J Physiol 593.18 (2015) pp 4151–4164

The Journal of Physiology

TOPICAL REVIEW

Chloride transporters and receptor-mediated endocytosis in the renal proximal tubule

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Key points

- The reabsorptive activity of renal proximal tubule cells is mediated by receptor-mediated endocytosis and polarized transport systems that reflect final cell differentiation.
- Loss-of-function mutations of the endosomal chloride–proton exchanger ClC-5 (Dent's disease) cause a major trafficking defect in proximal tubule cells, associated with lysosomal dysfunction, oxidative stress and dedifferentiation/proliferation.
- A similar but milder defect is associated with mutations in *CFTR* (cystic fibrosis transmembrane conductance regulator).
- Vesicular chloride transport appears to be important for the integrity of the endolysosomal pathway in epithelial cells.

Abstract The epithelial cells lining the proximal tubules of the kidney reabsorb a large amount of filtered ions and solutes owing to receptor-mediated endocytosis and polarized transport systems that reflect final cell differentiation. Dedifferentiation of proximal tubule cells and dysfunction of receptor-mediated endocytosis characterize Dent's disease, a rare disorder caused by inactivating mutations in the *CLCN5* gene that encodes the endosomal chloride–proton exchanger, ClC-5. The disease is characterized by a massive urinary loss of solutes (renal Fanconi syndrome), with severe metabolic complications and progressive renal failure. Investigations of mutations affecting the gating of ClC-5 revealed that the proximal tubule dysfunction may occur despite normal endosomal acidification. In addition to defective endocytosis, proximal tubule cells lacking ClC-5 show a trafficking defect in apical receptors and transporters, as well as lysosomal dysfunction and typical features of dedifferentiation, proliferation and oxidative stress. A similar but milder defect is observed in mouse models with defective CFTR, a chloride channel that is also expressed in the endosomes of proximal tubule cells. These data suggest a major role for endosomal chloride transport in the maintenance of epithelial differentiation and reabsorption capacity of the renal proximal tubule.

Olivier Devuyst is Full Professor of Medicine and Physiology at the University of Zurich. Dr Devuyst and his group investigate the molecular mechanisms of membrane transport and the pathophysiology of inherited renal tubular diseases, using a translational approach from human genetics to model systems. These studies, which have generated more than 250 peer-reviewed articles, are funded by national and international agencies, including Belgian and Swiss Research Councils, the European Union (FP6, FP7), several foundations and the National Institutes of Health. **Alessandro Luciani** is a senior postdoctorate in the Institute of Physiology at the University of Zurich. Following his MSc and PhD studies in the Universities of Naples and Foggia, Dr Luciani received his training in cell biology at the San Raffaele Scientific Institute (IERFC), Milan, Italy. His studies established that impairment of lysosomally mediated clearance of aggregated/misfolded proteins is an important pathogenic mechanism in cystic fibrosis. His current projects focus on the impact of defects in the endolysosomal compartment and autophagy in epithelial cells of the kidney. He recently obtained personal fellowships from the ERA-EDTA (Impulsion Grant) and the Cystinosis Research Foundation (USA).

DOI: 10.1113/JP270087

(Received 17 December 2014; accepted after revision 16 March 2015; first published online 27 March 2015)
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Abbreviations AMN, amnionless; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; HNF1α, hepatocyte nuclear transcription factor-1α; KI, knock-in; KO, knock-out; LMW, low molecular weight; V-ATPase, vacuolar H^+-ATPase; ZO-1, zonula occludens-1; ZONAB, zonula occludens-1-associated nucleic acid binding protein.

Introduction

The epithelial cells lining the proximal tubules of the kidney reabsorb a large amount of filtered solutes owing to a particularly efficient endolysosomal pathway, which reflects final cell differentiation. Dysfunction of the endosomes and/or lysosomes, secondary to congenital or acquired disorders, often leads to generalized dysfunction of the proximal tubule cells, causing a massive urinary loss of solutes (renal Fanconi syndrome) and severe metabolic complications, which can result in chronic kidney disease (Eckardt *et al.* 2013). Over the last 20 years, studies of inherited disorders of chloride transporters in the proximal tubule, including Dent's disease and cystic fibrosis, have provided novel insights into fundamental mechanisms regulating vesicular trafficking and function in epithelial cells. The relevance of these mechanisms is increasingly recognized for more frequent, acquired disorders, in which excess of filtered proteins cause overload of the endosomes and lysosomes and dysfunction of the proximal tubule. Furthermore, common genetic variants in components of the endocytic machinery are also identified in association with the risk of proteinuria and chronic kidney disease in the general population (Devuyst *et al.* 2014).

In this review, we address the role of chloride transporters in the endolysosomal pathway of the proximal tubule in relation to epithelial differentiation, transcriptional regulation and maintenance of cell traffic and polarity. In particular, we discuss the insights gained from the study of Dent's disease, a rare, X-linked disorder associated with loss-of-function mutations in the *CLCN5* gene that encodes ClC-5, a Cl^-–H^+ exchanger that is enriched in endosomes of epithelial cells lining the renal proximal tubule. We mention cystic fibrosis, which is also associated with manifestations of proximal tubule dysfunction. Finally, we elaborate on the potential links between abnormalities in endosomes and lysosomes and dysfunction of epithelial cells and summarize the clinical and physiological perspectives linked to the role of chloride transporters in the renal proximal tubule.

Endocytosis and vesicular chloride transport in the renal proximal tubule

In most mammals, the maintenance of body fluid homeostasis and normal plasma electrolyte levels depends critically on the appropriate handling of water and solutes by the kidneys. This essential function relies on the expression, trafficking and interactions of specific transport systems operating in the apical *versus* basolateral plasma membrane domains of the epithelial cells lining the kidney tubules. In particular, the cells lining the proximal tubules play a pivotal role in the reabsorption of a large amount of filtered ions and solutes through their apical brush border area and their polarized transport systems reflecting final cell differentiation. In these cells, the sustained activity of the basolateral Na^+–K^+-ATPase drives the massive reabsorption of glucose, amino acids and ions via specific sodium-dependent cotransporters that are expressed in the apical membrane (Kriz & Kaissling, 2000).

In addition to glucose, amino acids and ions, a significant amount (at least several grams per day) of albumin and low-molecular-weight (LMW) plasma proteins is continuously filtered through the glomerular basement membrane, to be reabsorbed by proximal tubule cells. These LMW proteins include albumin (66 kDa) and transferrin (81 kDa), as well as hormones (parathyroid hormone, insulin and growth hormone), carrier proteins (e.g. retinol, vitamin D and folate), enzymes (cytochrome *c* and lysozyme), cell surface antigen components (β_2-microglobulin) and immunoglobulin light chains. Most of these proteins are reabsorbed and metabolized by proximal tubule cells, because human urine is virtually devoid of plasma proteins in physiological conditions. This massive uptake of proteins accounts for ~80% of the total metabolic clearance of small proteins and peptides. It also plays an important role in hormone homeostasis, conservation of essential vitamins (vitamins D, A and B_{12}) and trace elements and the provision of a protein-free milieu for the cells lining distal nephron segments (Christensen & Birn, 2002).

The uptake of albumin and LMW proteins by proximal tubule cells essentially involves receptor-mediated, clathrin-dependent endocytosis, and potentially, fluid-phase endocytosis (Dickson *et al.* 2014; Fig. 1). Receptor-mediated endocytosis requires two multiligand receptors, megalin and cubilin, and the co-operating protein amnionless (AMN) that are expressed at the brush border of the cells (Nielsen & Christensen, 2010). In contrast to megalin, which is a member of the low-density lipoprotein receptor family, cubilin, also known as the intestinal intrinsic factor–vitamin B_{12} receptor, is a

highly conserved membrane-associated protein with little structural homology to known endocytic receptors. It is characterized by the absence of a transmembrane domain. High-affinity binding of purified megalin to cubilin N-terminal region has been shown *in vitro*, suggesting that megalin participates in the endocytosis and intracellular trafficking of cubilin (Christensen & Birn, 2002). The apical sorting of cubilin and its participation in receptor-mediated endocytosis critically depend on its reciprocal interaction with AMN (Coudroy *et al.* 2005). Cubilin contributes ligand-binding regions of the receptor complex, whereas AMN ensures the membrane anchorage, biosynthetic processing and recycling of the complexes at the plasma membrane (Fyfe *et al.* 2004). Ligand binding and interactions between both receptors induce their internalization into coated vesicles and their subsequent delivery to endosomes and lysosomes for ligand processing and receptor degradation or recycling.

Receptor-mediated endocytosis depends on the integrity of the actin cytoskeleton and the microtubules (Gekle, 2005), whereas progression along the endocytic apparatus requires a sustained vesicular acidification from early endosomes to lysosomes (Faundez & Hartzell, 2004). The drop in pH in the successive endocytic compartments triggers receptor–ligand dissociation and modulates vesicle trafficking, endosomal fusion events and coat formation (Hurtado-Lorenzo *et al.* 2006). In proximal tubule cells, endosomal acidification is driven by the electrogenic vacuolar H^+-ATPase (V-ATPase; Fig. 2), whose inhibition by pharmacological agents such as bafilomycin A-1 or toxic agents such as cadmium severely

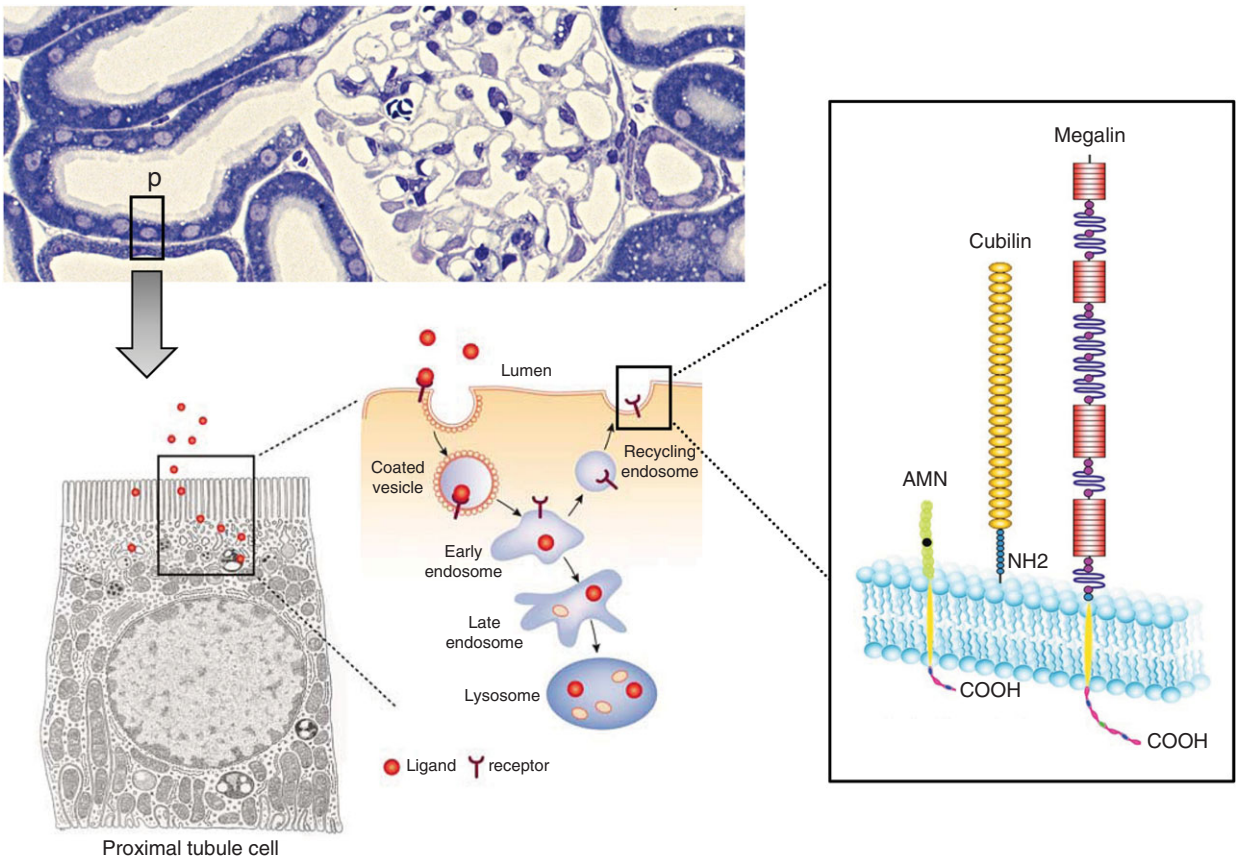

Figure 1. Reabsorption of low-molecular weight (LMW) proteins by proximal tubule cells
Albumin and LMW proteins (red symbols) are continuously filtered across the glomerular filtration barrier, to be reabsorbed and processed by the epithelial cells lining the proximal tubule (p). The LMW ligands first interact with the non-selective megalin–cubilin–amnionless (AMN) receptor complex at the apical membrane. After internalization, receptor–ligand complexes progress along the clathrin-dependent endocytic pathway. The endosomes undergo a progressive acidification (more acidic pH values in darker blue) that results in the dissociation of the receptor–ligand complexes, with the receptors (inset) being recycled to the apical membrane, whereas the ligand is directed to acidic lysosomes for degradation. Other possible pathways for albumin handling by proximal tubule cells, including fluid-phase endocytosis and transcytosis back into the circulation, are not detailed. Adapted from Devuyst & Pirson (2007) and Nielsen & Christensen (2010), with light microscopy picture kindly provided by Dr B. Kaissling.

impairs the uptake of albumin and LMW proteins *in vitro* and *in vivo* (Herak-Kramberger *et al.* 1998). The translocation of protons from the cytoplasm into the endosomes generates a transmembrane electrical potential ($\Delta\Psi$), with rapid inhibition of V-ATPase activity. The V-ATPase activity will thus depend on the dissipation of the $\Delta\Psi$, either by cation leakage or by Cl^- transport (Plans *et al.* 2009). In most cases, vesicular acidification depends on a Cl^- conductance. The intravesicular Cl^- concentration increases progressively from early endosomes (20–40 mM) to lysosomes (>80 mM; Stauber & Jentsch, 2013). It could directly affect the V-ATPase activity (Moriyama & Nelson, 1987) as well as other ionic movements and vesicle recycling independently of its effect on pH (Faundez & Hartzell, 2004; Stauber & Jentsch, 2013).

Dent's disease, a rare congenital disorder of the proximal tubule

In view of their essential role in reabsorbing water and electrolytes and processing filtered proteins, the proximal tubule cells are particularly sensitive to defective mechanisms of epithelial differentiation and polarized transport. Dysfunction of the apical transport processes leads to a loss of LMW proteins and solutes (e.g. phosphate, glucose and amino acids) in the urine, a clinical entity referred to as renal Fanconi syndrome (Igarashi, 2009). In turn, these losses cause severe clinical problems, including dehydration and electrolyte imbalance, rickets, muscular weakness, growth retardation and progressive renal failure. Proximal tubule dysfunction and renal Fanconi syndrome may result from congenital disorders, such as Dent's disease, Lowe's syndrome and cystinosis, or may be acquired, for instance after exposure to exogenous susbstances (toxins or drugs) or associated with autoimmune disorders (Igarashi, 2009; Devuyst & Thakker, 2010). Proximal tubule cells also play a central role in proteinuric conditions, because abnormally filtered proteins, such as albumin or immunoglobulin free light chains, are internalized by the megalin–cubilin complex, triggering various signalling pathways and transcription cascades that are associated with tubulo-interstitial damage and renal failure (Baines & Brunskill, 2011). In the following sections, we discuss the clinical, genetic and mechanistic aspects of Dent's disease, taken as a model of proximal tubule dysfunction due to defective endosomal chloride transport.

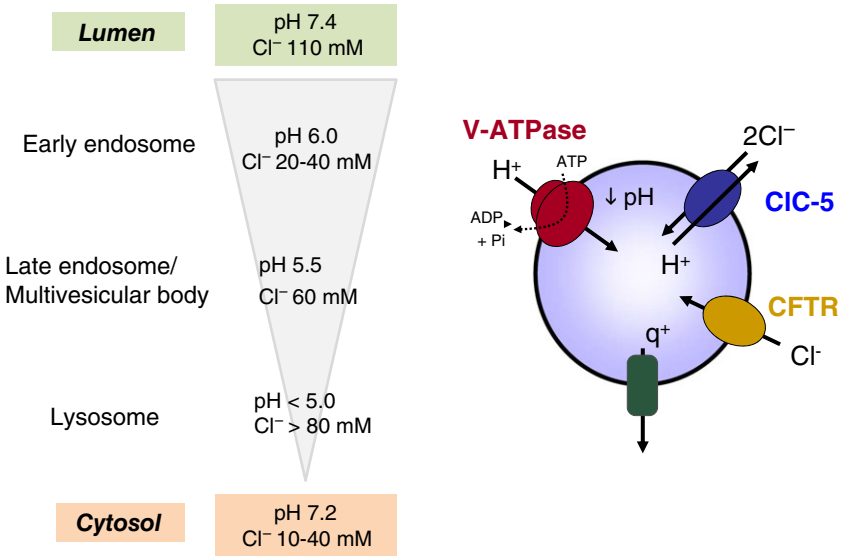

Figure 2. Vesicular acidification and chloride concentrations along the endolysosomal pathway
The endocytic pathway in proximal tubule cells involves coated pits and coated vesicles, followed by early endosomes that form recycling endosomes or mature to late endosomes and lysosomes. The luminal pH drops from 7.4 in the tubule lumen to 6.0 in early endosomes, 5.5 in late endosomes and below 5.0 in lysosomes. Such vesicular acidification is necessary for dissociation of the ligand–receptor complex, recycling of receptors to the apical membrane and progression of ligands into lysosomes. In parallel, the chloride concentrations drop from 110 mM in the extracellular space to 20–40 mM in early endosomes, 60 mM in late endosomes and more than 80 mM in lysosomes, i.e. much higher than the 10–40 mM in the cytosol. Right panel illustrates that endosomal acidification is achieved by ATP-driven transport of cytosolic H^+ through the vacuolar H^+-ATPase (V-ATPase, or proton pump). ClC-5 operates as a Cl^-–H^+ exchanger that facilitates acidification (countercurrent through the $2Cl^-$:$1H^+$ stoichiometry. Together with cation leakage (q^+), the Cl^- channel CFTR, which is also enriched in the endosomal fraction containing ClC-5, could participate in the electrical shunt that is necessary for sustained vesicular acidification. Adapted from Faundez & Hartzell (2004); Jouret & Devuyst (2009) and Stauber & Jentsch (2013).

Clinical and genetic aspects. Dent's disease is a rare, X-linked renal tubulopathy characterized by LMW proteinuria associated with hypercalciuria, which may provoke nephrolithiasis, nephrocalcinosis and renal failure. Dent's disease may also be associated with aminoaciduria, phosphaturia, glycosuria, uricosuria, kaliuresis and impaired urinary acidification and is often complicated by rickets or osteomalacia. These features are generally found in males only, who may present from early childhood with bone pain, rickets or symptoms of renal stones. Low-molecular-weight proteinuria is the most consistent manifestation of Dent's disease, detected in almost all affected males and obligate female carriers (Devuyst & Thakker, 2010). The urinary loss of retinol-binding protein may cause vitamin A deficiency and impaired night vision (Becker-Cohen *et al.* 2012). Hypercalciuria and nephrocalcinosis are also highly prevalent, although there is considerable inter- and intra-familial variability in the occurrence of nephrolithiasis, which occurs in approximately half of the affected males (Devuyst & Pirson, 2007). Progression to end-stage renal failure occurs between the third and fifth decades of life in 30–80% of affected males (Scheinman, 1998). These manifestations of Dent's disease may occur occasionally in female carriers. The occurrence of these predominantly renal manifestations and their association with *CLCN5* mutations (see next subsection) is referred to as 'Dent's disease 1'.

A small subset of patients with Dent's disease present extrarenal manifestations, such as mental developmental delay, hypotonia and cataract, in association with mutations of the oculocerebrorenal syndrome of Lowe (*OCRL*) gene (Hoopes *et al.* 2005). The occurrence of these extrarenal manifestations associated with *OCRL* mutations is referred to as 'Dent's disease 2'.

Dent's disease and *CLCN5* mutations. Dent's disease 1 (OMIM #300009) is caused by inactivating mutations in the *CLCN5* gene that is located on chromosome Xp11.22 and encodes a 746-amino-acid electrogenic $2Cl^-$–H^+ exchanger, ClC-5 (Lloyd *et al.* 1996; Picollo & Pusch, 2005; Scheel *et al.* 2005). ClC-5 belongs to the CLC family of Cl^- channels/transporters that have been discovered and characterized by Jentsch and colleagues (Jentsch, 2008). Based on sequence homology, ClC-5 belongs to a cluster of three isoforms (ClC-3, ClC-4 and ClC-5) that are mainly located in intracellular vesicles. ClC-5 contains 18 α-helices, with two phosphorylation sites and one N-glycosylation site. Crystal structures of bacterial and eukariotic CLCs revealed that the protein forms diamond-shaped homodimers composed of two repeated halves that span the membrane in opposite orientations. Each subunit has its own pore responsible for the selective coupling of the Cl^- flux to H^+ countertransport (Dutzler *et al.* 2002; Feng *et al.* 2010). The *CLCN5* mutations are scattered through the coding region, with no evidence for mutational hot spots (Wu *et al.* 2009). The majority of *CLCN5* mutations (missense and nonsense) are predicted to result in truncated or absent ClC-5 protein, which would lead to complete loss of antiporter function. Heterologous expression in *Xenopus laevis* oocytes or HEK 293 cells has revealed that most *CLCN5* mutations lead to a loss of Cl^- conductance (Lloyd *et al.* 1996). Further detailed studies of the *CLCN5* missense mutations revealed that these may lead to impaired processing and folding, with endoplasmic reticulum retention and degradation by quality-control mechanisms (class 1), delay in processing and reduced stability (class 2), and normal trafficking but altered electrical activity (class 3; Lourdel *et al.* 2012). Of note, the majority of the missense mutations of ClC-5 are clustered at the interface between the two subunits (Wu *et al.* 2003).

There is genetic heterogeneity for Dent's disease, with approximately 50–60% of patients harbouring *CLCN5* mutations, ~15% with *OCRL* mutations and the remaining 25–35% having neither *CLCN5* nor *OCRL* mutations. Dent's disease 2 (OMIM #300555) defines patients with Dent's disease who present extrarenal manifestations and harbour mutations in *OCRL*, the gene that is mutated in the oculocerebrorenal syndrome of Lowe (Hoopes *et al.* 2005; Shrimpton *et al.* 2009). The *OCRL* gene encodes an inositol 5-phosphatase, which preferentially hydrolyses the 5-phosphate of phosphatidylinositol 4,5-bisphosphate. Disease-causing mutations in OCRL result in loss of 5-phosphatase activity, with phosphatidylinositol 4,5-bisphosphate accumulating in proximal tubule cells of patients with Lowe syndrome (Zhang *et al.* 1998). A number of lines of evidence suggest that OCRL plays a role in the endocytic pathway and the co-ordination of membrane dynamics with remodelling of the actin cytoskeleton (Erdmann *et al.* 2007; Mehta *et al.* 2014). Thus, it seems likely that the *OCRL* mutations in Lowe syndrome or Dent's disease 2 patients lead to disruptions in the endosomal and/or lysosomal trafficking, i.e. an abnormality similar to that observed in Dent's disease 1 (see next subsection).

ClC-5 in the proximal tubule. The clinical presentation of Dent's disease 1 reflects the predominant expression of ClC-5 in the cells lining the proximal tubule segments of the nephron. Of note, a sizeable expression is also detected in cells lining the thick ascending limb of Henle's loop and in the α-type intercalated cells of the collecting ducts (Devuyst *et al.* 1999). In proximal tubule cells, ClC-5 co-distributes with the V-ATPase in early endosomes (Günther *et al.* 1998; Devuyst *et al.* 1999), where it was predicted to ensure the Cl^- permeability necessary to sustain vesicular acidification (Fig. 2).

The segmental expression of ClC-5 parallels that of the V-ATPase and is essentially achieved during early nephrogenesis in the mouse, following the onset of glomerular filtration (Jouret *et al.* 2004). The hepatocyte nuclear transcription factor-1α (HNF1α), which is predominantly expressed in proximal tubule segments, directly regulates the expression of ClC-5 (Tanaka *et al.* 2010). *In situ* hybridization demonstrated that the expression of *Clcn5* strikingly overlaps with that of *Hnf1α* in the developing kidney as well as in absorptive epithelia, including the digestive tract and yolk sac. Multiple binding sites for HNF1α were mapped in the 5′-regulatory sequences of the mouse and human *Clcn5/CLCN5* genes. The transactivation of the *Clcn5/CLCN5* promoter by HNF1α was verified *in vitro* and *in vivo* (chromatin immunoprecipitation) in mouse kidney. The expression of *Clcn5* was reduced in the proximal tubule segments of HNF1α-null kidneys and it was rescued upon transfection of HNF1α-null cells with wild-type but not with mutant HNF1α (Tanaka *et al.* 2010).

Studies in two independent strains of ClC-5 knock-out (KO) mice provided critical insights into the mechanisms of proximal tubule dysfunction in Dent's disease (Piwon *et al.* 2000; Wang *et al.* 2000). These two strains recapitulated the major features of Dent's disease 1, including LMW proteinuria and other manifestations of proximal tubule dysfunction, but also showed significant differences that are summarized in Table 1. *In vitro* experiments showed defective acidification of vesicles isolated from ClC-5 KO mice, supporting a role for ClC-5 in acidification of early endosomes (Günther *et al.* 2003; Novarino *et al.* 2010).

In 2005, it was discovered that, instead of being a simple Cl^- channel, ClC-5 was in fact an electrogenic, $2Cl^-$–H^+ exchanger exploiting the H^+ gradient to move Cl^- ions into endosomes (Picollo & Pusch, 2005; Scheel *et al.* 2005; Fig. 2). In order to gain a better understanding of the biological role of this exchange activity and its relevance for Dent's disease, Novarino *et al.* (2010) generated a knock-in (KI) mouse model carrying a point mutation (E211A) affecting a glutamate residue that is essential for the gating of CLC exchangers. By replacing this glutamate with an alanine, ClC-5 is converted into a pure, uncoupled Cl^- conductor. At variance with the severe defect observed in ClC-5 KO, the E211A mutant ClC-5 did not affect endosomal acidification. However, despite the normal endosomal acidification, the KI mice showed the same renal phenotype as KO mice and patients with Dent's disease, including LMW proteinuria, glucosuria, hyperphosphaturia and hypercalciuria. Furthermore, both the KI and the KO mice showed a similar impairement in proximal tubular endocytosis, with reduced levels of the endocytic receptors megalin and cubilin and internalization of the sodium–phosphate cotransporter NaPi-2a, indicating a trafficking defect (Novarino *et al.* 2010).

The studies summarized above demonstrated that proximal tubule dysfunction in Dent's disease may occur despite normal endosomal acidification. Instead of the simple Cl^- shunt hypothesis, the disease may be caused by defective exchange activity, i.e. uncoupling of Cl^- from H^+ gradients and defective endosomal Cl^- accumulation. In fact, ClC-5 could mediate endosomal acidification independently of the V-ATPase in the early endosomes, with luminal H^+ uptake driven by the favourable gradient for Cl^- (Scheel *et al.* 2005; Fig. 2). Due to the coupling of the Cl^- gradient to vesicular pH, ClC-5 could maintain a high endosomal Cl^- concentration, similar to a CLC nitrate–proton exchanger found in *Arabidopsis thaliana* (atClC-a; De Angeli *et al.* 2006). Of interest, lysosomes harbouring an uncoupling mutation in the ClC-7 exchanger also show a lower Cl^- concentration despite normal lysosomal pH (Weinert *et al.* 2010). Given that ClC-4 can associate with ClC-5 in the proximal tubule (Suzuki *et al.* 2006), where they may form a functional heterodimer, it has been suggested that it may also contribute to endosomal acidification based on cellular inactivation studies (Mohammad-Panah *et al.* 2003). This hypothesis has not been substantiated by the genetic inactivation of ClC-4 in mice; deletion of ClC-4 (or of ClC-3) did not interfere with endocytosis *in vivo* and in proximal tubule cells (Rickheit *et al.* 2010). Also, it must be noted that the expression levels of ClC-3 and ClC-4 were unchanged in kidneys from ClC-5 KO mice (Maritzen *et al.* 2006). One can thus conclude that ClC-5 plays a unique role among CLC exchangers in proximal tubule endocytosis (Rickheit *et al.* 2010).

Recent studies in conditionally immortalized proximal tubule cells from patients with distinct mutations involving different domains of ClC-5 showed differing effects on endosomal acidification, uncoupled to defects in receptor-mediated endocytosis (Gorvin *et al.* 2013). At this stage, the role of the vesicular Cl^- (e.g. regulation of other transport systems, interaction with other proteins involved in the organelle, or importance for vesicle recycling) remains speculative (Stauber & Jentsch, 2013).

ClC-5 and traffic in the proximal tubule. Studies in *Clcn5* KO mice have demonstrated that inactivation of ClC-5 is associated with a severe trafficking defect in proximal tubule cells, with loss of megalin and cubilin at the brush border, impaired endocytosis and lysosomal processing of endocytosed ligands and defective internalization of NaPi-IIa and NHE3. These changes are reflected by the inappropriate loss of solutes that are normally reabsorbed by proximal tubule cells, resulting in renal Fanconi syndrome (Piwon *et al.* 2000; Wang

Table 1. Phenotype of the two ClC-5 knock-out mouse models

Jentsch model (Piwon *et al.* 2000, 2003)	
Methods	Targeting part of exon 5 and exon 6; C57BL/6 J background
Renal phenotype	Low-molecular weight proteinuria (albumin, vitamin D-binding protein, retinol-binding protein); defective receptor-mediated endocytosis (β_2-microglobulin, lactoglobulin, horseradish peroxidase); defective fluid-phase endocytosis (fluorescein isothiocyanate–dextran)
	Cell-specific endocytic defect demonstrated in proximal tubules of heterozygous *Clcn5*$^{+/-}$ females
	Polyuria (+35%) and phosphaturia (+35%); no hypercalciuria
Bone and mineral phenotype	Increased urinary levels of parathyroid hormone (1.7-fold) and 25(OH)-vitamin D_3 (14-fold); reduced serum levels of 25(OH)-vitamin D_3 (3-fold) and 1,25$(OH)_2$-vitamin D_3 (2-fold)
Guggino model (Wang *et al.* 2000; Christensen *et al.* 2003; Silva *et al.* 2003; Nielsen *et al.* 2007)	
Methods	Targeting exon 6; C57BL/6 J background
Renal phenotype	Low-molecular-weight proteinuria (vitamin D-binding protein, Clara cell protein of 16 kDa, transferrin); defective receptor-mediated endocytosis (β_2-microglobulin, horseradish peroxidase); no defect in fluid-phase endocytosis (fluorescein isothiocyanate–dextran 40 kDa)
	Renal Fanconi syndrome: polyuria (+42%); hypercalciuria (2.3-fold); phosphaturia (+34%); glucosuria (1.8-fold); generalized amino-aciduria
	Defective lysosomal processing of low-molecular-weight ligands (β_2-microglobulin); enzymuria (pro-cathepsin B, cathepsin B)
	Intrarenal calcium deposits (von Kossa positive) compatible with nephrocalcinosis
Bone and mineral phenotype	Elevated 1,25$(OH)_2$-vitamin D_3 levels (2-fold); normal serum parathyroid hormone levels
	Increase in bone turnover markers (serum alkaline phosphatase, serum osteocalcin and urinary deoxypyridinoline)

et al. 2000; Christensen *et al.* 2003). Importantly, mice lacking ClC-5 do not show ultrastructural alterations of the endocytic apparatus (Christensen *et al.* 2003), a finding confirmed in a series of human renal biopsies from patients with Dent's disease and established mutations in *CLCN5* (Moulin *et al.* 2003). In order to circumvent the poor differentiation and limited capacity of endocytosis of immortalized proximal tubule cell models (Dickson *et al.* 2014), we established and extensively validated a technique to grow primary cell cultures from micro-dissected tubular segments obtained in various mouse lines (Terryn *et al.* 2007). The primary proximal tubule cells are grown on filters and retain their morphological differentiation and specific properties, including transport processes and receptor-mediated endocytosis. The use of this cell culture system allowed us to show that the endocytic defect observed in ClC-5 KO mice was retained in primary cultures of proximal tubule cells (Reed *et al.* 2010) and could be rescued upon transfection with HNF1α (Tanaka *et al.* 2010).

The variability in the urinary loss of vitamin D-binding protein, 25(OH)-vitamin D_3 and parathyroid hormone could produce opposite effects in the proximal tubule, leading to variable levels of 1,25$(OH)_2$-vitamin D_3 that could explain the different phenotypes observed in the Jentsch and Guggino mice (Piwon *et al.* 2000; Silva *et al.* 2003; Table 1). Likewise, renal hypercalciuria and kidney calcifications were observed in one strain of ClC-5 KO mouse (Wang *et al.* 2000) but not in the other (Piwon *et al.* 2000). It must be noted that significant increases in the mRNA expression of 25(OH)-vitamin D_3 1α-hydroxylase (Cyp27b1) and some of its target genes were detected in the ClC-5 KO mouse kidneys (Maritzen *et al.* 2006).

Recently, Nielsen *et al.* (2007) demonstrated that megalin was mediating an efficient pathway to reabsorb circulating and continuously filtered lysosomal enzymes (such as cathepsin B), providing a major source of acid hydrolases to lysosomes in proximal tubule cells. Accordingly, the lack of ClC-5 and the ensuing defect in megalin/cubilin were associated with impaired lysosome biogenesis and function, as evidenced by defective LMW processing/degradation and urinary loss of lysosomal enzymes (Christensen *et al.* 2003; Nielsen *et al.* 2007).

Despite their vulnerability, proximal tubule cells are able to recover from an ischaemic or toxic insult, while surviving cells dedifferentiate and proliferate to restore tubular integrity eventually (Bonventre, 2003). A similar process occurs in Dent's disease, with a 4-fold increase in the proliferative activity of proximal tubule cells (assessed by proliferating cell nuclear antigen, KI-67, and cyclin E) paralleled by dedifferentiation (expression of osteopontin and the mesodermal marker carbonic anhydrase type III). The induction of dedifferentiation was also linked to an increased production of superoxide anion and the induction of type I superoxide dismutase and thioredoxin, pointing to increased oxidative stress and solicitation of cell oxidative defenses in ClC-5 KO kidneys (Gailly *et al.* 2008). Of note, these modifications occurred at a

time when no visible alterations in cell morphology or renal failure were observed in ClC-5 KO mice, nor was there any change in the apoptotic rate. The evidence of oxidative stress in other diseases of the proximal tubule, including nephropathic cystinosis (Raggi *et al.* 2014), raises the possibility of a link between oxidative stress and defective endolysosomal pathway. Albumin is known to exert a potent survival activity in mouse proximal tubule cells, most probably through scavenging of reactive oxygen species (Iglesias *et al.* 1999), so that a reduced capacity of albumin uptake may be deleterious. Of interest, mutations of ClC-7, another CLC exchanger that is expressed in late endosomes and lysosomes of the proximal tubule cells, or in its β-subunit OSTM1 result in osteopetrosis, neurological manifestations and lysosomal storage disease (Kornak *et al.* 2001; Lange *et al.* 2006). ClC-7 KO mice show severe neurodegenerative manifestations, but apparently no renal phenotype despite electron-dense deposits in lysosomes of proximal tubule cells (Kasper *et al.* 2005; Lange *et al.* 2006).

A hypothetical model of proximal tubule dysfunction secondary to the loss of ClC-5 can be proposed as follows (Fig. 3). The defective Cl^- transport in early endosomes leads to impaired trafficking and recycling of apical receptors, defective receptor-mediated endocytosis and ensuing urinary loss of LMW ligands (Christensen *et al.* 2003). The functional loss of ClC-5 is also associated with impaired lysosomal function, which in turn might compromise autophagy, the cytoprotective mechanism for the degradation of damaged organelles through lysosome-mediated self-digestion (Moreau *et al.* 2010). The defective lysosome-mediated clearance of autophagosomes containing ubiquitinated proteins and dysfunctional mitochondria may lead to oxidative stress as observed in ClC-5 KO kidneys (Gailly *et al.* 2008). It was recently shown that oxidative stress disrupts the integrity of the junctional complex [i.e. zona occludens-1 (ZO-1) protein; Yu *et al.* 2012]. In turn, these changes might promote the release of ZO-1-associated nucleic acid binding protein (ZONAB), a transcription factor known to cause proliferation (increased transcription of cyclin D1 and proliferating cell nuclear antigen) and apical dedifferentiation (repression of the transcription of megalin and cubilin) in proximal tubule cells (Lima *et al.* 2010; Raggi *et al.* 2014). By analogy, defective autophagy has been reported in epithelial cells in cystic fibrosis, as part of a complex perturbation of the post-translational network consequent to defective cystic fibrosis transmembrane conductance regulator (CFTR) function (Luciani *et al.* 2010). In particular, altered Beclin-1 protein levels associated with reduced abundance of the endosomal phosphatidylinositol 3-phosphate pool in CFTR-depleted epithelial cells abrogate the recycling of apical receptors and impair Rab5/Rab7 transition, delaying the endosomal–lysosomal trafficking and altering the lysosome identity (Villella *et al.* 2013). These findings may explain defective membrane trafficking and recycling pathways in proximal tubule cells lacking ClC-5 (Christensen *et al.* 2003).

Defective proximal tubule endocytosis in cystic fibrosis

Cystic fibrosis (CF; OMIM #219700) is an autosomal recessive disease affecting 1 in 2,500 live births in Caucasians. Cystic fibrosis is a potentially lethal, multisystem disease resulting from the accumulation of thick mucus that obstructs the airways, pancreatic ducts, intestine, bile ducts and genital tract (Rowe *et al.* 2005). Cystic fibrosis is due to loss-of-function mutations in the *CFTR* gene (also named *ABCC7*) that encodes a 1480-amino-acid Cl^- channel called CFTR (Kerem *et al.* 1989; Riordan *et al.* 1989). More than 1000 CF-associated mutations have been reported thus far in *CFTR*, and they have been classified into five groups according to their structural or functional consequences for Cl^- transport (Rowe *et al.* 2005). The in-frame deletion of three bases encoding a phenylalanine residue at position 508 (ΔF508) accounts for ~70% of the mutations in patients with CF. This mutation affects the processing and maturation of CFTR (Cheng *et al.* 1990).

Structure and renal distribution of CFTR. The CFTR belongs to the ATP-binding cassette (ABC) family of integral membrane transporters (Gadsby *et al.* 2006). The protein contains two transmembrane domains (TMD1 and TMD2) and two nucleotide-binding domains (NBD1 and NBD2), separated by a regulatory (R) domain (Riordan *et al.* 1989). Each membrane domain contains six α-helices, which are involved in the Cl^- pore. The CFTR is regulated by cAMP-dependent phosphorylation of the R domain via protein kinase A, followed by gating events initiated by ATP binding to NBD1 and NBD2 and resulting in transepithelial Cl^- transport (Sheppard & Welsh, 1999). Complex interactions modulate both the channel activity and the intracellular trafficking of CFTR, which participates in macromolecular complexes at the plasma membrane (Guggino & Stanton, 2006).

A sizeable expression of CFTR has been detected in all nephron segments of the rat and human kidney (Jouret & Devuyst, 2009). Detailed studies in mouse kidney (Jouret *et al.* 2007) revealed that CFTR is mainly expressed in the apical area of proximal tubule cells (pars recta, S3 segment), with a subcellular co-distribution with ClC-5, V-ATPase and Rab5a compatible with endosomal enrichment. The localization of CFTR in the proximal tubule was also confirmed in the human kidney (Devuyst *et al.* 1996; Morales *et al.* 1996).

Proximal tubule phenotype in mice and humans with defective CFTR. The fact that CFTR is enriched in apical endosomes of proximal tubule cells suggested a possible involvement in endocytosis. Indeed, *Cftr*$^{-/-}$ (*Cftr*^{tm1Cam}) mice showed a significant increase in the urinary excretion of the LMW Clara cell protein (16 kDa) in comparison to control mice. The *Cftr*$^{-/-}$ mice also showed a defective uptake of ^{125}I-β_2-microglobulin and a decrease of cubilin expression in proximal tubule cells, with urinary loss of cubilin ligands, such as transferrin (Jouret *et al.* 2007). These changes were paralleled by an abnormal shedding of cubilin in the urine, which could be due to the improper processing or trafficking in relation to the lack of CFTR.

Almost 90% of Caucasian CF patients have at least one ΔF508 allele, with retention of ΔF508-CFTR in the endoplasmic reticulum (ER) and subsequent degradation via the ubiquitin–proteasome pathway (Rowe *et al.* 2005). Nevertheless, the ΔF508-CFTR can function as a cAMP-regulated Cl^- channel in distinct permissive conditions (Pasyk & Foskett, 1995). Mice homozygous

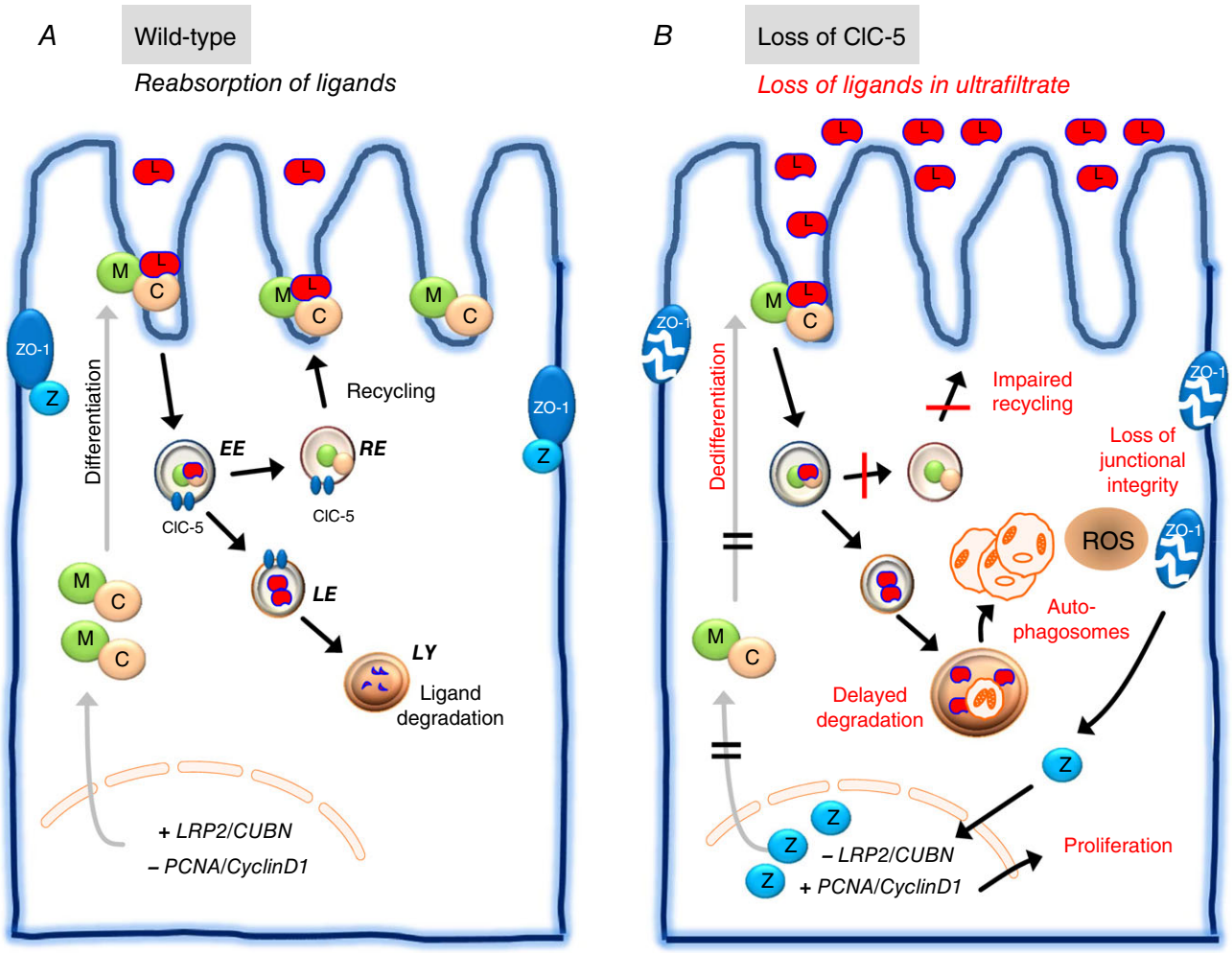

Figure 3. Potential mechanisms involved in the proximal tubule dysfunction secondary to the loss of ClC-5
The functional loss of ClC-5 leads to defective Cl^- transport in the early endosomes, associated with impaired trafficking and recycling of apical receptors, defective receptor-mediated endocytosis and urinary loss of LMW ligands. The loss of ClC-5 is also associated with impaired lysosomal function, which might compromise the lysosomal mediated-degradation and clearance of autophagosomes containing ubiquitinated proteins and dysfunctional mitochondria, leading to excessive production of reactive oxygen species (ROS). These changes might disrupt the integrity of the junctional complex proteins [i.e. zonula occludens-1 (ZO-1)] and release the ZO-1-associated nucleic acid-binding protein (ZONAB) transcription factor (Z). In turn, ZONAB translocates to the nucleus to promote cell proliferation [proliferating cell nuclear antigen (PCNA) and cyclin D1] and potentially repress the transcription of megalin (LRP2)/cubilin (CUBN). It is important to stress that Dent's disease is caused not by a defective endosomal acidification but rather by a defective endosomal accumulation of Cl^- ions (see main text for details). Abbreviations: EE, early endosomes; L, low-molecular-weight ligands; LE, late endosomes, LY, lysosomes; RE, recycling endosomes.

for a ΔF508 mutation ($Cftr^{\Delta F/\Delta F}$) have a typical CF manifestations, including growth retardation, hypertrophy of intestinal goblet cells and higher basal nasal potential difference. The $Cftr^{\Delta F/\Delta F}$ tissues also show a residual Cl^- conductance, suggesting that at least some of the mutant ΔF508-CFTR reaches the plasma membrane. The mRNA abundance of ΔF508-CFTR was ∼2-fold reduced in $Cftr^{\Delta F/\Delta F}$ kidney *versus* control kidney, with a variable expression in the apical area of proximal tubule cells (Jouret *et al.* 2007). Study of a large cohort of CF patients harbouring at least one ΔF508 mutation showed a significant albuminuria and LMW proteinuria *versus* healthy control subjects and patients with chronic lung inflammation due to active asthma (Jouret *et al.* 2007). These changes may affect long-term renal function, because proximal tubule dysfunction can trigger tubulo-interstitial injury and chronic kidney disease. Moreover, the urinary loss of transferrin could participate in the iron deficiency and lower circulating transferrin levels that are observed in CF patients (O'Connor *et al.* 2002).

Functional differences between CFTR and ClC-5 in the proximal tubule

In mouse kidney, CFTR is strongly detected in the apical area of proximal tubule cells lining the S3 segment (pars recta), whereas the distribution of megalin and ClC-5 is much more extensive, including the S1 and S2 segments (pars convoluta), as well as the pars recta. The segmental location of the ΔF508-CFTR was similar to that of normal CFTR, although the signal was weaker (Jouret *et al.* 2007). The functional counterpart of the distribution of CFTR and ClC-5 in the proximal tubule was provided by comparing endocytosis and gentamycin handling in $Cftr^{\Delta F/\Delta F}$ *versus* $Clcn5^{Y/-}$ (ClC-5 KO) mice. The $Cftr^{\Delta F/\Delta F}$ mouse, which bears the ΔF508 mutation, shows a residual Cl^- conductance (Raggi *et al.* 2011). Aminoglycosides are among the most commonly used antibiotics worldwide, because of their activity against Gram-negative bacteria combined with a low rate of resistance. However, the clinical use of aminoglycosides is limited by their renal toxicity, which is caused by the megalin-mediated accumulation in proximal tubule cells (Lopez-Novoa *et al.* 2011).

The $Cftr^{\Delta F/\Delta F}$ mice showed a significant albuminuria and LMW proteinuria, but much milder than the changes observed in $Clcn5^{Y/-}$ mice. These differences were also reflected by gentamicin uptake; in comparison to control animals, $Cftr^{\Delta F/\Delta F}$ and $Clcn5^{Y/-}$ mice showed a 15 and 85% decrease in gentamycin accumulation in the kidney, respectively (despite similar peak gentamycin blood concentrations). Studies on primary cultures of $Cftr^{\Delta F/\Delta F}$ and $Clcn5^{Y/-}$ mouse proximal tubule cells confirmed the reduction in gentamycin uptake, with many fewer gentamycin-positive vesicles in the early endosomal and lysosomal compartments (Raggi *et al.* 2011). These studies demonstrated that the functional loss of ClC-5 or CFTR is reflected by a decrease of receptor-mediated endocytosis, which is much more important in pronounced effect in the $Clcn5^{Y/-}$ mice compared with the $Cftr^{\Delta F/\Delta F}$ mice. The difference probably reflects specific expression profiles of the transporters in the proximal tubule, distinct activities of these segments, as well as the residual activity of the mutant ΔF508-CFTR in the epithelial cells (Jouret *et al.* 2007; Raggi *et al.* 2011).

Clinical and physiological perspectives

Since the discovery of ClC-5 and its association with Dent's disease in 1996, a large amount of research has yielded valuable insights pointing to the clinical and physiological importance of these transport proteins.

Chloride transporters encoded by members of the *CLCN* gene family are involved in rare disorders affecting various tubular segments of the kidney. The Cl^- channel ClC-Kb is involved in salt-losing tubulopathies affecting the distal nephron (Bartter and Gitelman syndromes), whereas the Cl^-–H^+ exchanger ClC-5 is involved in Dent's disease, a model for generalized dysfunction of the proximal tubule (renal Fanconi syndrome). Dent's disease is particularly important because it may lead to multisystemic complications and a risk of developing chronic kidney disease. Investigations on mouse and cellular models of Dent's disease pointed to the importance of receptor-mediated endocytosis for the reabsorptive function of the proximal tubule in general and the processing of albumin and LMW proteins and the metabolism of vitamins in particular. Defective proximal tubule transport functions can readily be detected by urinalysis (glucose, amino acids, phosphate, uric acid, albumin and LMW proteinuria). These markers of proximal tubule dysfunction evidenced a defective renal endocytosis component in cystic fibrosis, which is explained by the distribution of CFTR in the endosomes of proximal tubule cells. The consequences of the severe defect in receptor-mediated endocytosis associated with loss-of-function mutations of ClC-5 include dedifferentiation and oxidative stress in proximal tubule cells, which is probably instrumental in the progression to renal failure. The moderate but significant defect in LMW protein handling by the kidney of CF patients should be kept in mind because the spectrum of CF manifestations has broadened considerably over the last decade, reflecting improved care and increased survival.

The seminal studies on the function and structure of ClC-5 (and of CFTR) illustrate the importance of

identifying critical residues involved in gating, as well as the power of genetic engineering in the mouse. The availability of detailed mouse models provided information on issues linking receptor-mediated endocytosis with vesicular acidification, trafficking and recycling and cell differentiation, which have puzzled cell biologists for decades. In particular, these studies point to the physiological requirement for chloride along the endocytic apparatus which is instrumental for the uptake capacities of proximal tubule cells.

Despite the advances summarized above, many questions remain open regarding the molecular basis of proximal tubule dysfunction caused by the functional loss of ClC-5. For instance, the link between potential changes in vesicular chloride concentrations and membrane traffic in proximal tubule cells remains to be defined. The role of other channels and transporters present in endosomes, as well as the compensatory mechanisms in case of loss of function are unknown. The regulation of megalin/cubilin expression and the mechanisms responsible for the switch between proliferation and differentiation associated with a defective endolysosomal pathway need to be characterized. Clinical issues include the following: deciphering the link between dysfunction of the proximal tubule cell and progression of kidney disease; mechanisms of hypercalciuria and kidney stones; and factors involved in the (individual and familial) variability of the phenotype of Dent's disease. The evidence of genetic heterogeneity in Dent's disease and the fact that inherited forms of renal Fanconi syndrome remain unexplained suggest that new genes relevant for proximal tubule function and, potentially, for the endosome–lysosome function, remain to be discovered. Insights into these areas will lead to a better understanding of the fundamental processes affecting epithelial cell differentiation, regulation of transport mechanisms and role of tubular cells in renal disease progression.

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Additional information

Competing interests

None declared.

Author contributions

Both authors contributed to the manuscript and approved its final version.

Funding

The work of the authors has been supported by the Belgian agencies FNRS and FRSM; the Foundation Alphonse et Jean Forton; Concerted Research Actions; the Association Belge de Lutte contre la Mucoviscidose; the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 305608 (EURenOmics); the Cystinosis Research Foundation (Irvine, CA, USA); the Swiss National Science Foundation project grant 310030_146490 and NCCR Kidney.CH program; and MINZ and RADIZ, the KFSP programs of the University of Zurich.

Acknowledgements

The authors wish to thank R. Beauwens (Brussels), E. Christensen (Aarhus), P. Courtoy (Brussels), W. B. Guggino (Baltimore), T. Jentsch (Berlin) and R. V. Thakker (Oxford) for fruitful discussions and/or collaborations over the years.

Supporting information

The following supporting information is available in the online version of this article.

Figure S1
Figure S2