Pflugers Arch - Eur J Physiol (2009) 458:23–37
DOI 10.1007/s00424-008-0597-z

ION CHANNELS, RECEPTORS AND TRANSPORTERS

Physiological roles of CLC Cl^-/H^+ exchangers in renal proximal tubules

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Received: 31 July 2008 / Revised: 22 September 2008 / Accepted: 26 September 2008 / Published online: 14 October 2008
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Abstract The CLC gene family encodes Cl^- channels or Cl^-/H^+ exchangers. While our understanding of their structure–function relationship has greatly benefited from the crystal structure of bacterial homologues, human inherited diseases and knock-out mice were crucial in deciphering their physiological roles. Several vesicular CLC Cl^-/H^+ exchangers are expressed in the proximal tubule (PT). ClC-5 mutations cause Dent's disease which is associated with low molecular weight proteinuria and kidney stones. ClC-5 knock-out mice revealed impaired endocytosis as the primary defect in Dent's disease. It extends to receptor-mediated and fluid-phase endocytosis and entails changes in calciotropic hormones that result in kidney stones. No renal functions could be assigned so far to ClC-3 and ClC-4, which are also expressed in PTs. Loss of ClC-7 or its β-subunit Ostm1 entails lysosomal storage in the PT, in addition to the neuronal lysosomal storage and osteopetrosis that are the hallmarks of ClC-7/Ostm1 loss in mice and men.

Keywords Vesicular pH · Hyperphosphaturia · Vitamin D · Megalin · Gene disruption · CLCN5

Introduction into the structure and function of CLC Cl^- channels and transporters

Originally cloned from the electric organ of *Torpedo* [1], which was known to contain high concentrations of a peculiar Cl^- channel from reconstitution experiments [2, 3], CLC genes were subsequently identified in virtually all organisms with the exception of some bacteria. For instance, *Escherichia coli* possesses two different isoforms, the yeast *S. cerevisiae* just one, while seven have been found in the plant *Arabidopsis thaliana* and nine in mammals. CLC proteins either transport Cl^- (or other anions) across biological membranes by passive diffusion in a channel-like manner, or exchange Cl^- (or other anions like NO_3^-) for H^+. CLCs invariably display a "double-barrel" architecture in which two identical or homologous subunits associate with each other to form a dimer. Importantly, each of the two monomers has its own permeation pathway. The "double-barreled" organization was first deduced from single channel recordings from reconstituted native [2, 3] or cloned [4] ClC-0. At least in that channel, each pore can close and open independently from the other one (this is called protopore gate or "fast" gate because of its kinetics in the *Torpedo* channel), but both pores can also be closed together by a common gate (which is slow in ClC-0). Biophysical analysis of mutants assembled to concatemers proved this concept [5, 6] and demonstrated that each ion translocation pathway is entirely contained within a single monomer of the dimer [7]. The homodimeric architecture was beautifully confirmed by the crystal structure of bacterial CLC proteins [8], which gave extremely important insights into the structure and function of these fascinating molecules. These high-resolution structures revealed a complex arrangement of 17 twisted membrane-embedded α-helices per subunit with a twofold axis of symmetry perpendicular to the membrane plane [8]. Both subunits contact each other in a wide interface. Two internal and an external Cl^- binding sites were identified on each subunit [8]. A negative side chain of a glutamate occupies the external Cl^- binding site in the crystal, thereby

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blocking the permeation pathway. This glutamate plays a crucial role in the gating of CLC channels by the permeant anion [9, 10], as well as for the coupling of H^+ to Cl^- flux [11–13]. Neutralizing this "gating" glutamate almost abolishes gating of several CLC channels and converts exchangers into pure anion conductances. Gating of ClC-0 can be driven by transmembrane pH gradients [14], which provide a conceptual link to the Cl^-/H^+ exchange of other CLCs.

Mammalian CLCs can assemble to either homodimers or heterodimers with their close homologues in heterologous expression systems [7, 15, 16]. In vivo heteromerization has been demonstrated only for ClC-4 and ClC-5 [17], but the physiological relevance of these findings remains unclear. Levels of ClC-3 and ClC-4 mRNAs and proteins were not significantly changed in kidneys lacking ClC-5 [18] (and own unpublished observations). CLCs also possess extended cytoplasmatic carboxytermini. In eukaryotic CLCs as well as in CLCs from some *archae*, they contain two copies of the so-called cystathionine beta-synthase (CBS) domains. Some, but not all CBS domains of CLC proteins can bind adenosine triphosphate (ATP) and other nucleotides [19–22]. Such a binding may or may not influence gating of CLC channels, with data being sometimes controversial [23–25]. An effect on slow gating was also observed with chimeras within the C-terminus of ClC-0 [26], and with several point mutations in CBS domains [27]. These domains, which form intramolecular dimers that bind to the CBS dimers of the second subunit of the CLC dimer, may also be important for trafficking [27, 28]. At least some mammalian CLCs require accessory β-subunits, which are important for trafficking to their target membrane, for gating or for protein stability [29–31]. Barttin associates specifically with ClC-K isoforms, whereas Ostm1 associates only with ClC-7.

Roles of CLC proteins in physiology and disease

The nine mammalian CLCs can be ordered based on sequence homology (Fig. 1). The first cluster contains ClC-1, ClC-2, and the kidney- and inner ear-specific, highly homologous ClC-Ka and ClC-Kb isoforms. Members of this branch are plasma membrane chloride channels. They are important for establishing the appropriate conductance of skeletal muscle, for the transport of ions across several epithelia, or probably for the homeostasis of extra or intracellular ion concentrations [32–38]. Loss of function mutations in those channels or in barttin, the common β-subunit of both ClC-K isoforms, causes severe diseases in men and mice. These

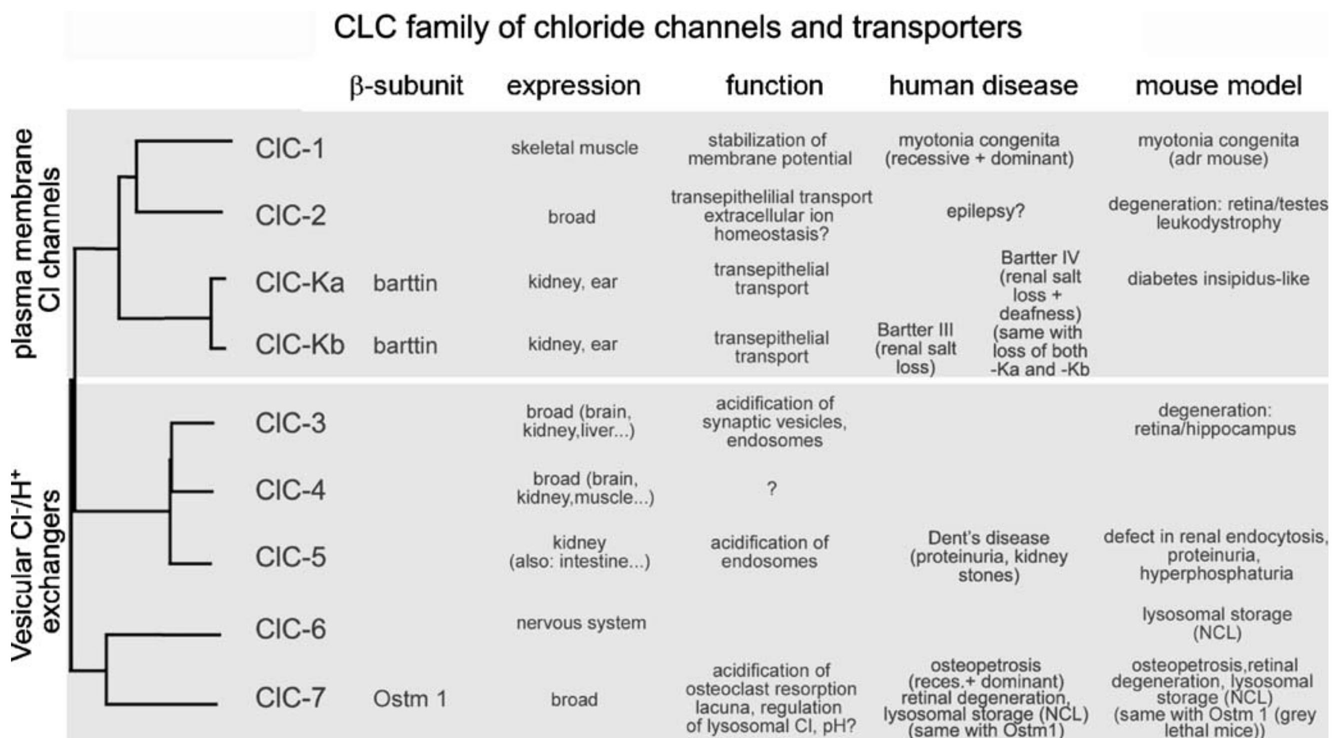

Fig. 1 Overview of the mammalian CLC family of chloride channels and transporters, indicating known β-subunits, tissue distribution, and human and mouse pathologies observed upon the disruption of the respective gene. The combined disruption of both ClC-Ka and ClC-Kb causes Bartter syndrome type IV, a disorder which is more frequently observed with loss-of-function mutations of the common β-subunit barttin

include myotonia, leukoencephalopathy, blindness, infertility, deafness, and renal salt loss [32–34, 39, 40].

The other two clusters of the CLC tree contain ClC-3 to ClC-5 and ClC-6 and ClC-7, respectively (Fig. 1). Those CLCs are mainly located in intracellular compartments from the endosomal–lysosomal pathway and, in the case of ClC-3 [41], also on synaptic vesicles [38, 42]. Owing to their predominantly intracellular localization, not all members of these two branches have been characterized biophysically. ClC-4 and ClC-5, which reach the plasma membrane to some degree, were shown to function as electrogenic Cl^-/H^+ exchangers [12, 13] similar to bacterial ecClC-1 [11]. Currents resembling those of ClC-4 and ClC-5 were reported for ClC-3 [12, 43], but expression levels were too low to determine H^+-transport [12]. As ClC-3 to ClC-7 all contain a particular "proton" glutamate that seems to be a hallmark of CLC Cl^-/H^+ exchangers, ClC-3, ClC-6, and ClC-7 are thought to be antiporters as well. Accordingly, a subcellular liver fraction enriched for lysosomes was recently shown to display $2Cl^-/H^+$ exchange activity [44]. It seems reasonable to assume that this exchange is mediated by ClC-7/Ostm1, which, in contrast to other CLC proteins, is prominently expressed on lysosomes [30, 45, 46].

It had been proposed that intracellular CLCs provide an electrical shunt to facilitate vesicle acidification by the H^+-ATPase [42, 47]. Synaptic vesicles lacking ClC-3 [41] and endosomes lacking ClC-3 [48] or ClC-5 [49, 50] displayed reduced luminal acidification. In contrast, the lysosomal pH of cells lacking ClC-7 or Ostm1 was not changed [30, 46], although the resorption lacuna of osteoclasts failed to acidify in ClC-7 knock-out (KO) mice [45]. In the "classical" picture of vesicular acidification (Fig. 2, left), the current of the electrogenic H^+-ATPase is shunted by a Cl^- channel. This picture, however, must now be changed in view of the Cl^-/H^+ exchange activity of vesicular CLCs (Fig. 2, right). These highly electrogenic exchangers will still facilitate acidification, but more ATP is needed to reach the same value of pH [42]. The most important difference, however, may be the direct coupling of Cl^- to pH gradients that predicts a larger luminal accumulation of chloride than with an uncoupled anion conductance. This concept is in accord with the function of AtClC-a from *A. thaliana* which accumulates nitrate in plant vacuoles [51].

Mutations in some intracellular CLCs cause human diseases. Loss of ClC-5 leads to a renal disorder called Dent's disease [52, 53] and disruption of ClC-7 or of its β-subunit Ostm1 causes osteopetrosis and lysosomal storage disease [30, 45, 46]. In addition, the disruption of ClC-3 in mice led to severe neurodegeneration [41, 54, 55], while that of ClC-6 entailed a mild form of lysosomal storage disease in the nervous system [56].

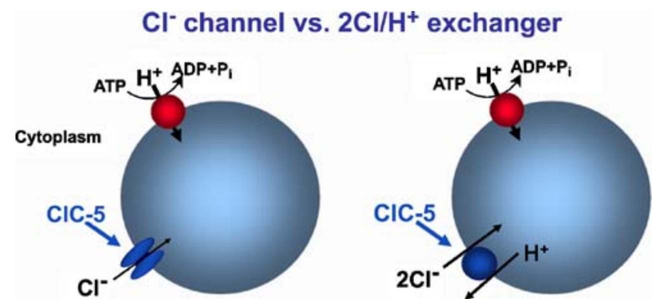

Fig. 2 Schematic drawing of a vesicle (endosome) that coexpresses ClC-5 and the proton pump (V-type H^+-ATPase). *Left*, the "classical" Cl^- channel hypothesis: ATP hydrolysis is used to actively pump H^+ into the vesicle, leading to the generation of an inside-positive voltage that has to be dissipated by the influx of chloride that was believed to occur through a Cl^- channel. *Right*, charge neutralization is not provided by a Cl^- channel, but by the Cl^-/H^+ exchanger ClC-5 that may have a $2Cl^-/1H^+$ stoichiometry similar to the bacterial EcClC-1. However, this remains to be demonstrated. With this coupling ratio, a transport cycle of ClC-5 would transport three negative charges into the vesicular lumen. Electroneutrality requires that this is accompanied by the pumping of three protons by the H^+-ATPase. Thus, one out of three pumped protons leaves the vesicle, implying that 50% more metabolic energy is needed to achieve the same value of pH

This review focuses on the physiological roles of CLC proteins in kidney proximal tubules. Among the vesicular CLCs, ClC-3, ClC-4, ClC-5, and ClC-7, but not ClC-6 [56] could be detected in that highly endocytic epithelium. The "kidney isoforms" ClC-Ka and ClC-Kb [57] and their β-subunit barttin [29] are prominently expressed in the thin and thick loop of Henle and intercalated cells of the distal nephron [29, 58–60] and in the inner ear [29], but not in the proximal tubule. Although the broadly expressed chloride channel ClC-2 is expressed in the kidney [61] and has been detected in proximal tubules [62–64], its renal function is unclear. No renal abnormalities were reported for ClC-2 KO mice [36, 40, 65, 66]. The ClC-1 chloride channel is highly specific for skeletal muscle [67].

ClC-5 and ClC-7, vesicular Cl^-/H^+ exchangers important for proximal tubular function

The importance of CLC proteins for the function of the proximal tubule has been illustrated by Dent's disease [53] and by knock-out mouse models for ClC-5 [68, 69] and ClC-7/Ostm1 [30, 45, 46]. The Cl^-/H^+ exchanger ClC-5 plays a crucial role in proximal tubular endocytosis. Its loss changes intrarenal hormonal balance and metabolism, thereby leading to severe secondary effects like kidney stones and nephrocalcinosis that are a hallmark of Dent's disease. The loss of ClC-7/Ostm1, by contrasts, leads to an accumulation of lysosomal storage material in the PT [30, 46].

Expression pattern and structure–function of ClC-5

ClC-5 is prominently expressed in renal and intestinal epithelia [70, 71] and can be found in other tissues to a minor degree. In the kidney, ClC-5 is mainly present in proximal tubule cells and in acid-secreting α- and base-secreting β-intercalated cells of the collecting duct [47, 72]. Minor amounts of ClC-5 were also detected in the thick ascending limb [73]. In both proximal tubules and intercalated cells, the majority of ClC-5 resides on intracellular vesicles in the apical cell pole where it colocalizes with the V-type H^+-ATPase [47, 71]. In part depending on the fixation procedure, minor amounts of ClC-5 can also be detected on the highly infolded brush-border plasma membrane of proximal tubules. In transfected cells, ClC-5 is similarly predominantly expressed on intracellular vesicles [47]. Colocalization with endocytosed proteins and its recruitment to the large vesicles formed by coexpression with the constitutively active Q79L mutant of rab5 identified these as endosomes [47]. Also in transfected cells, some ClC-5 protein was detected at the plasma membrane, compatible with the notion that it reaches that membrane in a recycling pathway. This plasma membrane expression, which was confirmed by an immunological detection of inserted extracellular epitopes into ClC-5 [74], facilitated the study of its biophysical properties. Currents mediated by ClC-5 and its close homologue ClC-4 (which are now known to be mediated by Cl^-/H^+ exchange [12, 13]) closely resemble each other. Similar currents were also seen with ClC-3 [43]. These currents are strongly outwardly rectifying and become apparent only at voltages more positive than about +20 mV [70, 75]. Upon stepping to these voltages, the largest component of the resulting outward current appears instantaneously, with a smaller component being activating within tens of milliseconds. The strong rectification of ClC-4 or ClC-5 currents poses a dilemma, as it predicts that these antiporters should be nearly inactive at the physiological voltages being present at the plasma membrane and endosomes (which are thought to be inside-positive due the activity of the electrogenic proton pump). There are several hypotheses to solve this problem: either a small, almost undetectable activity at negative voltages suffices for their physiological function, or their voltage dependence is altered in native cells by some so far unknown modulation or protein interaction [42]. Finally, there might be situations in which vesicular voltages, which are poorly known, reverse. Currents of ClC-4 and ClC-5 were inhibited by extracellular (intraluminal) acidic pH and displayed a $NO_3^- > Cl^- > Br^- > I^-$ conductance sequence. Ion permeabilities could not be deduced from reversal potentials, however, owed to the strong current rectification. Therefore, this technique could not be used to test for a possible coupling of Cl^- to other ions, a method that led to the discovery that the *E. coli* ecClC-1 is a $2Cl^-/H^+$ exchanger rather than a Cl^- channel [11]. Motivated by those findings, Picollo and Pusch [12] and our group [13] showed independently that ClC-4 and ClC-5 are Cl^-/H^+ exchangers as well. These results were obtained from simultaneous measurements of either intra- or extracellular pH and currents upon expression in either *Xenopus* oocytes or cultured cells [12, 13]. As current reversal potentials cannot be detected, however, the exact stoichiometry of coupling could not be determined. It seems likely that it is $2Cl^-/1H^+$ as in ecClC-1.

Neutralization of the key “gating glutamate” residue abolished rectification [75] and coupling of anion flux to proton countertransport in ClC-4 and ClC-5, resulting in pure Cl^- conductances [12, 13]. Mutating another glutamate (the “proton glutamate”) that is located close to the cytoplasmic surface to non-protonatable residues abolished Cl^-/H^+ exchange [76]. Neutralizing additionally the “gating glutamate” yielded uncoupled Cl^- currents, suggesting that the supply of protons to the central exchange site located at the “gating glutamate” proceeds through this “proton glutamate” that is not located in the Cl^- permeation pathway and that was first identified in ecClC-1 [77]. Furthermore, the analysis of concatemers in which one wild-type (WT) subunit forms a dimer with subunit mutated in either of these crucial glutamate residues revealed that Cl^-/H^+ exchange is carried out independently by each subunit [76]. Noise analysis of ClC-5, which gave results similar to ClC-4 [78], suggested that this exchange takes place in bursts that resemble the gating of channels [76].

Loss of ClC-5 leads to human Dent’s disease

Loss of function mutations in ClC-5 underlie Dent’s disease [52, 53], an X-linked renal Fanconi-like syndrome characterized by low molecular weight (LMW) proteinuria, hyperphosphaturia, hypercalciuria, and aminoaciduria. The clinically more important symptoms are kidney stones, nephrocalcinosis, and rickets [79]. Occasionally, these symptoms lead to renal insufficiency and therefore might require kidney transplantations. Although many of these symptoms are variable, LMW proteinuria is consistently present.

More than 80 different *CLCN5* mutations have been reported among patients with Dent’s disease. Some of these mutations truncate the protein, resulting in a complete loss of function. Several human missense mutations were studied in heterologous expression and were found to abolish or severely reduce ClC-5 currents. In several cases, this reduction could be attributed to impaired plasma membrane expression as a result of defective intracellular trafficking [80–82]. Many missense mutations are clustered

at or near the interface of the dimer [83]. The reason for this clustering remains unclear. In principle, such mutations may impair the association of ClC-5 monomers to functional dimers, or might affect the function of fully assembled dimers.

ClC-5 KO mice as models for Dent's disease reveal defective endocytosis as basic defect

The mechanisms underlying the pathology of Dent's disease were elucidated using ClC-5 KO mouse models [68, 69]. These KO mice exhibit the most common feature of Dent's disease, namely LMW proteinuria, but differ quantitatively in other aspects like vitamin D metabolism and hypercalciuria. LMW proteins (<70 kDa) are normally filtered by the glomerulus into the primary urine and are almost completely recaptured by receptor-mediated endocytosis in proximal tubule cells. The selective loss of LMW proteins in the ClC-5 KO indicates a defect in proximal tubular endocytosis, a hypothesis consistent with the localization of ClC-5 on apical endosomes in the PT [47, 72]. Indeed, in vivo endocytosis experiments revealed defective receptor-mediated as well as fluid-phase endocytosis by proximal tubule cells [68] (Fig. 3a). Reduced endocytosis was later confirmed in primary cell cultures derived from KO proximal tubules [84]. Piwon et al. [68] demonstrated a cell autonomous impairment of endocytosis by analyzing the endocytosis rate in chimeric tubules in which cells expressing or lacking ClC-5 can be studied side-by-side within the same tubule. Such chimeric tubules are found in heterozygous females because *Clcn5* is located on X-chromosomes, one of which is always inactivated in female cells in a random fashion. Injection of fluorescently labeled proteins or dextran clearly showed a reduction of both receptor-mediated as well as fluid-phase endocytosis in cells lacking ClC-5 (Fig. 3a), with receptor-mediated endocytosis being more severely impaired. This might be explained by an approximately twofold, cell autonomous reduction in the expression of the multiligand receptor megalin in cells lacking ClC-5 [68]. Immunohistochemistry showed a narrower band of megalin staining in KO cells [68], which is probably owed to a diminished expression in the apical brush border as revealed by immunogold studies in electron microscopy [85] (Fig. 3b). Megalin is an apical multiligand scavenger receptor, which, together with its associated co-receptor cubulin, is crucial for receptor-mediated endocytosis of a broad range of substances in the proximal tubule [86–88]. After ligand binding, megalin is internalized. It is recycled back to the plasma membrane after its ligand has dissociated in an acidic endosomal compartment. Protein ligands are destined for lysosomal degradation, but other substances like vitamin D, which is co-endocytosed with its binding protein, are recovered. The cell autonomous decrease in megalin expression in cells devoid of ClC-5 and its changed subcellular localization might be explained by defective recycling of the receptor back to the plasma membrane. As might be expected for a co-receptor closely associated with megalin, cubulin protein expression is also reduced in PTs from ClC-5 KO mice [85]. Megalin-dependent apical endocytosis is a major pathway for delivering lysosomal enzymes like cathepsin B to proximal tubular lysosomes [89]. As a consequence, the intracellular concentrations of lysosomal enzymes are reduced in PTs lacking either megalin or ClC-5 [89]. In contrast to megalin KO mice, however, the proximal tubular endocytotic apparatus of *Clcn5*$^{-/-}$ mice shows no morphological alterations at the electron microscopical level [85]. Thus, there is no reason to suspect that the defect in PT endocytosis in ClC-5 KO mice is owed to gross structural changes of the endosomal/lysosomal system.

Piwon et al. [68] also observed a reduced rate of endocytosis of apical plasma membrane transporters in KO PT cells. Immunohistochemistry showed a significant delay in the parathyroid hormone (PTH)-induced relocation of the apical Na^+-phosphate cotransporter NaPi-2a and the apical Na^+/H^+ exchanger NHE3 to intracellular vesicles. Taken together, these results point to an important and broad role of ClC-5 in proximal tubule endocytosis. But how does the lack of a chloride-proton exchanger impair endocytosis?

The colocalization of ClC-5 with the proton pump in apical endosomes of PT cells suggested a role of ClC-5 in vesicular acidification [47, 72]. Indeed, analysis of either renal cortical endosomes in suspension, or of primary cultures of proximal tubular cells that had taken up ion-sensitive dyes by endocytosis, showed that disruption of ClC-5 led to reduced luminal acidification and chloride accumulation [49, 50]. The role of ClC-5 in endosomal acidification was at first viewed in terms of the "classical model" of vesicular acidification (Fig. 2, left). In this model, a chloride channel is needed to neutralize the currents of the electrogenic proton pump that transports protons into the vesicle. Without an electric shunt, the V-type H^+-ATPase would create a large voltage across the vesicular membrane that would inhibit further pumping. Because chloride is needed for proper acidification of vesicles [90], it was assumed that vesicular chloride channels are responsible for providing neutralizing anion currents for this pump. ClC-5 is a very good candidate for mediating this current, as indicated by its endosomal localization and the observed impairment of endosomal acidification. However, it is now known that ClC-5 is not a chloride channel, but rather an electrogenic chloride proton exchanger [12, 13]. This observation initially caused some

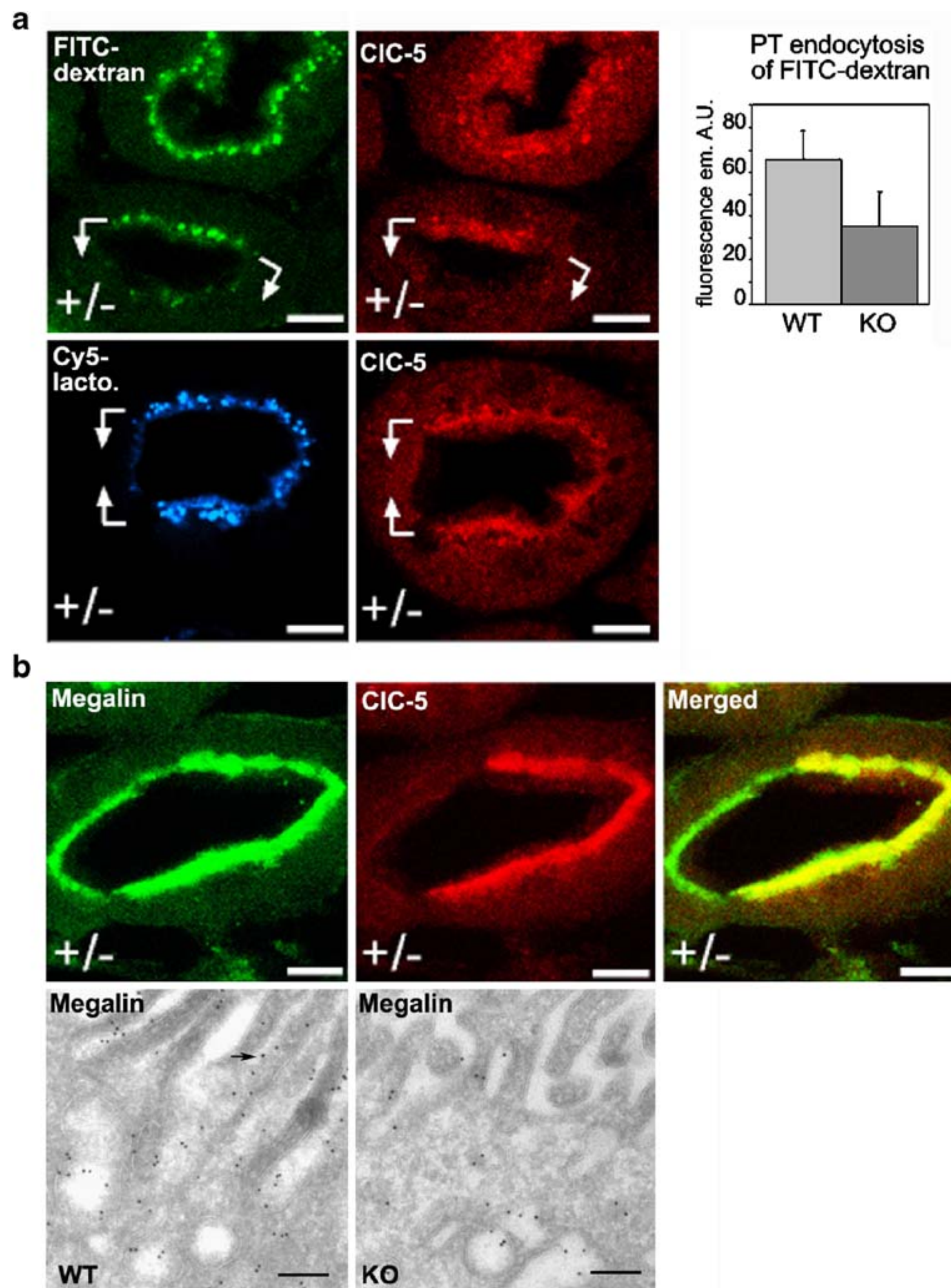

Fig. 3 **a** Impaired endocytosis in proximal tubules of *Clcn5*$^{y/-}$ male knock-out mice and heterozygous *Clcn5*$^{+/-}$ females. *Upper panel*, uptake of FITC-dextran in a chimeric PT from a *Clcn5*$^{+/-}$ female 7 min after intravenous injection of the coupled dye, *left*, and ClC-5 protein in the same tubules in *red*, *middle*. Cells lacking ClC-5 are shown between *arrows*. They display a (cell autonomous) impairment of endocytosis. *Lower panel*, similar experiment with an injection of Cy5-β-lactoglobulin to monitor receptor-mediated endocytosis. Taken from Piwon et al. [68]. *Right*, diagram showing the effect of ClC-5 disruption on fluid-phase endocytosis. WT and *Clcn5*$^{y/-}$ KO mouse kidneys in anaesthetized mice were allowed to endocytose FITC-dextran for 15 min, followed by extensive perfusion to wash out as much extracellular FITC-dextran as possible. Kidneys were then homogenized and FITC fluorescence determined spectroscopically. As it is impossible to remove all extracellular FITC-dextran, the difference shown underestimates the difference in fluid-phase endocytosis. **b** Altered expression of the apical scavenger receptor megalin in cells lacking ClC-5. *Upper panel*, a PT from a *Clcn5*$^{+/-}$ female stained for megalin (*green*) and ClC-5 (*red*) and merged. Megalin staining is reduced cell autonomously in cells lacking ClC-5 and the staining is less broad. Taken from Piwon et al. [68]. *Lower panel*, immunogold labeling of megalin on ultrathin frozen sections of renal cortices. *Arrows* indicate labeling at brush border, which is much reduced in *Clcn5* KO as compared with WT mice. Taken from Christensen et al. [85]. *Bars* indicate 10 μm for cryosections or 0.2 μm for ultrathin sections. Genotypes are indicated in the *lower left corner*

confusion concerning its involvement in endosomal acidification, as one might think that protons that are imported into endosomes in an energy-consuming process are free to leave them again via ClC-5. However, ClC-5 being a highly electrogenic exchanger, it can still electrically compensate currents of the H^+-ATPase. Assuming a $2Cl^-/1H^+$ stoichiometry, ClC-5 would transport three negative charges into the vesicular lumen, requiring the ATPase to transport three H^+ ions for electroneutrality. Hence, only one out of three protons imported by the H^+-ATPase will leave the vesicle through ClC-5. In comparison to the channel model, more metabolic energy is needed for acidification. Importantly, owed to the direct coupling of chloride gradients to vesicular pH gradients, ClC-5 might have a role in accumulating chloride in endosomes, resembling the function of atClC-a, which accumulates NO_3^- in plant vacuoles [51]. Another possible role of ClC-5 could be a direct, H^+-ATPase-independent acidification of early endosomes just after they bud off from the plasma membrane. Because the concentration of chloride should be initially close to 120 mM, the chloride concentration of normal extracellular space, and because cytoplasmic [Cl^-] is in the range between 10 and 40 mM, the vesicular Cl^- gradient might drive luminal proton uptake through ClC-5. This transport direction also fits well to the strong outward rectification of ClC-5. In the absence of mechanisms accumulating chloride in the lumen of those vesicles, such a direct, proton-pump-independent acidification by CLC proteins may only work in early endosomes because the vesicular chloride concentration would decrease along the endocytic pathway.

Progressive acidification along the endocytic pathway is important in several respects. Many receptor–ligand interactions are destabilized by acidic pH, enabling receptor recycling, with detached ligands being targeted to lysosomes. Both the formation of vesicular intermediates between early and late endosomes [91], as well as the binding of certain regulatory proteins like ARNO and Arf6 to endosomal membranes [92] is dependent on an acidic luminal pH. Recent data suggest that a certain subunit of the V-type H^+-ATPase plays a role in signaling acidic luminal pH to the external surface of the vesicular membrane where those factors bind [93]. When vesicular acidification is disturbed by specific inhibitors of the H^+-ATPase or by weak bases, the overall rate of endocytosis goes down [94, 95].

Hyperphosphaturia, hypercalciuria, and kidney stones as indirect consequences of impaired endocytosis

The proteinuria observed in Dent's disease patients and in ClC-5 KO mice is a consequence of impaired proximal tubular endocytosis that may be ascribed to impaired acidification of renal endosomes. But what is the molecular mechanism leading to hyperphosphaturia and kidney stones? Dent's disease patients as well as both ClC-5 KO mouse models show elevated urinary phosphate concentrations [68, 69, 79]. Under normal circumstances, the bulk of the filtered phosphate is reabsorbed by proximal tubules through the apical sodium-coupled phosphate transporter NaPi-2a [96]. Consistent with the hyperphosphaturia detected in ClC-5 KO animals, immunohistochemistry revealed reduced brush-border expression of NaPi-2a in most segments of KO proximal tubules, with the exception of early S1 segments [68]. Heterozygous females (*Clcn5*$^{+/-}$) that are chimeras for the expression of ClC-5 indicated that this relocation and degradation was not cell autonomous. An explanation for this observation is given by the parathyroid hormone-dependent regulation of NaPi-2a (Fig. 4a). Elevated PTH levels reduce the brush-border expression of NaPi-2a by stimulating its internalization and lysosomal degradation [97]. Piwon et al. [68] measured normal serum PTH concentrations in KO animals, whereas urinary PTH levels were elevated like in patients with Dent's disease [98]. PTH is a small peptide hormone which is filtered by the glomerulus into the primary urine, endocytosed by proximal tubule cells in a megalin-dependent manner, and later on degraded in lysosomes [99]. Due to impaired endocytosis in ClC-5 KO animals, PTH is not properly reabsorbed by proximal tubule cells and therefore accumulates in later nephron segments. This leads to an excessive stimulation of apical PTH receptors in the late proximal tubule, triggering the internalization of NaPi-2a from the brush-border membrane and ultimately increasing urinary phosphate concentrations. In early parts of the S1 segment, PTH concentration should reflect that of the serum, explaining the unchanged apical localization of NaPi-2a in that segment [68]. In summary, the hyperphosphaturia observed in patients with Dent's disease and in ClC-5 KO mice can be explained as an indirect consequence of impaired endocytosis.

The likely mechanism underlying the more variable symptoms in Dent's disease and ClC-5 KO mice, i.e., hypercalciuria and kidney stones, is more complicated. It involves PTH and vitamin D_3, hormones crucial for calcium homeostasis (Fig. 4b). Proximal tubular cells are essential for vitamin D metabolism. They convert the inactive precursor 25(OH)-vitamin D_3 to the active compound $1,25(OH)_2$-vitamin D_3, a reaction carried out by the mitochondrial enzyme 25(OH)-vitamin D_3-1α-hydroxylase. In addition, both the precursor and the active hormone can be hydroxylated to largely inactive metabolites by the vitamin D_3 24-hydroxylase that is also expressed in the PT. Both enzymes, the vitamin D_3 activating and degrading one, are regulated by PTH, with the transcription of the 25

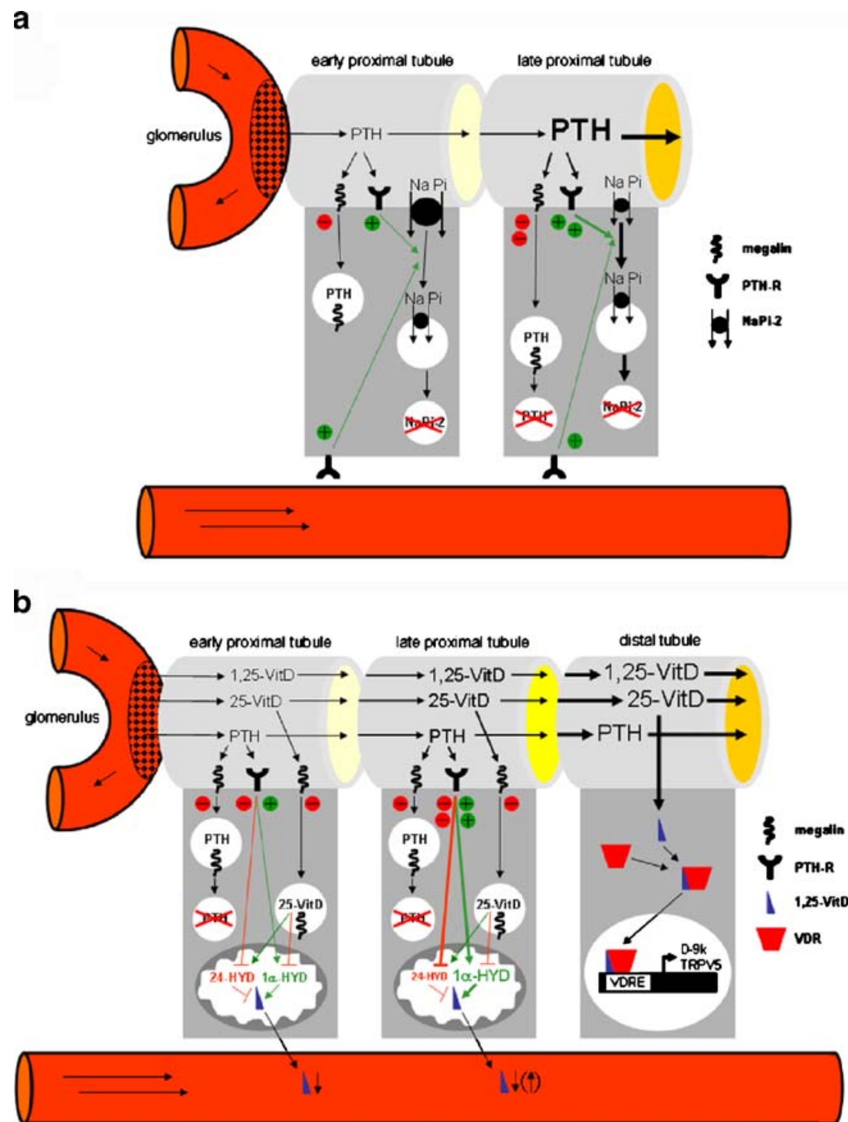

Fig. 4 Schematic drawing of the alteration of calciotropic hormone metabolism in proximal tubules of ClC-5 KO kidneys. **a** The defect in ClC-5-dependent endocytosis, which is further exacerbated by the secondary decrease in the apical scavenger receptor megalin, results in a progressive increase in luminal PTH concentration along the length of proximal tubules. This leads to an enhanced stimulation of luminal PTH receptors in later proximal tubule segments, resulting in increased endocytosis and lysosomal degradation of the apical NaPi-2 cotransporter. This in turn causes the phosphaturia observed in the KO mouse and in patients with Dent's disease. **b** 25(OH)$_2$-VitD$_3$, another key player in calcium homeostasis, is converted in the proximal tubule to its active form 1,25(OH)$_2$-VitD$_3$ by the mitochondrial enzyme 1α-hydroxylase. In serum, the precursor is bound to its carrier protein vitamin D binding protein (*DBP*). This rather small protein is filtered into the primary urine where it is normally endocytosed in a process that requires megalin and ClC-5. Elevated PTH in more distal segments of the proximal tubule stimulates the expression of 1α-hydroxylase. As the transcription of the activating enzyme 1α-hydroxylate is inhibited, and that of the inactivating enzyme 24-hydoxylase is stimulated by 1,25(OH)$_2$-VitD$_3$, the impaired endocytosis of VitD$_3$ bound to DBP that is observed in the KO reduces the concentration of this transcriptional regulator in PT cells. The resulting enzymatic changes should increase the levels of the active hormone 1,25(OH)$_2$-VitD$_3$, but this is counteracted by the decreased endocytic uptake of the precursor. In the ClC-5 KO, the synthesis of 1,25(OH)$_2$-VitD$_3$ thus depends on the balance of reduced supply and increased enzymatic activity. When this results in increased levels of serum 1,25(OH)$_2$-VitD$_3$, the resulting stimulation of intestinal calcium absorption must be balanced by increased renal calcium excretion, favoring the formation of kidney stones. In the distal tubule, 1,25 (OH)$_2$-VitD$_3$ enters by passive diffusion over the plasma membrane, binds the vitamin D$_3$ receptor which heterodimerizes with the RXR transcription factor to activate the transcription of 1,25(OH)$_2$-VitD$_3$-dependent genes (e.g., TRPV5, D-9k)

(OH)-vitamin D$_3$-1α-hydroxylase being augmented and the messenger RNA (mRNA) stability of the vitamin D$_3$ 24-hydroxylase being decreased [100–102]. Indeed, as predicted by increased urinary PTH concentrations in the ClC-5 KO, 25(OH)-vitamin D$_3$-1α-hydroxylase mRNA and protein levels are drastically elevated in KO mice, whereas vitamin D$_3$ 24-hydroxylase mRNA is markedly decreased [18, 49, 103]. Another, potentially even more important

factor for the changed expression levels of these enzymes is their regulation by the active hormone 1,25(OH)$_2$-vitamin D$_3$. The transcription of 25(OH)-vitamin D$_3$-1α-hydroxylase is inhibited, and that of vitamin D$_3$ 24-hydroxylase activated, by the active hormone. Both genes contain vitamin D$_3$ responsive elements in their promoter region [101, 102]. In proximal tubular cells, the main uptake route for vitamin D$_3$ metabolites (which are bound to their cognate binding protein) is megalin-dependent apical endocytosis [87]. As this uptake is severely decreased in proximal tubules lacking ClC-5, these cells will experience abnormally low intracellular levels of 1,25(OH)$_2$-vitamin D$_3$ and hence will upregulate the activating and downregulate the inactivating enzyme.

These changes of vitamin D$_3$ metabolizing enzymes would tend to increase vitamin D$_3$ serum levels in KO animals, as indeed observed in most patients with Dent's disease [79, 104]. However, the opposite was the case in ClC-5 KO mice from our lab [18, 68]. Only the ratio of 1,25(OH)$_2$-vitamin D$_3$ to 25(OH)-vitamin D$_3$ was elevated, a finding that agrees with the observed changes in the transcription of the abovementioned hydroxylases and that indicates a corresponding change in protein levels [49, 68]. The decreased serum level of 1,25(OH)$_2$-vitamin D$_3$ is owed to the marked urinary loss of vitamin D$_3$ and its binding protein as a consequence of the endocytic defect [18, 68].

Hence, the loss of ClC-5 has two opposing effects on 1,25(OH)$_2$-vitamin D$_3$ serum levels: an increased amount of the vitamin D$_3$-activating, and decreased amounts of the vitamin D$_3$-inactivating enzyme, which will conjure to increase the active hormone, but a decreased availability of the precursor. The delicate balance between these two effects may explain the differences between the two ClC-5 KO mouse models [68, 69] as well as the variability of Dent's disease. Whereas the KO mouse from our lab has decreased 1,25(OH)$_2$-vitamin D$_3$ serum levels, the KO from Guggino's lab as well as most Dent's disease patients have elevated serum levels of the active metabolite [49, 79, 104, 105]. Because the strategy used for generating these KO models as well as the genetic background is quite similar, the different phenotypes might be explained by nutritional factors. In this respect, it is interesting to note that the diet fed to mice of the Guggino group was enriched with vitamin D$_3$, which was not the case in our lab [49, 69]. Unfortunately, systematic studies investigating nutritional effects in these mice are still lacking.

1,25(OH)$_2$-vitamin D$_3$ is a key regulator of calcium homeostasis and stimulates intestinal calcium absorption. Because the extra calcium that is reabsorbed with higher levels of active vitamin D$_3$ must eventually be eliminated by the kidney, the increased 1,25(OH)$_2$-vitamin D$_3$ serum levels might indirectly lead to hypercalciuria and kidney stones. Indeed, most patients with Dent's disease as well as Guggino's hypercalciuric KO mouse showed elevated levels of 1,25(OH)$_2$-vitamin D$_3$. In contrast, the KO mice of Piwon et al. have decreased serum concentrations of this hormone and do not display hypercalciuria and kidney stones. In conclusion, hypercalciuria and kidney stones might be secondary due to the defective endocytosis of PTH and vitamin D, which indirectly results in higher 1,25 (OH)$_2$-vitamin D$_3$ serum levels that ultimately lead to hypercalciuria by stimulating intestinal Ca^{++} resorption. It should, however, be mentioned that Silva et al. concluded that Guggino's KO mouse had renal hypercalciuria that was not attributable to increased intestinal Ca^{++} absorption [105].

The impaired proximal tubule endocytosis in the ClC-5 KO leads to transcriptional changes not only in the proximal tubule, but also in more distal nephron segments. Genome-wide expression analysis of ClC-5 KO kidneys identified several 1,25(OH)$_2$-vitamin D$_3$ target genes as being upregulated, even though serum levels of 1,25(OH)$_2$-vitamin D$_3$ were reduced. All these upregulated genes are expressed in distal nephron segments, like the epithelial calcium channels TRPV5 and TRPV6 or the calcium binding proteins calbindin D28K and D9K [18]. This pattern agrees with the notion that these changes are due to local increases of 1,25(OH)$_2$-vitamin D$_3$ in the lumen of later nephron segments, which in turn is a consequence of decreased endocytosis in the PT. In line with this hypothesis, expression profiling of intestine and bone, the two other main 1,25(OH)$_2$-vitamin D$_3$ target organs, revealed that upregulation of 1,25(OH)$_2$-vitamin D$_3$ target genes is not systemic. Whereas bone turnover markers were elevated in the ClC-5 KO mouse from Guggino's laboratory [105], the microarray analysis of bones from the mouse generated by Piwon et al. did not reveal changes of mRNAs important for bone metabolism [18]. Therefore, the bone phenotype described by Silva et al. might not represent a direct, bone-intrinsic effect of ClC-5 disruption, but may be secondary to hypercalciuria and associated hormonal changes [105].

Interestingly, also mRNA levels of retinoic acid-dependent genes were differentially regulated in ClC-5 KO mice. Midkine, which is highly expressed in proximal tubule cells, was downregulated, whereas the mRNA encoding lipocalin 2, which is expressed in the distal nephron, was upregulated [103]. Together with the unchanged serum levels of retinol and the increased amount of retinol binding protein in the urine of KO mice [68], one can suggest a mechanism similar to that described for 1,25(OH)$_2$-vitamin D$_3$. Hence, the activation of genes in distal nephron segments by vitamins and hormones that are normally endocytosed in the proximal tubule may extend to other pathways like those activated by retinoic acid.

Heterogeneity of Dent's disease

Dent's disease is genetically heterogeneous. In addition to mutations in *CLCN5*, also some mutations in the *OCRL1* gene, which encodes a phosphatidylinositol 4,5-bisphosphat 5-phosphatase, cause a very similar, if not identical phenotype [106]. Most mutations in *OCRL1*, however, cause Lowe oculocerebrorenal syndrome, which in addition to renal symptoms is characterized by microphthalmia, cataracts, and mental retardation. Phosphatidylinositol 4,5-bisphosphat 5-phosphatase changes levels of vesicular lipids such as PIP2 which play a role in intracellular trafficking events. The OCRL protein visits late-stage clathrin-coated pits and has been shown to bind the rab5 effector APPL1 on early endosomes [107]. Thus, the lack of this phosphatase may impair proximal tubular endocytosis by a different mechanism. It remains unclear why *OCRL1* mutations causing Dent's disease do not precipitate the ocular and CNS symptoms seen in Lowe syndrome. Interestingly, in several families with Dent's disease, no mutation in either *CLCN5* or *OCRL1* could be identified, suggesting that mutations in at least another gene can cause a similar subtype of Fanconi's syndrome [108].

Further consequences of ClC-5 disruption and interactions with other proteins and pathways

Surprisingly, a immunohistochemical analysis by Moulin et al. suggested that the V-type H^+-ATPase displays a basolateral distribution in renal biopsies from four Dent's disease patients with confirmed mutations in *CLCN5* [109]. In proximal tubules, the proton pump is normally most highly expressed in apical endosomes below the brush border [47, 72]. The H^+-ATPase was also reported to be absent from the plasma membrane of α-intercalated cells of the collecting duct of two patients [109]. Those findings are in striking contrast to the unchanged subcellular localization of the proton pump in ClC-5 KO mice [68], a finding confirmed in another ClC-5 KO mouse model by the same authors who reported the changed localization in human patients [109]. The reason for this discrepancy is entirely obscure.

Dent's disease patients also present to a variable degree of glycosuria [79, 104], raising the possibility that apical glucose transporters may be missorted in the absence of ClC-5 in analogy to the changes seen with megalin [68]. However, no changes in the apical SGLT1, SGLT2 Na^+-glucose cotransporters, or the GLUT1 glucose permease were observed in ClC-5 KO mice [110]. These authors [110] rather observed in KO mice lower mRNA and protein levels for the basolateral glucose transporter GLUT2, a finding that is difficult to explain. Another study reported that ClC-5 KO mice expressed high levels of markers for proliferation or oxidative stress, and displayed increased protein expression and urine excretion of carbonic anhydrase type III [111]. The upregulation of those proteins seemed to be a general feature of proximal tubule dysfunction ("Fanconi syndrome").

ClC-5 localization and activity has been reported to be modified by several interaction partners and ATP. Between the two carboxyterminal CBS domains, the transporter carries a PY motif that remains accessible in a flexible loop as described in crystallography and NMR studies [19, 22, 74, 112]. PY motifs are potential binding sites for WW domain containing proteins [74, 113, 114]. By measuring the binding affinities to some peptides, a peptide corresponding to the PY motif region of ClC-5 strongly bound WWPII in vitro [114]. This protein is a HECT domain E3 ubiquitin ligase that is broadly expressed in several tissues including the kidney [74]. Point mutations destroying the PY domain of ClC-5 increased its plasma membrane expression and currents by almost twofold when expressed in *Xenopus* oocytes [74]. Likewise, coexpression of a dominant negative mutant of WWPII increased the currents of the WT transporter, but not of the PY-mutated version. These experiments suggested an ubiquitin-mediated regulation of ClC-5 via its PY domain, which would target the transporter for endocytosis. ClC-5 was later shown to also bind Nedd4 and Nedd4-2, two other WW domain containing ubiquitin ligases [115]. Tagged ubiquitin was shown to be conjugated to ClC-5 in the presence of albumin and proteasome inhibitors. Reduction of albumin uptake was achieved by knocking down Nedd4-2 in opossum kidney (OK) cells by 25% [115]. However, ClC-5 trafficking and ubiquitylation was not characterized under knock down conditions. Anyway, the role of ClC-5 ubiquitylation and of its PY motif remains to be clarified under more physiological conditions.

Other proteins that were shown to interact with ClC-5 include the actin-depolymerizing protein cofilin [116] and Na^+/H^+ exchanger regulatory factor-2 (NHERF-2) [117]. The latter, a PDZ containing protein, was shown to bind the ClC-5 C-terminus through its PDZ-2 domain. ClC-5 currents were unchanged when both proteins where coexpressed in *Xenopus* oocytes [117]. Knocking down NHERF-2 reduced albumin uptake in OK cells by 25%, while knocking down NHERF-1, which is a structurally related member of the same family, slightly increased albumin uptake [117]. Likewise, knocking down NHERF-2 decreased cell surface expression of ClC-5, whereas knocking down NHERF-1 had the opposite effect [117]. These studies in the opossum kidney OK cell culture line appear to contradict studies performed with NHERF-1 and NHERF-2 KO mice. Neither of those mice presented proteinuria and only NHERF-1 KO mice showed hyperphosphaturia, which

was attributed to an interaction of NHERF-1 with NaPi-2a, and hypercalciuria which remains unexplained [118].

Nucleotides bind to CBS domains of ClC-5 [22]. However, binding affinities did not differ between ATP, ADP, and AMP, virtually excluding that this binding serves to regulate ClC-5 activity in response to metabolic activity. Moreover, disruption of the nucleotide binding site on CBS domains did not alter ClC-5 currents in *Xenopus* oocytes. However, currents were changed when this mutation was done in the background of an uncoupling ClC-5 mutant in the "gating glutamate" [22]. These intriguing findings and their potential physiological significance deserve further investigation.

Another chloride channel, the cystic fibrosis transmembrane conductance regulator (CFTR), is expressed in the S3 segment of the proximal tubule. Upon fractionation on Percoll gradients, CFTR copurified with ClC-5 and the V-type H^+ ATPase [119]. Although cystic fibrosis patients do not show a clear kidney phenotype, CFTR KO mice present proteinuria that is minor when compared to ClC-5 KO mice. *Cftr*$^{-/-}$ mice also displayed decreased expression of the multiligand receptor cubilin [119].

Both ClC-3 and ClC-4 are also expressed in proximal tubular cells [17] (*and own unpublished results*). However, the corresponding KO mice do not show defective endocytosis as do mice devoid of ClC-5 [41] (*and own unpublished results*). Agreeing with these findings, sequencing the *CLCN4* gene (located on Xp22.3) in patients with Dent's disease that lacked mutations in *CLCN5* failed to reveal pathogenic mutations [106, 120]. ClC-6 and ClC-7 both reside on late endosomes [45, 56], with ClC-7 being the only CLC that is prominently expressed on lysosomes [30, 45, 46]. Whereas the ClC-6 protein is restricted to the nervous system [56], ClC-7 shows a ubiquitous expression pattern. Loss of ClC-7 or of its β-subunit Ostm1 [30] impairs the function of lysosomes and osteoclasts. The latter can no longer acidify their resorption lacuna, whereas lysosomes lacking ClC-7 reach their normal acidic luminal pH [30, 46]. The loss of ClC-7 in mice and men results in abnormally high bone density (osteopetrosis) [45] and lysosomal storage disease of the CNS of the neuronal ceroid lipofuscinosis type [45, 46]. Proximal tubule cells lacking ClC-7 have electron-dense intracellular deposits suggesting an improper lysosomal protein degradation [46]. These deposits may be particularly large in proximal tubules because of their high rate of endocytosis. The same phenotypes were found when the β-subunit of ClC-7, Ostm1, is lacking [30].

In summary, several vesicular CLC Cl^-/H^+ exchangers are expressed in proximal tubules. Mutations in ClC-5 and ClC-7/Ostm1 lead to distinct proximal tubular pathologies which have been investigated using KO mouse models. Of highest relevance for the proximal tubule is ClC-5, mutations in which underlie Dent's disease. The symptoms in Dent's disease may entirely be due to pathological cascades that result from a primary defect in endocytosis. This results in changes of calciotropic hormones that are endocytosed and metabolized in proximal tubular cells. Results from ClC-5 KO mice support the novel concept that changes of hormone concentrations in the lumen of the nephron can play important roles in kidney pathology and may also have physiological relevance. Finally, the discovery that vesicular CLCs are Cl^-/H^+ exchangers rather than Cl^- channels opens new perspectives for the cell biology of the endosomal–lysosomal system.

Acknowledgments We thank EI Christensen for allowing us to reproduce his EM picture in Fig. 3b. Work in our laboratory was supported by the Deutsche Forschungsgemeinschaft, the Bundesministerium für Bildung und Forschung (BMBF) in the framework of the National Genome Project (NGFN2), the European Union, the Prix Louis-Jeantet de Médecine, and the Ernst-Jung-Preis für Medizin to TJJ. VP is supported by Marie Curie Fellowship of the European Union.

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