Loss of the endosomal anion transport protein ClC-5 impairs renal endocytosis and underlies human Dent’s disease. ClC-5 is thought to promote endocytosis by facilitating endosomal acidification through neutralization of proton pump currents. However ClC-5 is a 2Cl-/H+ -exchanger rather than a Cl- channel. We generated mice carrying the uncoupling E211A (unc) mutation that converts ClC-5 into a pure Cl- conductor. ATP-dependent acidification of renal endosomes was reduced in ClC-5 knock-out mice, but normal in Clcn5unc mice. Surprisingly, however, their proximal tubular endocytosis was also impaired. Thus endosomal chloride concentration, which is raised by ClC-5 in exchange for protons accumulated by the H+-ATPase, may play a role in endocytosis.

Luminal acidification along the endocytic pathway serves many purposes (1) including the progression of endocytosis itself (2). It is performed by endosomal H+-ATPases that need a countercurrent for electroneutrality. Because this current depends on chloride, conventional wisdom suggests (1) that endosomal Cl- channels are involved (fig. S1A). ClC-5 was thought to embody this channel in proximal tubular endosomes (3-5). Disruption of ClC-5 impairs renal endosomal acidification in vitro (5) and drastically reduces proximal tubular endocytosis in mice and humans (4, 6, 7). The hyperphosphaturia and hypercalciura that lead to kidney stones in Dent’s disease have been attributed to impaired proximal tubular endocytosis of calcitropic hormones (4). Recently, however, it has been shown (8-10) that ClC-5 is a 2Cl/H+ exchanger rather than a Cl- channel. It seems counterintuitive that such an exchanger should neutralize pump currents because it mediates H+-efflux during ATP-driven acidification. The biological significance of proton coupling has remained enigmatic (11).

If the ClC-5 Cl/H+-exchanger could be converted into an uncoupled Cl- conductor, it should efficiently facilitate endosomal acidification. Phenotypes of mice carrying such a mutation can’t be attributed to impaired endosomal acidification, but specifically to a loss of coupling chloride- to proton-gradients. A mutation in the ‘gating’ glutamate of CLC exchangers (12) suffices to convert them into pure anion conductors (8, 9, 13-15). We inserted the corresponding, well-characterized E211A mutation (8, 9, 14) into the Clcn5 gene on mouse chromosome X and created Clcn5unc knock-in mice in which ClC-5 was converted into an uncoupled Cl- conductor (figs. S1A and S2). The mutant protein was expressed at WT levels (Fig. 1A). No change was observed in its subcellular localization in kidney proximal tubular and intercalated cells (Fig. 1B and fig. S3). The renal expression of the related CIC-3 and CIC-4 proteins was not affected, either (fig. S4).

To test whether the uncoupled ClC-5unc mutant supported endosomal acidification, we added ATP to endosomal fractions from renal cortex (containing mainly proximal tubules) of WT or Clcn5unc mice and monitored vesicular pH using acridine orange fluorescence. H+-ATPase-driven acidification of WT and Clcn5unc vesicles occurred with similar efficiency, but was severely reduced with ClC-5 knock-out (KO) endosomes as expected (5, 16) (Fig. 1C- E).

Despite maintaining active endosomal acidification, Clcn5unc mice displayed abnormalities found in CIC-5 KO (Clcn5-) mice and patients with Dent’s disease (4, 7, 17) like low molecular weight proteinuria (Fig. 2, A and B), hyperphosphaturia and hypercalciuria (table S1). Proteinuria of Clcn5- mice results from impaired proximal tubular endocytosis (4, 7) which was studied in chimeric tubules resulting from random X-chromosomal inactivation in female Clcn5+/+ mice (4). In those tubules, WT and KO cells were distinguished by ClC-5 antibodies (3, 4), but this approach cannot differentiate between cells expressing WT or uncoupled ClC-5 in Clcn5unc tubules. Rather than epitope-tagging the E211A mutant, which might interfere with its function, we generated knock-in mice in which the C-terminus of ClC-5 was converted to that of CIC-3 (fig. S1B,C and S5). The generation of this Clcn5* allele required only two amino acid exchanges and changed neither ClC-5.

Endosomal Chloride-Proton Exchange Rather Than Chloride Conductance Is Crucial for Renal Endocytosis

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However, cells expressing the allele or WT ClC-5 was indistinguishable (fig. S6B). From the plasma membrane, endocytic vesicles may contain of ClC-5(E211A) could not even partially substitute for Cl- megalin (Fig. 3C). Thus unexpectedly the anion conductance endocytosis (fig. S8), nor in the localization and abundance of detected in receptor-mediated (Fig. 2E) or fluid-phase might have affected endocytosis less than a loss of ClC-5. We increase of PTH and excessive stimulation of apical PTH-filtered parathyroid hormone (PTH), entailing a luminal current of and C) and cubilin (Fig. 3B), a finding ascribed to impaired recycling to the plasma membrane (4, 18). In Clcn5+ mice, the sodium-phosphate cotransporter NaPi-2a was shifted from the apical membrane to intracellular vesicles and its overall abundance was reduced (fig. S7), explaining the observed hyperphosphaturia. Similarly increased endocytosis of NaPi-2a in Clcn5+ mice was attributed to reduced endocytosis of filtered parathyroid hormone (PTH), entailing a luminal increase of PTH and excessive stimulation of apical PTH-receptors (4, 5).

Because the E211A mutation did not abolish CIC-5 currents and maintained endosomal acidification (Fig. 1C), it might have affected endocytosis less than a loss of CIC-5. We thus compared Clcn5+ and Clcn5- cells side-by-side in chimeric tubules of Clcn5+/-- females. No differences were detected in receptor-mediated (Fig. 2E) or fluid-phase endocytosis (fig. S8), nor in the localization and abundance of megalin (Fig. 3C). Thus unexpectedly the anion conductance of CIC-5(E211A) could not even partially substitute for Cl- /H+-exchange in supporting proximal tubular endocytosis and normal localization of apical receptors. Because the intramembrane E211A point mutation was unlikely to have changed CIC-5 protein interactions, the pathology of Clcn5+ mice is neither caused by the disruption of a macromolecular CIC-5-containing endocytic complex (19, 20), nor by the loss of interaction with KIF3B (21), mechanisms suggested to underlie Dent’s disease. The impairment of endocytosis in Dent’s disease is neither likely to result only from reduced ATP-dependent endosomal acidification (3), but is related to an uncoupling of Cl- from H+-ATPase-independent acidification by exchanging cystolic H+ for intravesicular Cl- at an early step of endocytosis (9). Shortly after pinching off from the plasma membrane, endocytic vesicles may contain the high extracellular chloride concentration. A selective lack of such an initial, Cl-gradient-driven endosomal acidification might explain the impaired endocytosis of both Clcn5+ and Clcn7+ mice. However, endosomal [Cl] was found to be initially low, a finding ascribed to surface charge effects (16). Furthermore, a lack of a Cl-gradient-driven acidification cannot be invoked for the severe lysosomal pathology of Clcn7+/+ (22, 23) and Clcn7+/- mice (24) because their lysosomal pH is normal (22-24) and because this mechanism would require a substantial Cl supply to lysosomes. The surprisingly similar pathologies of Clcn5+ and Clcn7+/- mice might be due to reduced endosomal Cl- accumulation in each mouse model. Whereas Cl- channels operating in parallel to an H+-ATPase would raise intravesicular Cl during active acidification, stoichiometric 2Cl-/H+ exchange would maintain high vesicular Cl- concentration also under steady state. Indeed, lysosomes containing the WT CIC-7 Cl- /H+ exchanger display higher [Cl-] than their Clcn7+/+ or Clcn7+/- counterparts (24). Furthermore, endosomal [Cl-] might regulate endosomal Ca2+ channels (25). The analysis of Clcn5+ and Clcn7+/- mice suggests that luminal anion concentration is important all along the endosomal-lysosomal pathway.

References and Notes
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Fig. 1. Renal endosomal acidification of knock-in mice converting ClC-5 into a chloride conductor. (A) Immunoblot for ClC-5 using kidney membranes of different genotypes with antibodies against the N-terminus (left) or the C-terminus (C5/05A) (right). [* indicates mutated C-terminus of ClC-5 (fig. S5)]. (B) Identical staining pattern using the C-terminal PEP5E ClC-5 antibody (J) in proximal tubules of WT and Clcn5 uncle mice. Scale bar, 5 μm. (C to D) Averaged traces of acridine orange fluorescence comparing ATP-driven acidification of renal cortical vesicles from WT (green) and Clcn5 uncle (red) (C), and WT (green) and Clcn5 (black) (D) mice. (Identical WT traces). Reduced fluorescence reflects vesicular acidification. The protonophore FCCP was added as control. F0, fluorescence at t=0. (E) Averaged total change in fluorescence. Data averaged from 25 (+/y), 13 (-/y) and 10 (unc/y) experiments with material obtained from ≥4 independent vesicle preparations per genotype. Error bars, SEM. *P ≤ 0.05; **P ≤ 0.01.

Fig. 2. Proteinuria and impaired endocytosis in Clcn5- and Clcn5 uncle mice. (A) Urinary proteins analyzed by SDS-PAGE and silver staining. (B) Immunoblot for vitamin D binding protein (DBP) and retinol binding protein (RBP) in urine. (C) In vivo 10-min uptake of Alexa Fluor 546-labeled β-lactoglobulin (red) showed decreased receptor-mediated endocytosis in Clcn5 uncle-expressing cells in a chimeric proximal tubule from a Clcn5 uncle/* female. Clcn5-UNC, but not the CIC-5* ClC/H+ -exchanger, is recognized by the C5/05A antibody (green). Right, overlay with additional brush-border staining for villin (blue). (D) Reduced fluid-phase endocytosis of Alexa Fluor 488-labeled dextran in Clcn5 uncle-expressing cells, in an experiment similar to (C). (E) Similar β-lactoglobulin-uptake of cells lacking ClC-5 or expressing Clcn5 uncle in a Clcn5 uncle/* female. Scale bar, 5 μm (C), 3.2 μm (D) and 6 μm (E).