosis was less severe in *Clcn7*$^{unc/unc}$ mice and neurodegeneration less severe in *Clcn7*$^{td/td}$ mice. We concluded that ClC-7 Cl^-/H^+-exchange cannot be functionally replaced by a mere Cl^- conductance, but that the ClC-7^{unc} Cl^- conductance partially rescues the osteopetrotic phenotype (Weinert et al, 2010). While the mere presence of the (non-transporting) ClC-7^{td} protein ameliorates neurodegeneration, a lysosomal Cl^- conductance seems detrimental (Weinert et al, 2014). Intriguingly, *Clcn7*$^{-/-}$ and *Ostm1*$^{-/-}$ mice, but neither *Clcn7*$^{unc/unc}$ nor *Clcn7*$^{td/td}$ mice display grey fur in an *agouti* background (Weinert et al, 2014). This suggests that ClC-7 protein-protein interactions, rather than ClC-7 ion transport, is needed for melanocyte function.

Outlook

Twenty-five years after the discovery of ClC-0 we look back on many exciting discoveries concerning their structure, function, and amazingly diverse physiological and pathological roles as described in more than two thousand papers. Cl^- channels have emerged from the dark ages and we now appreciate their diverse functions in the cell and the organism. Moreover, Cl^- channels have provided refreshing insights into diverse ways to build ion channels and transporters and the fine line separating them. More surprises are likely to follow.

Footnote 1

We proposed to indicate, if appropriate, the species by prefixes and individual isoforms by suffixes after a dash (e.g. hClC-1 for the human skeletal muscle Cl^- channel, atClC-a and ecClC-1 for particular CLCs from *Arabidopsis thaliana* and *E. coli*, respectively), but this nomenclature is not followed by everybody in the field. The official human gene nomenclature (HUGO) is *CLCN1*, *CLCN2* etc.

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Figure 1. Cloning of the *Torpedo* channel ClC-0 by hybrid depletion

Total electric organ RNA that was hybrid-depleted with single stranded DNA derived from pools of 12 clones from a highly size-selected cDNA library was expresed in *Xenopus* oocytes. Current 'fingerprints' were obtained using a symmetrical voltage clamp-protocol (*A*, inset) and recorded by a chart recorder. After current response had increased to steady-state magnitudes (due to opening of the slow gate), the response to low chloride was recorded at depolarizing potentials. Subsequent superfusion with acetylcholine (ACh) probed for the expression of the *Torpedo* AChR that was used as internal reference to avoid false positives due to RNA degradation. *A*, background currents in non-injected oocytes, no response to ACh. *B*, negative pool of clones that shows normal Cl^- channel and AChR expression. *C*, positive pool which contained a partial ClC-0 cDNA; reduction of Cl^- current with normal response to ACh. *D*, expression of full-length ClC-0 cRNA; large Cl^- currents and no response to ACh. Oocytes were measured in ND96 (in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$), except for the low chloride pulse (7 mM Cl^-). AChR currents were elicited by 1 mM acetylcholine in the presence of 10 µM atropine to block muscarinic receptors. Modified from (Jentsch et al, 1990).

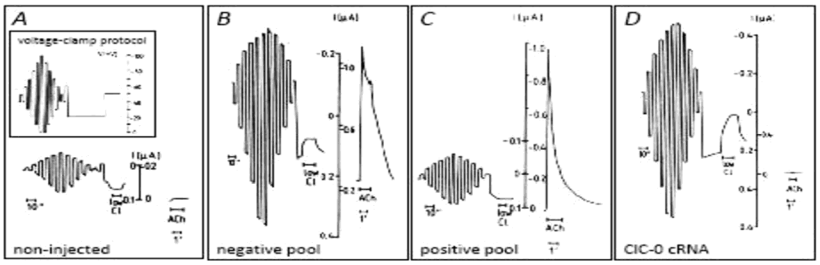

Figure 1

Figure 2. Modeling vesicular acidification with Cl^- channels and Cl^-/H^+-exchangers

Reductionist model calculations revealing differential effects of Cl^- channels or $2Cl^-/H^+$-exchangers on the acidification of vesicles (taken from (Weinert et al, 2010)). ATP is virtually added at t=0.

(-) model vesicles containing only a proton pump and a proton leak nearly instantaneously reach a high luminal potential (*B*) that is given by the energy supplied by ATP hydrolysis. Virtually no acidification occurs (*A*).

(unc) model vesicles containing additionally a Cl^- channel, as in the classical model of vesicular acidification and as realized in *Clcn5*$^{unc/y}$ and *Clcn7*$^{unc/unc}$ mice (Novarino et al, 2010; Weinert et al, 2010), acidify their lumen (*A*) and accumulate Cl^- (*C*). They reach a more moderate inside-positive potential (*B*).

(WT) model vesicles containing instead of a Cl^- channel a $2Cl^-/H^+$-exchanger (CLC antiport) rather surprisingly reach a more acidic steady-state pH than those containing a Cl^- channel (*A*). This is related to the fact that they reach a more negative luminal potential (*B*). They also accumulate more Cl^- (*C*) as expected from H^+-diven uptake of Cl^-.

For equations and parameter used see (Weinert et al, 2010).

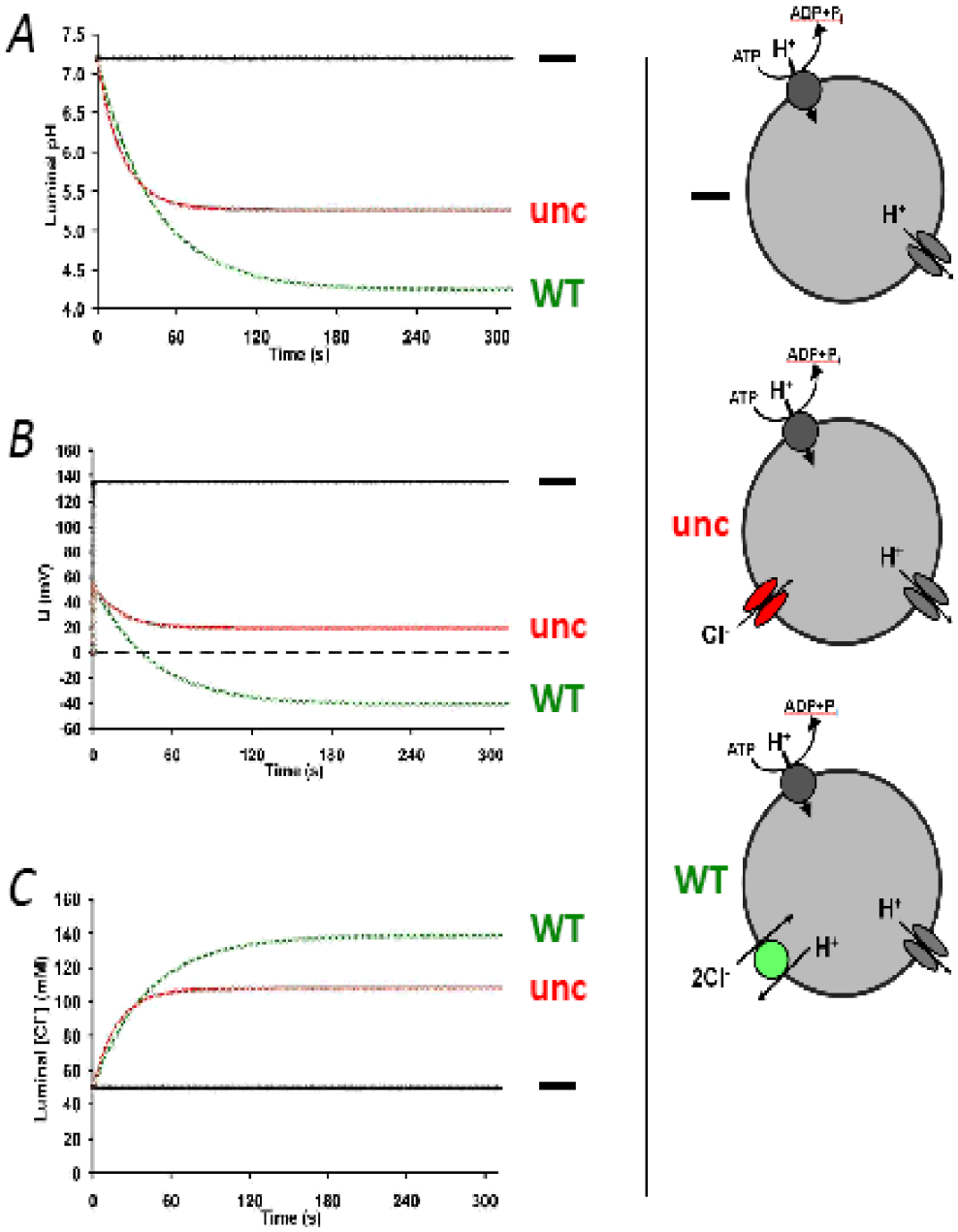

Figure 2

Figure 3. Role of ClC-K/barttin channels in transepithelial transport

A, Schematic diagram of NaCl reabsorption in the thick ascending limb (TAL) of Henle's loop, and (*B*) of K^+ secretion by marginal cells in the stria vascularis of the inner ear. Taken from (Estévez et al, 2001).

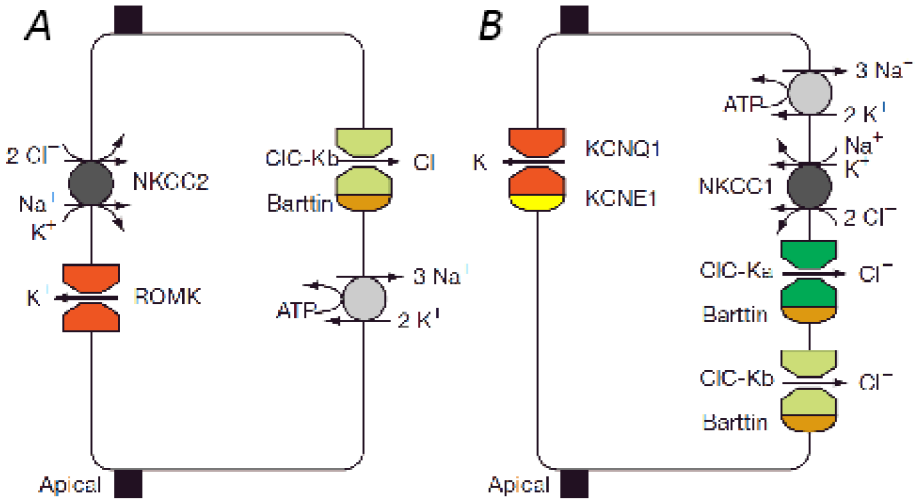

Figure 3

Table I: The CLC family of chloride channels and antiporters in mammals.

Table summarizing the expression patterns of mammalian CLC proteins, their established or presumed functions, and pathologies resulting from loss-of-function mutations in humans and mice. Associated β-subunits are given in red. Barttin and Ostm1 are obligatory β-subunits of both ClC-K isoforms and of ClC-7, respectively. Loss of barttin leads to Bartter syndrome IV that is associated with massive renal salt loss and deafness. The glial cell adhesion molecule GlialCAM can associate with ClC-2 and change its localization and properties in glial cells. It does not qualify as essential β-subunit. GlialCAM mutations lead to a distinct form of leukodystrophy. The HUGO gene names are *CLCN1* - *CLCN7*, *CLCNKA*, *CLCNKB*, *HEPACAM* (for GlialCAM), *BSND* (for barttin), and *OSTM1.* NCL, neuronal ceroid lipofuscinosis.