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► **To cite this version:**

S. Lourdel, Teddy Grand, Johanna Burgos, Wendy Gonzales, Pv Sepulveda, et al.. CIC-5 mutations associated with Dent's disease: a major role of the dimer interface. *Pflügers Archiv European Journal of Physiology*, 2012, 463 (2), pp.247-256. <10.1007/s00424-011-1052-0>. <hal-02452454>

HAL Id: hal-02452454

<https://hal.sorbonne-universite.fr/hal-02452454v1>

Submitted on 24 Jan 2020

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Journal:	<i>Pflugers Archiv-European Journal of Physiology</i>
Manuscript ID:	EJP-00246-2011.R1
Manuscript Type:	Invited Review
Date Submitted by the Author:	n/a
Complete List of Authors:	Lourdel, Stéphane; UPMC Univ Paris 06, UMR_S 872; INSERM, UMR_S 872; CNRS, ERL 7226 Grand, Teddy; UPMC Univ Paris 06, UMR_S 872; INSERM, UMR_S 872; CNRS, ERL 7226 Burgos, Johanna; Centro de Estudios Científicos (CECs) González, Wendy; Universidad de Talca, Centro de Bioinformática y Simulación Molecular Sepúlveda, Francisco; Centro de Estudios Científicos (CECs) Teulon, Jacques; UPMC Univ Paris 06, UMR_S 872; INSERM, UMR_S 872; CNRS, ERL 7226
Keywords:	Cl ⁻ channels, Cloned ion channels, Kidney, Electrophysiology, Renal proximal convoluted tubule

CIC-5 mutations associated with Dent's disease: a major role of the dimer interface

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Abstract

Dent's disease is an X-linked recessive disorder affecting the proximal tubules. Mutations in the $2\text{Cl}^-/\text{H}^+$ exchanger CIC-5 gene *CLCN5* are frequently associated with Dent's disease. Functional characterization of mutations of *CLCN5* have helped to elucidate the physiopathology of Dent's disease, and provided evidence that several different mechanisms underlie the CIC-5 dysfunction in Dent's disease. Modeling studies indicate that many *CLCN5* mutations are located at the interface between the monomers of CIC-5, demonstrating that this protein region plays an important role in Dent's disease. On the basis of functional data, *CLCN5* mutations can be divided into three different classes. Class 1 mutations impair processing and folding, and as a result, the CIC-5 mutants are retained within the endoplasmic reticulum and targeted for degradation by quality control mechanisms. Class 2 mutations induce a delay in protein processing and reduce the stability of CIC-5. As a consequence, the cell surface expression and currents of the CIC-5 mutants are lower. Class 3 mutations do not alter the trafficking of CIC-5 to the cell surface and early endosomes, but induce altered electrical activity. Here, we discuss the functional consequences of the three classes of *CLCN5* mutations on CIC-5 structure and function.

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Key words

Dent's disease; Chloride/proton exchanger; *CLCN5*; ClC-5; mutation.

For Peer Review

Genetics of Dent's disease

Dent's disease is a heterogeneous group of X-linked inherited disorders characterized by low molecular weight proteinuria, hypercalciuria, nephrocalcinosis, nephrolithiasis, and in many cases, renal failure [8,31,32,43,47,58]. Positional cloning analysis in families affected by Dent's disease has identified mutations of *CLCN5* (MIM #300009), which is located on chromosome Xp11.22 and encodes the electrogenic $2\text{Cl}^-/\text{H}^+$ exchanger CIC-5 [41,46]. Although approximately two-thirds of patients with Dent's disease exhibit mutations of *CLCN5*, mutations of the gene for the Lowe syndrome *OCRL1* located on chromosome Xq25, which encodes the phosphatidylinositol 4,5-bisphosphate 5-phosphatase OCRL1, have also been found in about 15% of patients with Dent's disease (MIM #300555) [24]. Of note, the other patients with Dent's disease do not harbour mutations in either of these genes, suggesting an involvement of other genes [59].

Pathophysiology of Dent's disease

CIC-5 belongs to the CIC family of membrane proteins originally thought to function as Cl^- channels and consequently this was presumed to be its function upon its first identification [49]. In the kidney, CIC-5 is predominantly present in the early endosomes of proximal tubule cells and, to a lesser extent, in the thick ascending limb of Henle's loop and in the intercalated cells of the collecting duct [9,11,21,45,50,55]. CIC-5 knock-out mouse models have provided important clues about the mechanisms underlying Dent's disease. These mice reproduce the most common features of Dent's disease, such as low molecular weight proteinuria [22,42,53]. Because of its co-distribution with v-type H^+ -ATPase in early endosomes, it has long been suggested that CIC-5 may provide Cl^- shunt conductance that

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3 permits efficient intraluminal acidification of these organelles by v-type H⁺-ATPase. CIC-5
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5 loss-of-function would therefore impair endosomal acidification [21-23,42]. This could
6
7 explain the defective endocytosis observed in Dent's disease, because progression along the
8
9 endocytic pathway depends on an acidic endosomal lumen. However, the direct measurement
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11 of pH in endocytic vesicles in CIC-5 knock-out mice showed that acidification was reduced,
12
13 but not abolished [22,23]. In addition, CIC-5 disruption also led to a trafficking defect of
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15 megalin and cubilin, two multiligands receptors for endocytosis that are heavily expressed at
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17 the brush border of proximal tubule cells [6,42].
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21 The discovery that CIC-5, together with other intracellular CIC proteins, functions as
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23 an obligatory 2Cl⁻/H⁺ exchanger presented the problem that shunting the electrical current of
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25 v-type H⁺-ATPase via Cl⁻ flux would entail wasteful H⁺ recycling. To unravel the role of CIC-
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27 5 in proximal tubule endocytosis, Novarino *et al.* have more recently generated mice that
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29 carry a mutation of a critical glutamate residue (i.e. the "gating glutamate", see below) that
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31 converts CIC-5 into a pure Cl⁻ conductor [40]. Surprisingly, despite normal endosomal
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33 acidification, the mice exhibited impaired proximal tubule endocytosis like CIC-5 knock-out
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35 mice. As observed in patients with Dent's disease and CIC-5 knock-out mice, these mice also
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37 showed low molecular weight proteinuria, hypercalciuria and hyperphosphaturia. This work
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39 indicates that modulation of the endosomal Cl⁻ concentration in the endosomes of the
40
41 proximal tubule cells during H⁺ transport by CIC-5 may play a crucial role in proximal tubule
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43 endocytosis. In addition to its prominent endosomal expression, small amounts of CIC-5 are
44
45 also found on the apical surface of proximal tubule cells, where it may play additional roles.
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47 This might arise from a role in the protein-protein interactions required for receptor-mediated
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49 endocytosis and in microtubular transport as a result of associations with other proteins such
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51 as cofilin, a protein involved in the depolymerization of actin in the vicinity of endosomes,
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3 with the PDZ-domain protein NHERF2, with the ubiquitin-protein ligase Nedd-4, and with
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5 KIF3B, a member of the kinesin superfamily [25,26,28,44].
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8 9 **Structure-function relationship of CIC 2Cl⁻/H⁺ exchangers**

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14 CIC-5 is a 746 amino-acid protein that belongs to the highly conserved CIC family of
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16 Cl⁻ channels and electrogenic 2Cl⁻/H⁺ exchangers that are expressed in prokaryotic and
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18 eukaryotic organisms [30]. In mammals, CIC-1, CIC-2, and the CIC-K/barttin complex all
19
20 function as plasma membrane Cl⁻ channels, whereas CIC-3 to -7 function as electrogenic 2Cl⁻
21
22 /H⁺ exchangers that reside mainly in the membranes of the endosomal/ lysosomal
23
24 compartments and in synaptic vesicles.
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28 X-ray crystal structures of three CIC 2Cl⁻/H⁺ exchangers (*E. coli* EcCIC-1, *S.*
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30 *typhimurium* StCIC, and *C. merolae* CmCIC) have provided significant insights into the
31
32 structure of CIC proteins, by demonstrating that they function as homodimers with separate
33
34 ion pathways within each subunit and suggesting molecular mechanisms for the ion
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36 permeation process [12-14,16]. In CIC 2Cl⁻/H⁺ exchangers, a glutamate residue acts
37
38 simultaneously as the outside Cl⁻ gate and the extracellular H⁺ acceptor [13,14,16].
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40 Interestingly, neutralizing this “gating glutamate” of EcCIC-1 (E148) and CmCIC (E210) by
41
42 mutation to alanine abolished H⁺ flux, and purely anionic conductance was observed [2,16].
43
44 Abolition of the rectification of the currents and similar loss of H⁺ flux were obtained when
45
46 equivalent mutations of the “gating glutamate” were introduced in human CIC-3 (E224A),
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48 CIC-4 (E224A), CIC-5 (E211A), and CIC-6 (E200A) [35,39,41,46]. Another glutamate facing
49
50 the intracellular medium has been identified as being an additional H⁺-binding site in EcCIC-1
51
52 (E203), in CIC-4 (E281), in CIC-5 (E268), and in CIC-6 (E267) [3,39,62]. Neutralization of
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3 this “proton glutamate”, which is strictly conserved among CIC 2Cl⁻/H⁺ exchangers, abolished
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5 H⁺ flux in EcCIC-1 [3], and Cl⁻ and H⁺ flux in CIC-4, CIC-5 and CIC-6 [39,62].
6

7 All eukaryotic CICs have a large cytoplasmic carboxy-terminus containing a pair of
8
9 cystathionine beta-synthase (CBS) domains. Recent data have provided evidence that CBS
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11 domains bind nucleotides like ATP and are involved in regulating the activity of several CIC,
12
13 including CIC-5 [36,56,64].
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18 **Spectrum of mutations of the *CLCN5* gene in Dent’s disease**

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22 To date, 148 *CLCN5* mutations have been reported in patients with Dent’s disease.
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24 They consist of about 36% nonsense mutations, 30% missense mutations, 18% deletional
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26 mutations, 7% splice site mutations and 6% insertional mutations [10]. An early modeling
27
28 study of CIC-5 by Wu *et al.* postulated that a majority of CIC-5 missense mutations involve
29
30 residues positioned at the interface of the homodimers [60]. Wu *et al.* speculated that mutated
31
32 residues clustering at the dimer interface of CIC-5 would cause a loss of electrical activity by
33
34 disrupting the assembly of the homodimers. This would lead to the formation of misfolded
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36 proteins in the endoplasmic reticulum, and their rapid degradation within the cell. However, at
37
38 that time, the subcellular localization of the CIC-5 mutants had not been investigated, and so
39
40 the model did not take into account the possibility that some of the mutants showed reduced
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42 currents as a result of a gating defect.
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48 Despite the large number of *CLCN5* mutations identified, their functional impact at the
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50 cellular and molecular levels in Dent’s disease remains unclear. The aim of this review is to
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52 describe the findings of a series of recent functional investigations using heterologous
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54 expression systems carried out by us and by others that have made it possible to divide the
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56 *CLCN5* mutations into different classes, and have helped to elucidate the pathophysiology of
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3 Dent's disease. We have based the classification of *CLCN5* mutations on the well-established
4
5 classification of mutations of the cystic fibrosis transmembrane conductance regulator gene
6
7 (*CFTR*) that encodes the CFTR Cl⁻ channel. ***CFTR* mutations can be divided into five classes**
8
9 **that reflect their molecular dysfunction [57,63].** *CFTR* class I mutations comprise nonsense,
10
11 frameshift and aberrant mRNA splicing mutations that lead to impairment of protein
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13 synthesis. Class II mutations cause a defect of processing in the endoplasmic reticulum and
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15 the Golgi apparatus. As a consequence, the CFTR mutants are misfolded, achieve partial N-
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17 glycosylation, and undergo retention in the endoplasmic reticulum, which is followed by their
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19 rapid degradation within the cell. Class III and class IV mutations lead to a normal amount of
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21 CFTR protein at the plasma membrane, but alter channel regulation and conduction,
22
23 respectively. Class V mutations result in a decreased amount of functional protein, in most
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25 cases resulting from aberrant mRNA splicing.
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29 To date, mutations in the *CLCN5* gene can be classified into three different classes that
30
31 are discussed below. However, additional classes of *CLCN5* mutations might exist, as only
32
33 20% of the identified mutations have been functionally investigated. In addition, some
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35 mutations have been only partially explored (Table 1). Therefore, many mutations remain to
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37 be functionally analyzed.
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40 41 42 **Class 1 mutations inducing defective protein processing**

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47 After their translation, ClC-5 proteins move through the endoplasmic reticulum and
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49 the Golgi apparatus, where proper folding of the proteins occurs with the help of chaperones
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51 and other modifications take place, such as glycosylation. These post-translational
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53 modifications allow the 2Cl⁻/H⁺ exchanger to traffic to the early endosomes and to the plasma
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55 membrane.
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3 A remarkable number of naturally-occurring *CLCN5* mutations (about 60%) that have
4 been functionally investigated in *Xenopus laevis* oocytes, in HEK-MSR cells, and in HEK293
5 cells can be assigned to a first class of *CLCN5* mutations that induce defective protein
6 processing (Table 1) [18,19,34,38,48,51]. Although more recent studies have demonstrated
7 that some mutations do not cluster at the dimer interface [18,19], the conclusions from the
8 modeling study by Wu *et al.* [60] remain partially unchallenged, because it appears that many
9 mutations are indeed localized at the interface between the monomers of ClC-5 (Fig. 1 and 2,
10 Table 1) [18,19,34,48]. Immunocytochemical and biochemical analyses have revealed that
11 this class of mutations results in the retention of ClC-5 within the endoplasmic reticulum,
12 where it is subjected to early degradation by quality control systems. As a consequence, the
13 mutant proteins are improperly N-glycosylated, and are non-functional due to defective
14 trafficking to the cell surface and to the early endosomes.
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29 The position of these *CLCN5* mutations in the protein sequence may explain their
30 deleterious impact on ClC-5. As outlined by their position in ClC-5 and in view of the
31 modeling study by Wu *et al.* [60], it looks as though the first subgroup of mutations may
32 **impair monomer dimerization**. Because ClC-5 normally functions as a homodimer, the
33 misfolded proteins could be then subjected to early degradation by the cells. Concerning the
34 second subgroup of mutations (away from the dimer interface), they may significantly affect
35 the stability of the α -helices, thus enhancing the degradation of the abnormal ClC-5 proteins.
36 These studies have provided evidence that the proper folding of several protein regions, such
37 as the interface, is crucial for the normal function of ClC-5 at the plasma membrane and in the
38 early endosomes.
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51 Defects in protein folding and processing **have** been shown to be a common cellular
52 mechanism involved in the pathogenesis of several inherited disorders [20]. **One well-known**
53 **example is that of the most common p.F508del *CFTR* class II mutation in cystic fibrosis,**
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3 which results in the misprocessing of CFTR, producing a lack of functional protein at the cell
4 surface [4]. Thus, the mechanisms that lead to ClC-5 dysfunction in this category of *CLCN5*
5 mutations are similar to those responsible for the *CFTR* class II mutations in cystic fibrosis.
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11 **Class 2 mutations inducing delayed protein processing and lower stability of the mature**
12 **protein**
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18 About 20% of the *CLCN5* mutations that have been functionally analyzed in
19 heterologous expression systems generate ClC-5 proteins displaying delayed processing and
20 reduced biological stability of the mature glycosylated form (Fig. 3, Table 1). As a
21 consequence, the cell surface expression and the currents mediated by ClC-5 at the plasma
22 membrane are reduced. However, the distribution of the mutants in the early endosomes is
23 normal [18,48]. These functional defects of ClC-5 resemble those observed with *CFTR* class V
24 mutations that lead to lower protein levels at the plasma membrane due to a reduced synthesis
25 of the CFTR Cl⁻ channel [63].
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36 Four mutations of this type (c.779G>T, p.G260V; c.1581A>T, p.E527D; c.1637A>G,
37 p.K546E and c.1639T>G, p.W547G) are located at the transporter dimer interface, and one
38 mutation (c.834G>C, p.L278F) at the periphery of the interface (Fig. 1 and 2, Table 1). As
39 shown by their complex N-glycosylation, plasma membrane and early endosome expression
40 in HEK-293 transfected cells, a significant fraction of these mutants is not retained in the
41 endoplasmic reticulum [18]. Despite lower abundance at the cell surface, the residual
42 electrical activity and early endosomal distribution resulting from the mutation located at the
43 edge of the dimer interface (p.L278F) are in accordance with the conclusions from the
44 modeling study by Wu *et al.* [60]. These authors hypothesized that mutations of amino acids
45 located at the periphery of the subunit interface may induce a relatively minor disruption of
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3 the protein structure. This could explain why the p.L278F mutation is associated with residual
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5 function in HEK-293 transfected cells. The residual plasma membrane expression induced by
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7 the other mutations (p.G260V, p.E527D, p.K546E and p.W547G) makes the functional
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9 consequences of amino acid substitutions at the dimer interface of CIC-5 very difficult to
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11 predict in the absence of functional studies.
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14 Smith *et al.* have investigated the effect of another mutant (c.1581A>T, p.E527D) on
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16 endosomal pH in HEK-MSR cells by means of a vesicular acidification assay [48]. The
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18 p.E527D mutation results in current abolition and reduced plasma membrane expression, but
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20 normal expression in early endosomes (Table 1). Expression of this mutant in HEK-MSR
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22 cells impairs endosomal acidification. To explain the abnormal intraluminal pH, the authors
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24 hypothesize that this occurred by loss of currents of the mutant CIC-5.
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28 Ion conduction mediated by CIC-5 at the plasma membrane is not thought to be
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30 physiologically relevant. However, as we have already mentioned, several studies have
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32 provided evidence that the CIC-5 population at the cell surface of proximal tubule cells is a
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34 rate-limiting step in receptor-mediated endocytosis of low molecular weight proteins by
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36 establishing protein-protein interactions with proteins that are essential for renal endocytosis
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38 [27]. According to this view, in contrast to class 3 mutants that are normally targeted to the
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40 cell surface, the lower abundance of these mutants at the plasma membrane could severely
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42 reduce the rate of receptor-mediated endocytosis by disrupting the protein-protein interactions
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44 required for the formation of the endocytic macromolecular complex. This type of reasoning
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46 could explain the low molecular weight proteinuria observed in patients, but this point
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48 remains to be investigated.
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Class 3 mutations inducing altered conduction without any change in subcellular distribution

In this third class of *CLCN5* mutations, CIC-5 mutants can be produced, transformed into a complex glycosylated form in the endoplasmic reticulum and in the Golgi apparatus, and then finally transported to the plasma membrane and to the early endosomes, much as wild-type CIC-5 proteins (Table 1). However, two-electrode voltage-clamp recordings in *Xenopus laevis* oocytes have demonstrated that the mutant proteins display either reduced or no current.

This class of *CLCN5* mutations account for approximately 20% of the mutations that we and others have analyzed [18,19,32,37,38,53,54,61]. These mutations are analogous to the *CFTR* class III and class IV mutations that alter regulation and conduction, respectively [57]. Even though two missense mutations (c.815A>G, p.Y272C and c.1558T>C, p.S520P) are positioned at the transporter interface (Fig. 1 and 2, Table 1), the resulting mutant proteins that were expressed in HEK293 cells and in the pig renal proximal tubule cell line, LLC-PK₁, escaped from endoplasmic reticulum retention and the subsequent degradation [18,53]. This finding breaks the rule that mutation of amino acids located at the subunit interface of CIC-5 necessarily cause protein-folding defects. A set of data for other CICs also support these latter findings. For instance myotonia congenita is underlain by various missense mutations of the *CLCN1* gene that encodes the skeletal muscle Cl⁻ channel, CIC-1. It appears that many of the mutations that occur at the dimer interface of CIC-1 alter the function of CIC-1 at the plasma membrane by inducing a dramatic shift in the voltage dependence of channel activation [33]. Similarly, several artificially designed mutations positioned at the subunit interface in the *Torpedo* electric organ Cl⁻ channel CIC-0 induce altered gating behavior by inverting the voltage sensitivity of channel activation [5]. Further experiments are needed to find out

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3 whether the p.Y272C and p.S520P mutations also cause altered gating behavior, as suggested
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5 by similarly located mutations in CIC-0 or CIC-1.
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7 The p.G212A mutation induces reduced electrical activity. The mutated glutamate at
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9 position 212 is strictly conserved in CICs. It lies very close to the ion pathway of CIC-5,
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11 because it directly follows the “gating glutamate”, a residue that is involved in the coupling of
12
13 the Cl⁻ flux to the H⁺ flux in CIC 2Cl⁻/H⁺ exchangers. The exact mechanism responsible for
14
15 the decreased currents of CIC-5 remains to be elucidated, but one can hypothesize that
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17 mutation of a residue very close to the ion pathway could severely interfere with the gating
18
19 process. Mutagenesis analysis from CIC-1 supports this hypothesis. Heterologous expression
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21 of the CIC-1 p.G233A mutation, which is located at the equivalent G212 position in CIC-5,
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23 caused a dramatic alteration of anion-selectivity sequence and gating of CIC-1 [15].
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27 As we have already mentioned, the first hypothesis advanced to explain the defect of
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29 receptor-mediated endocytosis in patients with Dent’s disease was that it resulted in altered
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31 endosomal acidification due to a loss of proton neutralization by CIC-5 [30]. However, recent
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33 work by Novarino *et al.* contradicts this hypothesis by suggesting that the endosomal Cl⁻
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35 concentration, raised by CIC-5 in exchange for H⁺ accumulated by H⁺-ATPase, could be an
36
37 important parameter in the physiology of the endosomal and lysosomal pathway, rather than
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39 vesicle acidification [40]. Thus, the altered currents of these CIC-5 mutants may induce severe
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41 impairment of endocytosis due to impaired function of v-type H⁺-ATPases in early
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43 endosomes, or a reduction in Cl⁻ accumulation in early endosomes. It is interesting to note that
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45 Wang *et al.* have demonstrated that the S520P (c.1558T>C, p.S520P) mutant, which still
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47 displays residual currents and normal cell surface expression, cannot be internalized from the
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49 plasma membrane into the early endosomes in LLC-PK1-transfected cells [54]. This suggests
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51 that disorders in endocytosis could be caused either by inefficient endosomal acidification or
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53 endosomal Cl⁻ accumulation due to lack of targeting of CIC-5 to the early endosomes.
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3 Analysis of the effects of these ClC-5 mutants on pH or Cl⁻ concentration in the early
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5 endosomes of proximal tubule cells would provide very useful information about the cellular
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7 mechanisms that lead to Dent's disease in this class of *CLCN5* mutations, but such
8
9 investigations remain unaddressed.
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12 Several mutations affecting the CBS1 and CBS2 domains also belong to this category
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14 of mutations. The p.R648X was shown to severely reduce currents [34], and the p.Y617X and
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16 p.R704X mutations displayed impaired electrical activity [32,37,54,61]. Data from the
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18 p.R718X mutation which causes truncation of the intracellular C terminus and loss of a part of
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20 CBS2 domain are not in accordance with these latter mutants that are able to traffic to the
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22 plasma membrane, because the R718X mutant protein is not detected at the cell surface due to
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24 retention in the endoplasmic reticulum. These findings raise the possibility that the p.R718X
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26 mutation may interfere with the proper folding of the C terminus of ClC-5 that is required to
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28 pass the process of protein quality control in the endoplasmic reticulum prior to trafficking to
29
30 the early endosomes and to the plasma membrane.
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34 Finally, four missense mutations (c.64T>G, p.W22G; c.731C>T, p.S244L; c.815A>G,
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36 p.Y272C; c.1558T>C, p.S520P) are not located in close proximity to the Cl⁻ or H⁺ transport
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38 pathways of the ClC 2Cl⁻/H⁺ exchangers [2,13,14,16,41,46,62] and do not belong to any
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40 region with assigned functional association.
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Mutations altering the expression at the plasma membrane and in the early endosomes

It is not clear whether two other mutations (c.170G>T, p.G57V and c.839G>C, p.R280P) should also be assigned to another class of mutations. These two mutations have been shown to lead to a reduction both in plasma membrane expression and electrical activity in HEK-MSR transfected cells [48]. However they also induce alterations in the endosomal distribution of the mutant proteins: the R280P mutant accumulates in the early endosomes, whereas the G57V mutant accumulates in the late endosomes. Surprisingly, wild-type CIC-5 was found to localize in both early and late endosomes in HEK-MSR transfected cells. The expression of wild-type CIC-5 in late endosomes is at variance with its distribution in kidney tissue. Indeed, when investigated by immunofluorescence with specific antibodies, late endosomes of proximal tubule cells have clearly been shown to be devoid of CIC-5 [9,21,45,55]. Further investigations are needed to unravel the correct classification of these two *CLCN5* mutations, and to exclude the possibility that late endosomal expression of wild-type CIC-5 is not the consequence of an intracellular trafficking defect in HEK-MSR transfected cells due to overexpression or some other mechanism.

Conclusion

Functional studies of *CLCN5* mutations have made it possible to distinguish between three different classes of mutations (Fig. 3). CIC-5 class 1 mutants are characterized by a failure to induce currents, because of defective processing during N-glycosylation, resulting in their retention at the endoplasmic reticulum. CIC-5 class 2 mutants display delayed protein processing and lower stability of their mature form. CIC-5 class 3 mutants are correctly targeted to the plasma membrane and early endosomes, but display reduced electrical activity.

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3 Further investigation is needed to elucidate the mechanisms (altered gating or conduction)
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5 leading to dysfunction of CIC-5.
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8 Furthermore, the localization of the mutations deduced from molecular modeling
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10 studies demonstrates that about half of them cluster in the helices that form the dimer
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12 interface of CIC-5 (Table 1). This highlights the fact that impaired integrity of this region of
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14 CIC-5 is a mechanism that can frequently underlie Dent's disease. This observation clearly
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16 contrasts with the conclusions of modeling and functional studies of the mutations of CIC-1
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18 that underlie myotonia congenita, and those of CIC-7 that cause recessive and dominant
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20 osteopetrosis. Although some mutations are localized at the dimer interface of CIC-1 and
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22 CIC-7, most of them appear to be excluded from this region [7,17,33,52].
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26 Numerous studies have demonstrated that pharmacological therapies can rescue
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28 dysfunctional channels and transporters, as is the case, for instance, for the CFTR protein-
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30 carrying mutations for cystic fibrosis [4]. For example, chemical (e.g., quinazoline),
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32 pharmacological (e.g., benzo[c]quinolizinium compounds) and molecular chaperones (e.g.,
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34 sodium 4-phenylbutyrate) have been shown to stabilize and facilitate the folding of class II
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36 CFTR mutants, thereby allowing them to escape from degradation in the endoplasmic
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38 reticulum and to be targeted to the plasma membrane. Furthermore, several compounds (e.g.,
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40 genistein) have also been found to directly activate class III and class IV CFTR mutants that
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42 display defective regulation and conduction at the plasma membrane, respectively. A major
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44 future challenge will be to search for specific therapeutic molecules that will be able to restore
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46 sufficient function of mutant CIC-5 in patients with Dent's disease.
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Acknowledgements

Work in our laboratories was funded in part by grants from the French ANR program (ANR-05-MRAR-033-01), the Fondation du Rein, and ECOS Conicyt C10S03. CECs is funded by Conicyt PFB. The English text has been edited by M. Ghosh.

For Peer Review

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Figure legends

Fig. 1 Location of the *CLCN5* mutations in a three-dimensional model of CIC-5 based on the structure of StCIC [13] viewed from the side of the membrane with the extracellular solution at the top. **a** Class 1 mutations inducing defective protein processing. **b** Class 2 mutations inducing delayed protein processing and lower stability of the mature protein. **c** Class 3 mutations inducing altered conduction without any change in subcellular distribution. Mutated residues are shown in spheres. The mutation numbers refer to those of Table 1. The helices involved in the formation of the dimer interface and those located at the edge are shown in yellow and in pale yellow respectively. Yellow numbers indicate mutated residues positioned at the dimer interface. A monomeric structure of the model was built using as template the crystallographic data of StCIC channel (PDB ID code 1KPL) using the ICM programme [1]. The dimeric structure and the figures shown here were produced using VMD software [29]

Fig. 2 Location of the *CLCN5* mutations in the three-dimensional model of CIC-5 viewed from the subunit interface [13]. **a** Class 1 mutations inducing defective protein processing. **b** Class 2 mutations inducing delayed protein processing and lower stability of the mature protein. **c** Class 3 mutations inducing altered conduction without any change in subcellular distribution. Mutated residues are shown in spheres. The mutation numbers refer to those of Table 1. The helices involved in the formation of the dimer interface and those located at the edge are shown in yellow and in pale yellow respectively. Yellow numbers indicate mutated residues positioned at the dimer interface

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2
3 **Fig. 3** Molecular mechanisms responsible for the impaired function of ClC-5 in Dent's
4 disease. A full description of each of the molecular mechanisms observed in the different
5 *CLCN5* mutation classes is provided in the text
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Table 1 Summary of the functional effects of *CLCN5* mutations in patients with Dent's disease

Number	Nucleotide change ^a	Amino acid change	Position in protein structure ^b	Currents	Surface expression	Intracellular localization		N-glycosylation	Reference
						E.R.	E.E.		
	WT			+	+	+	+	Complex	[31]
									[18]
									[17]
1	c.64T>G	p.W22G ^{e,i}	Helix A	-	+	N.T.	N.T.	N.T.	[41]
									[57]
2	88insACC	30insH ^f	Helix A	Reduced	N.T.	N.T.	N.T.	N.T.	[33]
3	c.170G>T	p.G57V ^f	Helix B, edge of interface	Reduced	Reduced	+	-	N.T.	[51]
4	c.536G>A	p.G179D ^c	Loop D-E	-	-	+	-	Core	[17]
5	c.599T>G	p.L200R ^c	Helix E	-	-	+	-	Core	[18]
6	c.608C>T	p.S203L ^c	Helix E	-	-	+	-	Core	[18]
7	c.635G>A	p.G212A ^e	Helix F	Reduced	+	+	+	Complex	[18]
8	c.557C>T	p.C219R ^c	Helix F	-	-	+	-	Core	[18]
9	c.661T>C	p.C221R ^c	Helix F	-	-	+	-	Core	[18]
10	c.674T>C	p.L225P ^c	Helix F	-	-	+	-	Core	[17]
11	c.731C>T	p.S244L ^e	Helix G	Reduced	+	+	+	Complex	[17]
12	c.779G>T	p.G260V ^d	Helix H, interface	-	Reduced	+	+	Complex	[17]
13	c.810C>G	p.S270R ^c	Loop H-I, interface	-	-	+	-	N.T.	[51]
14	c.815A>G	p.Y272C ^e	Loop H-I, interface	-	+	+	+	Complex	[17]
15	c.834G>C	p.L278F ^d	Helix I, edge of interface	Reduced	Reduced	+	+	Complex	[17]
16	c.837G>A	p.W279X ^f	Helix I, edge of interface	-	N.T.	N.T.	N.T.	N.T.	[34]
17	c.839G>C	p.R280P ^f	Helix I, edge of interface	Reduced	Reduced	+	Increased	N.T.	[51]
18	c.971T>G	p.L324R ^c	Helix J	-	-	N.T.	N.T.	N.T.	[37]
19	c.985G>C	p.G333R ^c	Helix J	-	N.T.	N.T.	N.T.	Core	[54]
20	c.1020C>A	p.N340K ^{c,i}	Helix J	-	-	+	-	Core	[17]

Table 1 Continued

Number	Nucleotide change ^a	Amino acid change	Position in protein structure ^b	Currents	Surface expression	Intracellular localization		N-glycosylation	Reference
						E.R.	E.E.		
21	c.1033C>T	p.R347X ^{c,i}	Helix J	-	-	N.T.	N.T.	N.T.	[41]
22	c.1385G>T	p.G462V ^c	Helix N	-	-	N.T.	N.T.	N.T.	[37]
23	c.1406T>C	p.L469P ^c	Helix N	-	-	+	-	Core	[18]
24	c.1517G>A	p.G506E ^c	Helix O, interface	-	-	N.T.	N.T.	N.T.	[37]
25	c.1534G>C	p.G512R ^f	Helix O, interface	-	N.T.	N.T.	N.T.	N.T.	[35]
26	c.1538G>A	p.G513E ^c	Helix O, interface	-	-	+	-	N.T.	[51]
26	c.1539G>A	p.G513R ^c	Helix O, interface	-	-	+	-	Core	[17]
27	c.1546C>T	p.R516W ^c	Loop O-P, interface	-	-	N.T.	N.T.	N.T.	[37]
28	1842insT	M517fsX528 ^c	Helix P, interface	-	-	N.T.	N.T.	N.T.	[37]
29	c.1558T>C	p.S520P ^e	Helix P, interface	Reduced	+	N.T.	N.T.	N.T.	[57]
30	1853-54delTT	L521RfsX526 ^{c,h}	Helix P, interface	-	-	N.T.	N.T.	N.T.	[37]
31	1567-1569delGTC	p.V523del ^f	Helix P, interface	Reduced	N.T.	N.T.	N.T.	N.T.	[61]
32	c.1571T>A	p.I524K ^c	Helix P, interface	-	-	+	-	N.T.	[51]
33	c.1581A>T	p.E527D ^{d,g}	Helix P, interface	-	Reduced	+	+	N.T.	[51]
34	c.1637A>G	p.K546E ^d	Helix Q, interface	-	Reduced	+	+	Complex	[17]
35	c.1639T>G	p.W547G ^d	Helix Q, interface	Reduced	Reduced	+	+	Complex	[17]
36	c.1851C>G	Y617X ^{e,i}	CBS 1	-	+	N.T.	N.T.	N.T.	[63]
37	c.1962C>T	R648X ^{e,i}	Between CBS 1-2	-	+	N.T.	N.T.	N.T.	[40]
38	c.2110C>T	R704X ^{e,i}	CBS 2	-	+	N.T.	N.T.	N.T.	[40]
39	C.2152C>T	p.718X ^{c,i}	CBS 2	-	-	+	-	Core	[34]

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5 ^a Nucleotide numbering refers to the cDNA numbering with +1 being the A of the ATG translation initiation codon in the reference sequence.

6 Codon 1 is the initiation codon. The GenBank accession number of human wild-type CIC-5 is NG_007159.2

7 ^b Position refers to the predicted topology of CIC-5 determined by Wu *et al.* [62]

8 ^c The mutation belongs to class 1 mutations inducing defective protein processing

9 ^d The mutation belongs to class 2 mutations inducing delayed protein processing and lower stability of the mature protein

10 ^e The mutation belongs to class 3 mutations inducing altered conduction without any change in subcellular distribution

11 ^f The mutation needs further investigation to allow its classification

12 ^g The mutation leads to an absence of endosomal acidification

13 ^h This mutation was functionally tested as L521R

14 ⁱ This mutation is not displayed on the tridimensional model of CIC-5 due to lack of sequence homology between StCIC and CIC-5

15 *E.E.*, early endosomes; *E.R.*, endoplasmic reticulum; *N.T.*, not tested; *WT*, wild-type

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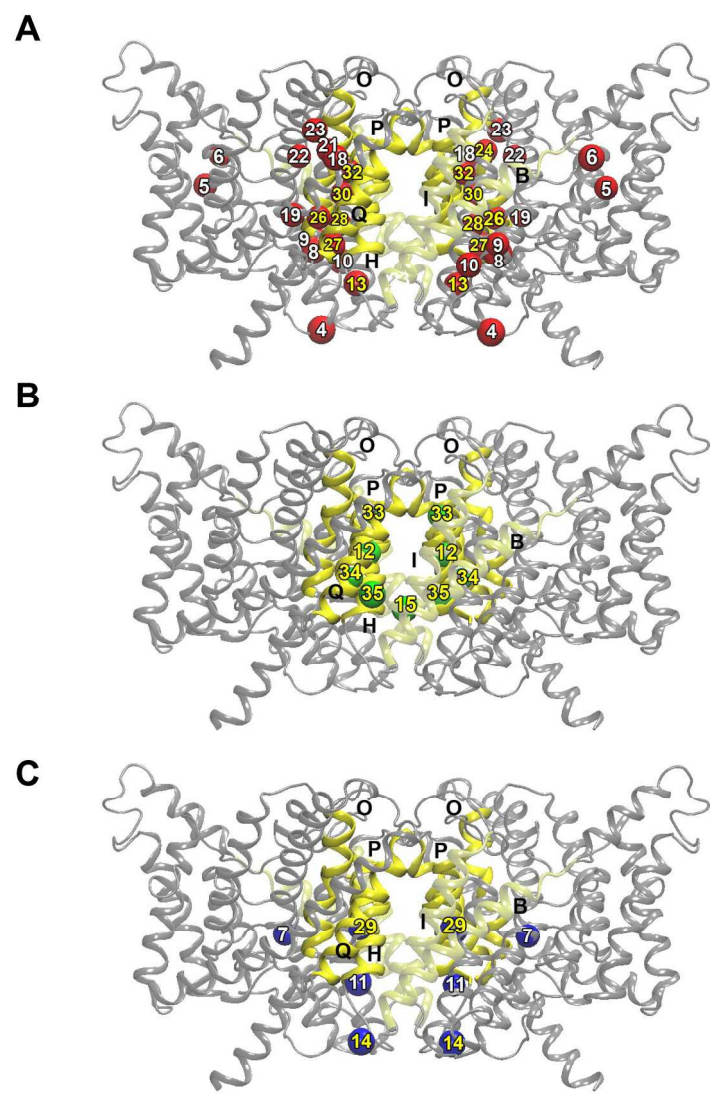


Figure 1

159x265mm (300 x 300 DPI)

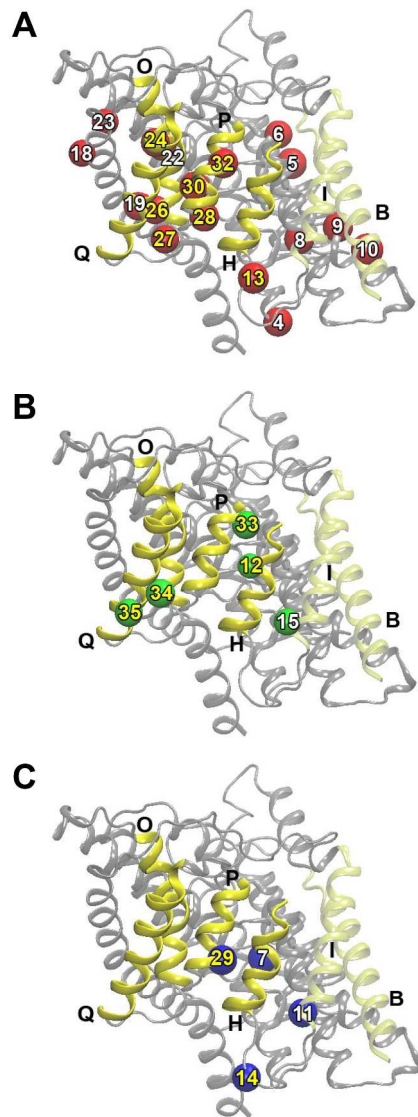


Figure 2

161x430mm (300 x 300 DPI)

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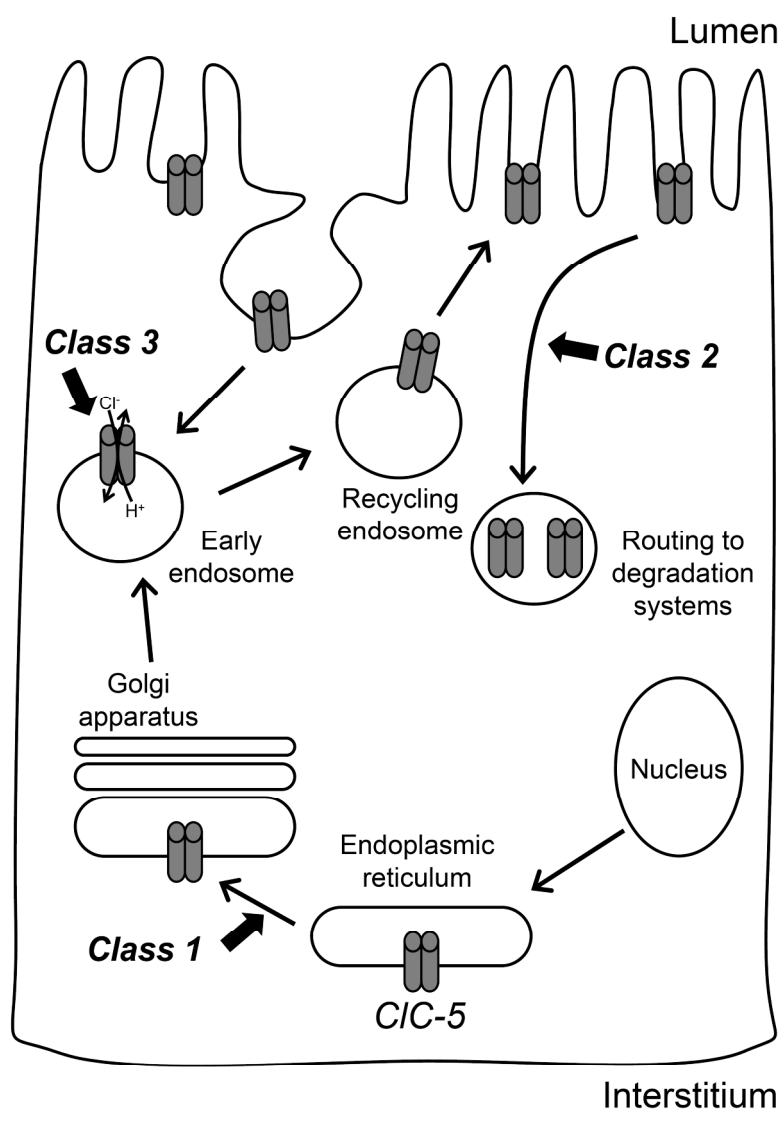


Figure 3

206x309mm (300 x 300 DPI)