

## Functional coupling of V-ATPase and CLC-5

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### Abstract

Dent's disease is an X-linked renal tubulopathy characterized by low molecular weight proteinuria, hypercalciuria and progressive renal failure. Disease aetiology is associated with mutations in the *CLCN5* gene coding for the electrogenic  $2\text{Cl}^-/\text{H}^+$  antiporter chloride channel 5 (CLC-5), which is expressed in the apical endosomes of renal proximal tubules with the vacuolar type  $\text{H}^+$ -ATPase (V-ATPase). Initially identified as a member of the CLC family of  $\text{Cl}^-$  channels, CLC-5 was presumed to provide  $\text{Cl}^-$  shunt into the endosomal lumen to dissipate  $\text{H}^+$  accumulation by V-ATPase, thereby facilitating efficient endosomal acidification. However, recent findings showing that CLC-5 is in fact not a  $\text{Cl}^-$  channel but a  $2\text{Cl}^-/\text{H}^+$  antiporter challenged this classical shunt model, leading to a renewed and intense debate on its physiological roles.  $\text{Cl}^-$  accumulation *via* CLC-5 is predicted to play a critical role in endocytosis, as illustrated in mice carrying an artificial  $\text{Cl}^-$  channel mutation E211A that developed defective endocytosis but normal endosomal acidification. Conversely, a recent functional analysis of a newly identified disease-causing  $\text{Cl}^-$  channel mutation E211Q in a patient with typical Dent's disease confirmed the functional coupling between V-ATPase and CLC-5 in endosomal acidification, lending support to the classical shunt model. In this editorial, we will address the current recognition of the physiological role of CLC-5 with a specific focus on the functional coupling of V-ATPase and CLC-5.

**Key words:** V-ATPase; CLC-5; Endocytosis; Endosomal acidification; Dent's disease; Gating glutamate; E211Q

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**Core tip:** Chloride channel 5 (CLC-5) mutations cause Dent's disease, which is characterized by renal proximal

tubulopathy due to defective endocytosis. Recent revelations that CLC-5 is a  $2\text{Cl}^-/\text{H}^+$  antiporter and not a  $\text{Cl}^-$  channel challenged the classical model proposing CLC-5 as a  $\text{Cl}^-$  shunt to facilitate V-ATPase-mediated endosomal acidification. Therefore, physiological roles of CLC-5 and its interaction with V-ATPase in endosomal acidification and/or endocytosis are intensely debated. Recent functional analysis of a novel pure  $\text{Cl}^-$  channel mutant from a Dent's disease patient indicated a possible functional coupling between V-ATPase and CLC-5 not only in endosomal acidification but also at the plasma membrane.

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## INTRODUCTION

Renal proximal tubular cells have a high capacity for the uptake of various proteins in primary urine *via* receptor-mediated endocytosis. Acidified condition in intracellular organelles such as endosomes and lysosomes is essential for the normal endocytic pathway consisting of ligand-receptor dissociation, recycling of the uncoupled receptors to the cell surface and transport of the released ligands to lysosomes for degradation<sup>[1,2]</sup>.

Intravesicular acidification is achieved mainly by the active  $\text{H}^+$ -pumping of vacuolar ATPase (V-ATPase)<sup>[1,3]</sup>. Conversely, chloride channel 5 (CLC-5), long believed to be a pure chloride channel as the name implies, colocalizes with V-ATPase and is suggested to be involved in V-ATPase-mediated endosomal acidification by shunting  $\text{Cl}^-$  to neutralize the positive charge due to  $\text{H}^+$  accumulation<sup>[4]</sup>. Indeed, mutations in CLC-5 cause Dent's disease, which is characterized by renal proximal tubulopathy due to defective endocytosis<sup>[5,6]</sup>, suggesting that V-ATPase and CLC-5 are functionally coupled in endosomes. However, recent reports demonstrating that CLC-5 is not a  $\text{Cl}^-$  channel but in fact functions as a  $2\text{Cl}^-/\text{H}^+$  antiporter demands re-evaluation of its physiological roles and the pathogenesis of Dent's disease<sup>[7-9]</sup>. Although the debate is ongoing on whether  $\text{Cl}^-$  accumulation<sup>[10]</sup> or CLC-5-induced V-ATPase activation is more important for normal endocytosis, we recently demonstrated that impaired endosomal acidification derived from inadequate V-ATPase activation by mutated CLC-5 may be the underlying pathology in Dent's disease<sup>[11]</sup>. Moreover, we identified such functional relationship between V-ATPase and CLC-5 even at the plasma membrane of mouse proximal tubules.

## CLC-5 AND DENT'S DISEASE

CLC-5, a member of the CLC family, was originally identified by cloning of a voltage-gated chloride channel, CLC-0, from *Torpedo marmorata* electric organ<sup>[12]</sup>. Several human mutations in corresponding genes of the

CLC family are known to cause genetic disorders such as myotonia congenita (*CLC-1*), Barter syndrome (*CLC-Kb*), osteopetrosis (*CLC-7*) and Dent's disease (*CLC-5*)<sup>[13,14]</sup>.

Dent's disease is an X-linked proximal renal tubulopathy arising from mutations in the *CLCN5* gene encoding for the electrogenic  $2\text{Cl}^-/\text{H}^+$  antiporter CLC-5<sup>[7-9]</sup> and is characterized by low molecular weight (LMW) proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis and slowly progressive renal failure<sup>[15,16]</sup>. In addition, affected patients present with various clinical signs of proximal tubular dysfunction including glycosuria, aminoaciduria, hyperphosphaturia and uricosuria, which is consistent with partial Fanconi syndrome<sup>[5]</sup>. These manifestations usually remain subtle or asymptomatic during childhood; however, Dent's disease leads to chronic renal insufficiency over a few decades. Furthermore, in the absence of effective therapy, early diagnosis for Dent's disease only allows for conservative therapy to prevent kidney stones and progression of chronic renal insufficiency<sup>[16]</sup>.

## TISSUE AND SUBCELLULAR DISTRIBUTION OF CLC-5

CLC-5 is abundantly expressed in renal and intestinal epithelial cells, though it is also expressed in brain, lung and, to a lesser extent, liver<sup>[17]</sup>. In the kidney, CLC-5 expression is highest in proximal tubules and intercalated cells of the collecting ducts<sup>[18]</sup>. Especially in proximal tubules, a major site for urinary LMW protein reabsorption *via* receptor-mediated endocytosis<sup>[17,19]</sup>, CLC-5 is predominantly located in early endosomes, colocalizing with V-ATPase<sup>[4,20]</sup>. Additionally, CLC-5 is also found to an extent in the apical membrane of proximal tubules, as suggested by its robust plasma membrane expression in HEK293 cells as well as in *Xenopus laevis* oocytes following heterologous overexpression<sup>[4,18]</sup>.

## FUNCTIONS OF INTRACELLULAR V-ATPASE

V-ATPase is a large multi-subunit complex that is ubiquitously expressed in intracellular organelles of eukaryotic cells such as endosomes, lysosomes, secretory granules and trans-Golgi network<sup>[21,22]</sup>. V-ATPase pumps  $\text{H}^+$  across membranes using energy generated by ATP hydrolysis and provides an acidic intraorganellar environment that is critical for normal endocytic pathway<sup>[22-24]</sup>. Thus, V-ATPase lowers luminal pH of early endosomes and promotes the dissociation of internalized ligand-receptor complexes, which is essential for both recycling of the uncoupled receptors to cell surface and transport of the released ligands first to late endosomes and then to lysosomes for protein degradation<sup>[24-27]</sup>.

## FUNCTIONS OF PLASMA MEMBRANE V-ATPASE

In addition to intracellular organelles, V-ATPase is also

highly expressed in plasma membrane of specialized cells in kidney and other tissues. Though V-ATPase is found over the entire length of the nephron, it is abundantly expressed in renal proximal tubular cells<sup>[2,28,29]</sup> and intercalated cells of the collecting duct<sup>[21,29,30]</sup>, playing a pivotal role in acid-base homeostasis.

Proximal tubular cells secrete  $H^+$  from cytosol to tubular lumen *via*  $Na^+/H^+$  exchangers (NHEs), mainly NHE3<sup>[31]</sup> on apical membrane, and reabsorb approximately 80% of the filtered  $HCO_3^-$ <sup>[32-34]</sup>. V-ATPase is assumed to be partially involved in this reabsorption process along the proximal tubules<sup>[26,35,36]</sup>. In the collecting duct, alpha-intercalated cells secrete  $H^+$  into the urine *via* apical membrane V-ATPase, while beta-intercalated cells export  $H^+$  into the vessel lumen *via* basolateral membrane V-ATPase, regulating final urine acidification. Genetic defects in specific V-ATPase subunits are known to cause renal tubular acidosis (RTA)<sup>[37-39]</sup>. Specifically, mutations in *ATP6VOA4* and *ATP6VOB1* coding for  $\alpha 4$  and B1 subunits, respectively, lead to distal RTA in humans<sup>[38,40]</sup>.

## NONCANONICAL FUNCTIONS OF INTRACELLULAR V-ATPASE

While the conventional functions of intracellular V-ATPase are well established, recent studies suggested that V-ATPase might have noncanonical functions as well<sup>[41]</sup>. For example, V-ATPase achieves the desired acidic endosomal pH in order to regulate the budding of endosomal carrier vesicles, where V-ATPase itself functions as not only a proton pump but also a pH sensor<sup>[1,42,43]</sup>. Luminal pH information is hypothesized to be detected by V-ATPase and transmitted to the cytosolic side *via* conformational changes in its transmembrane  $\alpha 2$  isoform. This in turn results in the recruitment of ADP-ribosylation factor (ARF)-6 (ARF-6) and ARF-nucleotide binding-site opener (ARNO), both of which are involved in endocytosis by regulating the formation of endosomal carrier vesicles. Conversely, lysosomal V-ATPase is also suggested to constitute an important component of the lysosomal-associated amino acid sensing machinery<sup>[44]</sup>. Although the precise mechanism underlying this function of V-ATPase is unknown, accumulation of amino acids in lysosomes activates Rag guanosine triphosphatases (GTPases)<sup>[45]</sup> that promote the translocation of the master growth regulator mechanistic target of rapamycin complex 1 (mTORC1) to the lysosomal membrane<sup>[46,47]</sup>. In this process, the interaction between V-ATPase and the regulator that anchors Rag GTPases to the lysosomes is necessary for amino acid-induced signal transduction<sup>[48]</sup>. Therefore, lysosomal V-ATPase performs pivotal roles as both a proton pump and an amino acid sensor transmitting signals to activate mTORC1, which is essential for lysosomal function<sup>[49]</sup>.

## FUNCTIONAL COUPLING OF V-ATPASE AND CLC-5

As indicated above, active  $H^+$  pumping by V-ATPase

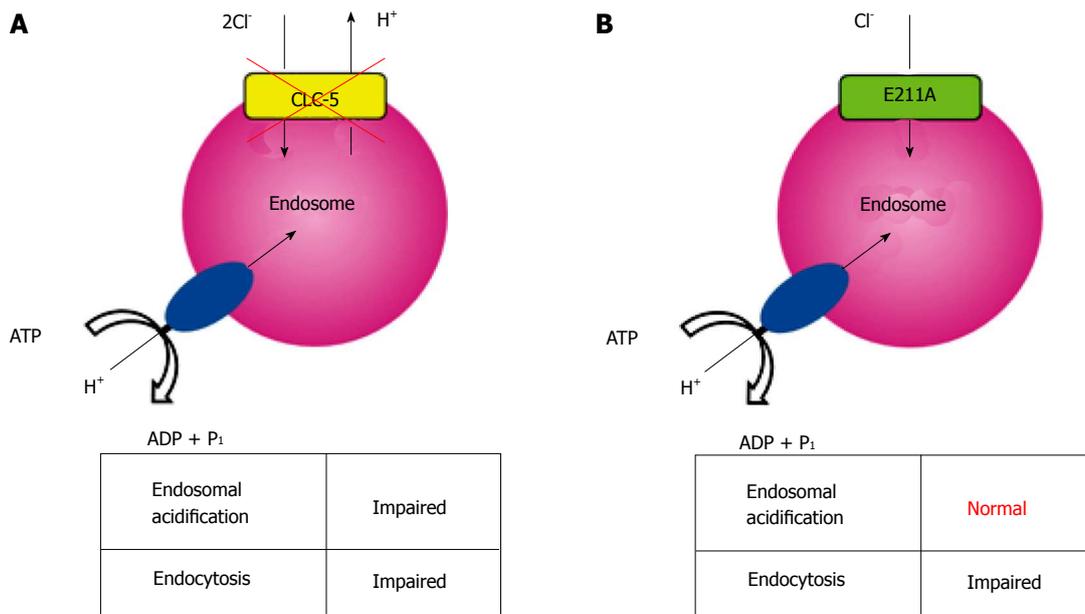
contributes to the preservation of an acidic luminal pH within intracellular organelles including endosomes and lysosomes, which is required for normal endocytic process. In contrast, two different strains of CLC-5 knockout mice developed LMW proteinuria, typical symptoms of Dent's disease due to defective endocytosis<sup>[50,51]</sup>. Furthermore, Günther *et al.*<sup>[6]</sup> showed that endosomes isolated from CLC-5 knockout mice were acidified at a significantly lower rate and to a lesser extent than those from wild-type mice. Subsequent analyses verified the lower luminal concentrations of  $Cl^-$  and  $H^+$  in early endosomes isolated from proximal tubules of CLC-5 knockout mice<sup>[52]</sup>. These observations, in conjunction with the colocalization of CLC-5 with V-ATPase in early endosomes, strongly suggest that V-ATPase and CLC-5 are functionally coupled during endosomal acidification and/or endocytosis.

Indeed, since its identification as a member of the CLC family, CLC-5 was considered to be a  $Cl^-$  channel<sup>[53]</sup> that provided  $Cl^-$  to counter and dissipate positive charge ( $H^+$ ) accumulation generated by V-ATPase, thereby facilitating efficient endosomal acidification. This  $Cl^-$  shunt model facilitated by functional V-ATPase coupling with CLC-5 was considered essential for normal endocytosis<sup>[25]</sup>. However, recent studies demonstrating that CLC-5 is not a  $Cl^-$  channel but a  $2Cl^-/H^+$  antiporter forced comprehensive reevaluation of these physiological roles of CLC-5<sup>[7-9]</sup>, given that the  $2Cl^-/H^+$  antiporter would result in ineffective acidification due to parallel  $H^+$  efflux at the expense of wasted energy (ATP) by V-ATPase. Thus, physiological roles of CLC-5 as a  $2Cl^-/H^+$  antiporter and its interaction with V-ATPase in endosomal acidification and/or endocytosis remain unknown and have become an important issue in the field.

## PHYSIOLOGICAL ROLES OF CLC-5 IN ENDOCYTOSIS: $Cl^-$ ACCUMULATION OR V-ATPASE ACTIVATION?

Analysis of a mutation in the so-called gating glutamate at position 211 (E211), a conserved residue that serves as a central gate for  $H^+$ -coupled  $Cl^-$  transport<sup>[54,55]</sup>, may provide a key in understanding the complex nature of CLC-5 functions. Novarino and colleagues generated mice carrying the E211A mutation which deprived  $H^+$  transport of CLC-5, altering it to a simple  $Cl^-$  conductance<sup>[10]</sup>, and these mice developed defective endocytosis similar to that observed in CLC-5 knockout mice. Surprisingly, however, endosomal acidification was preserved in E211A mice in contrast to CLC-5 knockout mice (Figure 1). Therefore, they proposed that endosomal  $Cl^-$  accumulation rather than endosomal acidification might be critical for renal endocytosis. However, mutations in the gating glutamate, such as that is present in E211A mutant mice, have not yet been identified in patients with Dent's disease.

We recently analyzed a previously unrecognized mutation of E211 to glutamine (E211Q) that was found in a typical Dent's disease patient<sup>[56]</sup> and confirmed that



**Figure 1 Effects of CLC-5 inactivation on endosomal acidification and endocytosis.** A: In CLC-5 knockout mice, both endosomal acidification and endocytosis are impaired, resulting in a Dent's disease-like phenotype; B: E211A, an artificial mutation of the gating glutamate of CLC-5, converts its function from a 2Cl<sup>-</sup>/H<sup>+</sup> antiporter into a pure Cl<sup>-</sup> conductance. E211A knock-in mice exhibit defective endocytosis and manifestations similar to those observed in Dent's disease manifestations but retain normal endosomal acidification.

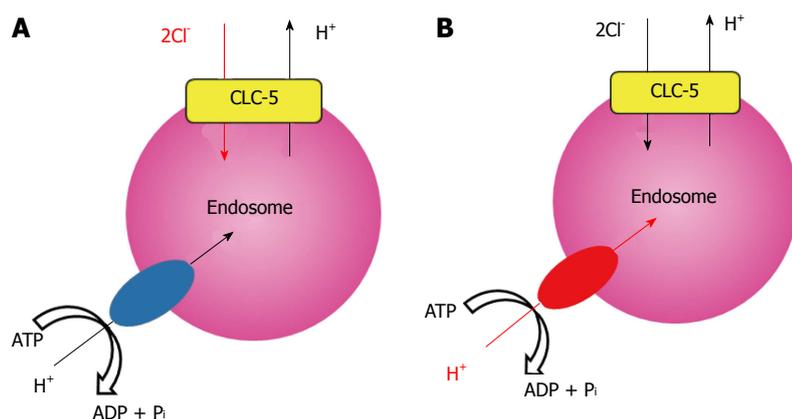
functional coupling between V-ATPase and CLC-5 occurred in endosomal acidification<sup>[11]</sup>. Electrophysiological studies in *Xenopus laevis* oocytes demonstrated that the disease-causing mutant E211Q had similar characteristics to the artificial mutant E211A. Thus, E211Q mutation also abolished H<sup>+</sup> transport of CLC-5 and altered it to a simple Cl<sup>-</sup> conductance, which was supported by the molecular modelling of CLC-5 mutants<sup>[11]</sup>. Upon heterologous overexpression in HEK293 cells, both Cl<sup>-</sup> channel mutants, E211Q and E211A, enhanced bafilomycin-sensitive endosomal acidification. However, acidification was greater in endosomes expressing wild-type CLC-5. Because CLC-5-induced endosomal acidification reflected V-ATPase activity, these results indicated that the 2Cl<sup>-</sup>/H<sup>+</sup> exchange mode of CLC-5 was required for maximal endosomal acidification<sup>[11]</sup>. Indeed, simulation studies on lysosomal acidification *via* CLC-7, another CLC member with Cl<sup>-</sup>/H<sup>+</sup> exchange function, lent support to the model in which the Cl<sup>-</sup>/H<sup>+</sup> exchange mode was more advantageous than the Cl<sup>-</sup> channel mode in intravesicular acidification<sup>[57,58]</sup>. Our results revealed that the conversion of CLC-5 from a 2Cl<sup>-</sup>/H<sup>+</sup> antiporter into a Cl<sup>-</sup> conductance is the definite cause of Dent's disease. More importantly, we showed that impaired endosomal acidification *via* inadequate V-ATPase activation should be considered as a pivotal component of the aetiology in Dent's disease. These potential roles of CLC-5 in endosome are summarized in Figure 2.

Based on the accumulating data, the cause of discrepancy in endosomal acidification by Cl<sup>-</sup> channel mutants between mice proximal tubules and HEK293 cells remains unclear. Significant basal acidification was still found in endocytic vesicles isolated from CLC-5 knockout mouse kidneys<sup>[6]</sup>. In contrast, in the absence of endogenous CLC-5<sup>[59]</sup>, the basal V-ATPase activity

in HEK293 cells was quite subtle. Therefore, a small difference in endosomal acidification generated by the E211A mutant that was detectable in HEK293 cells might have been overlooked in proximal tubules obtained from E211A mice. Of course, genetically altered mice could have potentially developed compensatory mechanisms. However, a convincing model for the interaction between CLC-5 and V-ATPase in endosomes continues to be a focus of intense debate.

## FUNCTIONAL COUPLING OF V-ATPASE AND CLC-5 AT THE PLASMA MEMBRANE

Physiological significance of CLC-5 at the plasma membrane is not clear<sup>[14]</sup>. Therefore, we also investigated the impact of CLC-5 on plasma membrane V-ATPase function by measuring hypotonicity-induced V-ATPase activity as previously reported<sup>[60,61]</sup>. Heterologous overexpression of both E211Q and E211A mutants in HEK293 cells led to the moderate activation of membrane V-ATPase. However, wild-type CLC-5 induced even higher V-ATPase activation, which was in harmony with the degree of endosomal acidification. This V-ATPase activation by hypotonicity was observed even in isolated mouse proximal tubules. Furthermore, siRNA-mediated gene silencing for CLC-5 strongly reduced V-ATPase activity, suggesting the presence of tight functional coupling between V-ATPase and CLC-5 even in apical membrane of intact proximal tubules. Although the detailed mechanisms by which CLC-5 activates the membrane V-ATPase are unknown, noncanonical roles of V-ATPase may allow several possible explanations. In addition to the abovementioned multiple



**Figure 2 Potential roles of CLC-5 in endosomes.** A: In this model, Cl<sup>-</sup> accumulation by CLC-5 is critical for normal endocytosis; B: In contrast, 2Cl<sup>-</sup>/H<sup>+</sup> exchange mode of CLC-5 activates V-ATPase which is required for maximal endosomal acidification.

V-ATPase functions, it is known that CLC-5 mediates the assembly with other proteins, and several binding proteins have already been proposed<sup>[14]</sup>. Therefore, it is possible that the 2Cl<sup>-</sup>/H<sup>+</sup> exchange mode of CLC-5 induces V-ATPase activation by recruiting unknown cellular factors and/or by directly modifying the function of V-ATPase<sup>[11]</sup>.

## CONCLUSION

Loss-of-function mutations in CLC-5 were definitely shown to cause Dent's disease phenotypes in humans as well as in mouse models, suggesting the indispensability of CLC-5 for normal endocytic pathway. However, it remains unclear whether Cl<sup>-</sup> accumulation or V-ATPase-mediated acidification by CLC-5 is more important for normal endocytosis. Our recent study focusing on disease-causing mechanisms of the E211Q mutant of CLC-5 revealed that impaired endosomal acidification caused by inadequate CLC-5-induced V-ATPase activation may play a key role in the aetiology of Dent's disease. However, future studies are necessary to clarify the potentially critical role of endosomal Cl<sup>-</sup> accumulation, as suggested by the findings in mice carrying the E211A mutation.

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