

## REVIEW

## Dent disease: A window into calcium and phosphate transport

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**Abstract**

This review examines calcium and phosphate transport in the kidney through the lens of the rare X-linked genetic disorder Dent disease. Dent disease type 1 (DD1) is caused by mutations in the *CLCN5* gene encoding CIC-5, a Cl<sup>-</sup>/H<sup>+</sup> antiporter localized to early endosomes of the proximal tubule (PT). Phenotypic features commonly include low molecular weight proteinuria (LMWP), hypercalciuria, focal global sclerosis and chronic kidney disease; calcium nephrolithiasis, nephrocalcinosis and hypophosphatemic rickets are less commonly observed. Although it is not surprising that abnormal endosomal function and recycling in the PT could result in LMWP, it is less clear how CIC-5 dysfunction disturbs calcium and phosphate metabolism. It is known that the majority of calcium and phosphate transport occurs in PT cells, and PT endocytosis is essential for calcium and phosphorus reabsorption in this nephron segment. Evidence from CIC-5 KO models suggests that CIC-5 mediates parathormone endocytosis from tubular fluid. In addition, CIC-5 dysfunction alters expression of the sodium/proton exchanger NHE3 on the PT apical surface thus altering transcellular sodium movement and hence paracellular calcium reabsorption. A potential role for NHE3 dysfunction in the DD1 phenotype has never been investigated, either in DD models or in patients with DD1, even though patients with DD1 exhibit renal sodium and potassium wasting, especially when exposed to even a low dose of thiazide diuretic. Thus, insights from the rare disease DD1 may inform possible underlying mechanisms for the phenotype of hypercalciuria and idiopathic calcium stones.

**KEYWORDS**

CIC-5, Dent disease, endocytosis, renal calcium transport, renal phosphate transport, sodium/proton exchanger NHE3

**1 | INTRODUCTION**

Dent disease (DD) is a rare monogenic, X-linked recessive disorder characterized by features of incomplete Fanconi syndrome including low molecular weight proteinuria (LMWP). Hypercalciuria is commonly observed, sometimes associated with calcium nephrolithiasis, nephrocalcinosis and/or hypophosphatemic rickets.<sup>1</sup> Focal global

sclerosis and progressive CKD are also common features.<sup>2</sup> It is unclear whether this global sclerosis and CKD commonly observed in DD are the result of defects at the level of the podocytes or are a result of distal nephron pathology including nephrocalcinosis. Approximately 60% of cases are caused by mutations in the *CLCN5* gene that encodes a Cl<sup>-</sup>/H<sup>+</sup> antiporter, primarily localized to early endosomes of the proximal tubule (DD1; MIM#300009). Mutations

in the *OCRL1* gene, encoding a phosphatidylinositol 4,5-bisphosphate 5-phosphatase localized at the Golgi apparatus, early endosome and lysosome of proximal tubular cells account for another 15%-20% of cases (DD2; MIM#300555). The remaining 20% of DD cases remain genetically unresolved.

## 2 | ALTERED CALCIUM-PHOSPHATE METABOLISM IN DENT DISEASE

Most affected males are hypercalciuric, and this can be marked in young children. However, in teenagers and adults the hypercalciuria is typically comparable to that observed in routine calcium stone formers.<sup>3</sup> Nephrocalcinosis with onset in childhood is common, while nephrolithiasis (calcium oxalate and/or phosphate) occurs in only about 25%. Although hypercalciuria is common, urinary oxalate and citrate excretions are typically normal.

Detailed studies on calcium and phosphate metabolism in DD are few.<sup>4,5</sup> Blanchard et al<sup>5</sup> retrospectively analysed 118 genetically confirmed male DD patients (109 DD1 and 9 DD2) and reported that: (a) hypercalciuria was present in almost all patients younger than 3 years old, but calcium excretion declined with age so that the calcium excretion was normal in only 40% of patients under 30 but in 85% of those over the age of 30; (b) mean plasma phosphate was low in all patients, especially after 12 years of age, even with advanced CKD; (c) serum 1,25-dihydroxyvitamin D [ $1,25(\text{OH})_2\text{D}$ ] was normal or in the upper range of normal and remained normal even in the late stages of kidney disease; (d) serum 25-hydroxyvitamin D [ $25(\text{OH})\text{D}$ ] was slightly below normal, and parathormone (PTH) was normal; and (e) plasma potassium decreased with age and hypokalemia was present in 50% patients over the age of 18.

## 3 | LOCALIZATION AND FUNCTION OF CLC-5

CIC-5 is a 746-aminoacid protein with multiple membrane-spanning domains and intracellular N- and C-terminal domain. CIC-5 belongs to the family of voltage-gated chloride channels, which is comprised of cell surface channels and intracellular  $\text{Cl}^-/\text{H}^+$  exchangers.<sup>6</sup>

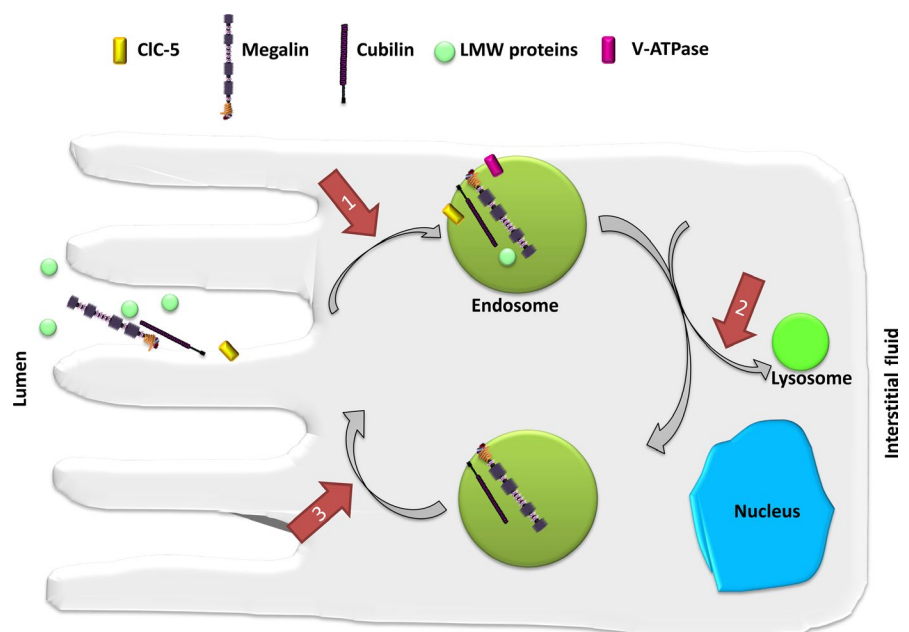
CIC-5 is highly expressed in the kidney, primarily in the proximal tubular cells (PTCs) of the S3 segment, in the  $\alpha$ -intercalated cells of the cortical collecting duct of mouse and rat kidney, but also in the cortical and medullary thick ascending limb of Henle's loop (mTAL).<sup>7-10</sup>

In the PTCs, CIC-5 plays an important role during receptor-mediated endocytosis of albumin and LMW proteins that make it through the glomerulus and colocalizes with vacuolar  $\text{H}^+$ -ATPase (V-ATPase).<sup>11,12</sup> There CIC-5 contributes to the acidic pH within the endosomes important for ligand:receptor dissociation and subsequent recycling of the receptor to the apical membrane and degradation of the ligand within endosomes<sup>13,14</sup> (Figure 1).

The prevalent view was that CIC-5 supports efficient acidification of endosomes either by providing a  $\text{Cl}^-$  conductance to counterbalance the accumulation of positively charged  $\text{H}^+$  pumped in by V-ATPase, either directly in parallel with V-ATPase by acting as a  $\text{Cl}^-/\text{H}^+$  exchanger.<sup>15</sup> Recently, however, experimental evidence indicates that the endosomal  $\text{Cl}^-$  concentration might play a role in endocytosis independent of endosomal acidification, thus suggesting another possible mechanism by which CIC-5 dysfunction may cause disease and impair endocytosis.<sup>7</sup>

CIC-5 may have additional functions since about 8% is located at the PTC plasma membrane.<sup>16</sup> CIC-5 plays a key role in the formation/function of the endocytic complex that includes the megalin/cubilin, the sodium-hydrogen antiporter 3 (NHE3)

**FIGURE 1** Receptor-mediated endocytosis of LMW proteins in the proximal tubule. LMW proteins undergo receptor-mediated endocytosis involving megalin and cubilin. Receptors and ligands are internalized by the formation of endosomes, which are progressively acidified by V-ATPase. Chloride influx via CIC-5 facilitates acidification by maintaining electroneutrality of ion transport. Endosomal recycling brings receptors back to the apical surface. The absence or dysfunction of CIC-5 can potentially disrupt endosome cycling at three points (indicated by arrows): (1) reduced rate of receptor internalization; (2) disrupt progression of endosomes to lysosomes; and (3) disrupt recycling of endosomes to the cell surface



and the V-ATPase. Evidence suggests that the large intracellular CIC-5 C-terminus plays a crucial function to mediate the assembly, stabilization and disassembly of the endocytic complex via protein-protein interactions. The Hryciw group demonstrated that the C-terminus of CIC-5 also binds the actin-depolymerizing protein cofilin.<sup>17</sup> When the nascent endosome forms, recruitment of cofilin by CIC-5 appears required to localize dissolution of the actin cytoskeleton, thereby allowing the endosome to pass into the cytoplasm. It has been demonstrated both in mouse models and in humans that loss of CIC-5 causes defective PTC trafficking of megalin and cubilin.<sup>18–20</sup> Thus, CIC-5 may serve two roles during receptor-mediated endocytosis: (a) vesicular acidification and receptor recycling; and (b) participation in the non-selective megalin-cubilin receptor complex at the apical membrane.

Protein endocytosis in the PTCs relies on active receptors that include not only megalin and cubilin, but also amnionless, disabled-2.<sup>21</sup> Megalin and cubilin bind many protein ligands from tubular fluid including the commonly measured LMW proteins  $\beta$ 2-microglobulin,  $\alpha$ -1 microglobulin and retinol-binding protein (RBP). Whereas megalin binds certain ligands independently, it binds others, such as albumin, jointly with the coreceptor cubilin. Other megalin ligands play key roles in systemic phosphate and calcium metabolism including vitamin D-binding protein and PTH.

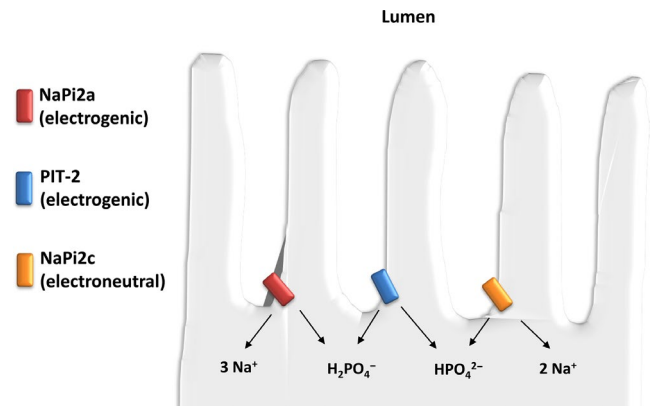
In the collecting duct, CIC-5 protein localizes to the  $\alpha$ -intercalated cells which are key for acid-base homeostasis. CIC-5 co-localizes with the V-ATPase in the apical and subapical vesicles.<sup>12</sup> Accordingly, CIC-5 might be important for insertion and recycling of these vesicles, and when CIC-5 function is lost, defective expression of V-ATPase may result causing impaired urinary acidification.

When CIC-5 expression was silenced by an antisense CIC-5 transfection in a collecting duct cell model (mIMCD-3), endocytosis was arrested, and calcium oxalate crystals agglomerated on the CIC-5 silenced cells, suggesting a possible physiological role for CIC-5 during crystal clearance in the collecting duct. Thus, loss of CIC-5 function in this segment may contribute to nephrocalcinosis risk.<sup>22–24</sup> The role of CIC-5 in mTAL remains circumstantial and speculative.<sup>25</sup> Studies suggest that endocytosis takes place in murine mTAL where V-ATPase is also present<sup>26</sup> albeit at a lesser extent than in PTCs, thus suggesting a possible role for CIC-5 during endocytosis and exocytosis processes in this nephron segment.

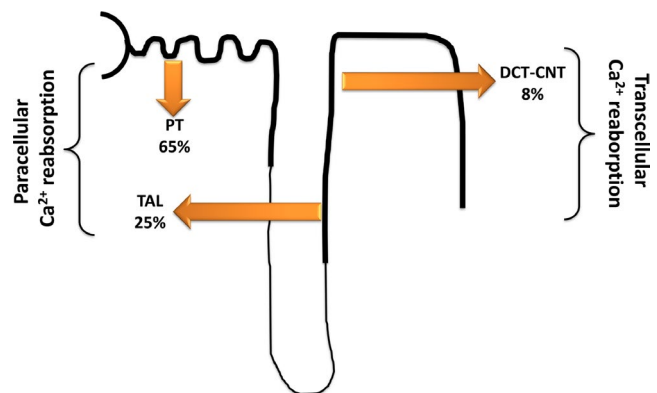
#### 4 | LOCALIZATION AND FUNCTION OF CALCIUM AND PHOSPHATE TRANSPORTERS IN THE KIDNEY

Approximately 80% of filtered phosphate is reabsorbed from the urine via proximal tubular transporters, mostly in juxtamedullary nephrons<sup>27,28</sup> (Figure 2).

About 98% of filtered calcium is reabsorbed along the nephron, paracellularly in the PT and in the TAL of the loop of Henle and transcellularly in the distal convoluted tubule and connecting tubule (Figure 3). No reabsorption occurs in the collecting duct; therefore,

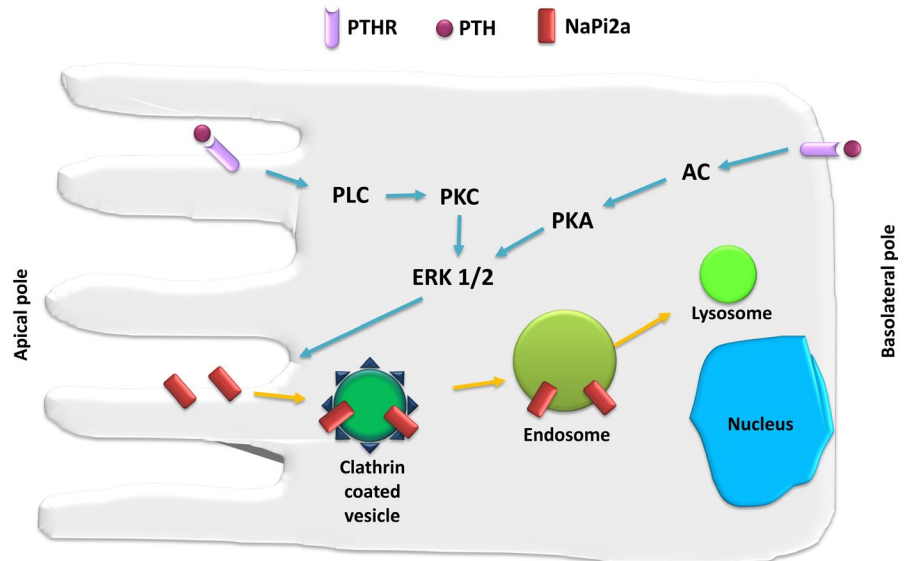


**FIGURE 2** Renal phosphate reabsorption in the proximal tubule. Phosphate is reabsorbed via three sodium phosphate cotransporters: NaPi2a, NaPi2c and PIT-2. In humans, NaPi2a and NaPi2c are believed to play the most important role in phosphate reabsorption. They are positioned at the apical membrane of renal proximal tubular cells and take advantage of an inward electrochemical gradient for sodium to move phosphate from the filtrate into the cell. The amount of phosphate reabsorbed is dependent on the abundance of the sodium phosphate cotransporters and variations in their number at the brush border membrane are a primary regulatory pathway for urinary phosphate excretion



**FIGURE 3** Renal calcium reabsorption along the nephron. The majority of filtered calcium is reabsorbed in the proximal tubule (PT) and thick ascending limb (TAL), with the final highly regulated percentage in the distal convoluted tubule (DCT) and in the connecting tubule (CNT). In the PT, calcium is mainly reabsorbed paracellularly, partially driven by activity of the sodium/proton exchanger 3 (NHE3), which allows transcellular sodium entry at the apical brush border, while the Na-K-ATPase pumps sodium out of the cell at the basolateral side. In TAL, calcium is reabsorbed by specialized and controlled paracellular pathways involving claudin 16, 19 and 14. The driving force for calcium is produced by the combined action of the basolateral Na-K-ATPase, the Na-K-Cl cotransporter (NKCC2) and the outward rectifying ROMK channel on the apical membrane. In DCT-CNT, calcium enters the cell at the apical side through TRPV5 channels, binds intracellular calbindin-D-28k and exits the cell at the basolateral side by the Na-Ca exchanger (NCX1) and the Ca-ATPase PMCA<sup>29</sup>

**FIGURE 4** PTH-induced endocytosis of NaPi2a. In the proximal tubule, PTH binds to apical and basolateral PTH receptors. Stimulation of either receptor is known to rapidly induce phosphaturia by decreasing apical phosphate transporter activity. Activation of phospholipase C (PLC) by apical PTH receptors leads to stimulation of ERK1/2 kinases and internalization of NaPi2a. Basolateral PTH receptors are linked to adenylate cyclase (AC), protein kinase A (PKA) and ERK1/2. NaPi2a is internalized via clathrin-coated vesicles, transported to endosomes and targeted to lysosomes for degradation



any calcium delivered there is subject to precipitation depending most importantly upon the tubular fluid pH.<sup>29</sup> In the PT, the majority of calcium is reabsorbed by passive and hormone-independent paracellular transport. The claudin family of epithelial tight junction proteins are crucial for paracellular calcium permeability. Active calcium transport in the S3 segment accounts for up to 20%-30% of PT calcium flux, and however, the precise pathway of this transport is not well defined. Several studies detected apical PT calcium-sensing receptor (CaSR) expression, a cation-sensing G protein-coupled receptor also present in the parathyroid gland.<sup>30,31</sup> CaSR expression in the PT appears to be regulated by vitamin D, and activation of CaSR may in turn alter PT expression of the vitamin D receptor. Activation of the CaSR in the kidney has other effects on transport, including increased sodium reabsorption and proton secretion in the mouse PT.

Thus, the majority of calcium and phosphate transport localizes in PTCs where CIC-5 plays an important role in endocytosis. Do these endocytic processes in turn impact PT calcium and phosphate transport?

## 5 | PROXIMAL TUBULAR ENDOCYTOSIS AND THE REGULATION OF PHOSPHATE AND CALCIUM HANDLING

### 5.1 | Renal phosphate handling

NaPi2a is the predominant phosphate transporter in the PT.<sup>27</sup> Major physiological regulators of NaPi2a expression include PTH, dopamine and fibroblast growth factor-23 (FGF 23), each of which stimulates NaPi2a endocytic removal and degradation as well as decrease mRNA expression.<sup>32</sup> PTH regulates NaPi2a membrane expression by stimulating NaPi2a endocytosis (Figure 4). This differs from regulation of many other transport proteins that requires modification of the protein itself.<sup>33</sup>

NaPi2a, apical PTH receptors and PLC $\beta$ 1 are organized in a macromolecular complex via the scaffolding protein Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor (NHERF1).<sup>33</sup> Phosphorylation of NHERF1 is followed by dissociation of the NaPi2a/NHERF-1 complex, with NHERF1 remaining at the apical membrane, while NaPi2a is internalized. PTH-induced inactivation of NaPi2a is facilitated by megalin.<sup>34</sup> NHERF1 is necessary for PTH-induced internalization of NaPi2a via apical but not basolateral PTH receptors.<sup>35</sup> A similar mechanism has been proposed for FGF23 and dopamine regulation of NaPi2a expression.

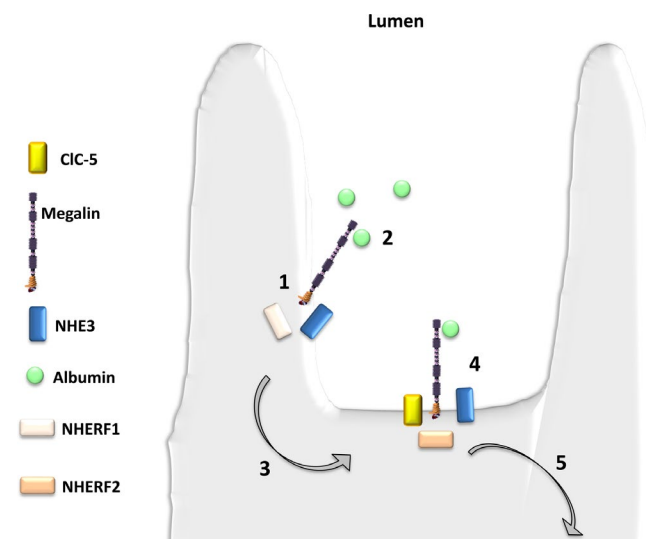
A low-phosphate diet has the opposite effect, stimulating insertion of NaPi2a into the apical membrane and inhibiting endocytosis. Because endocytosed cotransporters are degraded in lysosomes, recovery of NaPi2a to basal levels when PTH stimulation is removed depends on de novo synthesis. Thus, apical retention/removal of NaPi2a is a highly regulated process.<sup>33</sup>

Since the scaffold protein NHERF1 plays an important role regulating NaPi2a apical trafficking, as either a chaperone, a scaffolding protein or both,<sup>32</sup> a link between CIC-5 and NHERF1 whereby CIC-5 influences PT apical NaPi2a expression seems possible.

NHERF scaffold proteins are also crucial for maintaining the macromolecular complex responsible for LMW proteins uptake at the PT brush borders<sup>36</sup> (Figure 5). Evidence supports this series of events in animal models.<sup>37,38</sup> The presence of albumin increased levels of plasma membrane-associated NHERF2, and silencing NHERF2 led to significant reduction in apical membrane CIC-5, the number of actin clusters and albumin uptake. This in vivo data support a key role for CIC-5 nucleation of the endocytic complex, presumably by tethering of the complex via NHERF2 to the actin cytoskeleton. These data collectively suggest that NHERF2 is necessary for maintaining CIC-5 at the plasma membrane. However, the significance of these findings is still not clear, since NHERF1 or NHERF2 KO mice lacked LMWP, the hallmark of CIC-5 dysfunction.<sup>39</sup> Intriguingly, however, NHERF1 null mice manifest decreased apical membrane NaPi2a, hyperphosphaturia and hypercalciuria.

## 5.2 | Renal calcium handling

Proximal tubular cells express cytosolic  $1\text{-}\alpha$ -hydroxylase enzyme and are the major source of circulating active  $1,25(\text{OH})_2\text{D}$  (Figure 6). These cells also contain 24-hydroxylase that converts  $1,25(\text{OH})_2\text{D}$  to the biologically inactive metabolite calcitroic acid.<sup>40,41</sup> PTCs sense circulating  $1,25(\text{OH})_2\text{D}$  to modulate activity of the  $1\text{-}\alpha$ -hydroxylase and 24-hydroxylase. Wang et al<sup>42</sup> demonstrated that in the absence



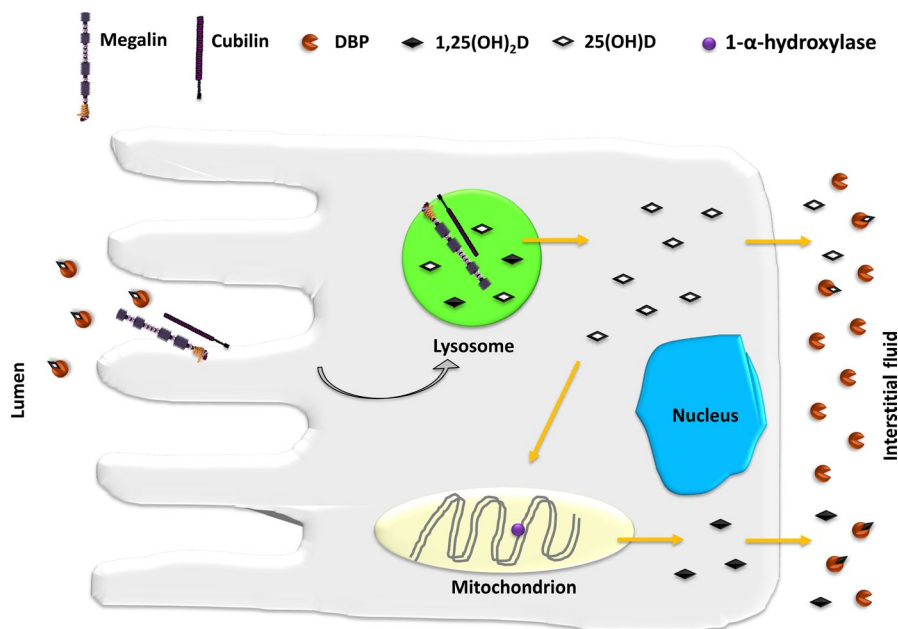
**FIGURE 5** Hypothetical role for NHERF1 in proximal tubular cell endocytosis. NHERF1 could potentially influence proximal tubular endocytosis via the following steps: (1) megalin, via an interaction with NHERF1, associates with NHE3 within microvilli; (2) albumin binds to megalin in the microvilli of proximal tubular cells; (3) the megalin albumin complex translocates to the intravillar cleft; (4) formation of a macromolecular complex containing megalin, NHE3 and CIC-5 occurs via NHERF2; and (5) the mature endosome is internalized which allows for ligand processing

of vitamin D, the vitamin D receptor (VDR) localizes to the interior face of the apical brush border. When  $1,25(\text{OH})_2\text{D}$  is administered to vitamin D-deficient mice, VDR bound to  $1,25(\text{OH})_2\text{D}$  translocates from the brush border to the cytoplasm and nucleus where it suppresses  $1\text{-}\alpha$ -hydroxylase and stimulates 24-hydroxylase.

Parathormone, a key modulator of both renal calcium and phosphate handling, is freely filtered by the glomerulus and reaches the luminal surface of PTCs where it can bind the PTH receptor or instead be reabsorbed via megalin-mediated endocytosis. Biochemical and in vitro studies demonstrated that PTH regulates  $1,25(\text{OH})_2\text{D}$  synthesis via G protein signalling.<sup>43</sup> A recent study revealed that the  $\alpha$ -subunit of the stimulatory G protein ( $\text{G}\alpha$ ) is essential for PTH action in the PT and that the loss of this signalling protein results in PTH resistance, leading to hypocalcemia, reduced serum  $1,25(\text{OH})_2\text{D}$  and elevated serum PTH. The  $\text{G}\alpha$  KO mice also demonstrated significantly elevated PT 24-hydroxylase mRNA.<sup>44</sup>

In a rat model of  $1,25(\text{OH})_2\text{D}$  deficiency, Silva et al<sup>45</sup> observed that  $1,25(\text{OH})_2\text{D}$  deficient and parathyroidectomized rats had low levels of cortical CIC-5 mRNA and protein, and lower serum and higher urinary calcium concentrations compared with control and  $1,25(\text{OH})_2\text{D}$ -deficient rats. Administration of PTH increased CIC-5 mRNA and protein expression to near-normal levels and decreased renal calcium leak. The authors concluded that PTH modulates cortical CIC-5 levels at both the mRNA and the protein level, but not in the medulla, suggesting that PTH, which is known to be a major mediator of calcium reabsorption in the kidney cortex, may act via regulation of CIC-5. They speculated that PTH could regulate CIC-5 expression in order to maintain the number of vesicles able to recycle membrane and endocytosed proteins, including NaPi2a.

Together, these data strongly support a pivotal role for PT endocytosis in renal calcium and phosphorus handling.



**FIGURE 6** Roles of megalin and cubilin in proximal tubule uptake and activation of  $25(\text{OH})\text{D}$ . Vitamin D-binding protein (DBP) binds  $25(\text{OH})\text{D}$  in the circulation, and the complex is freely filtered across the glomerulus to be then reabsorbed in the proximal tubule by receptor-mediated endocytosis involving megalin and cubilin. The complex is then delivered to lysosomes where DBP is degraded and the vitamin released into the cytosol.  $25(\text{OH})\text{D}$  is either secreted directly or trafficked to the mitochondrial membrane where it serves as a substrate for  $1\text{-}\alpha$ -hydroxylase and converted to  $1,25(\text{OH})_2\text{D}$ .  $1,25(\text{OH})_2\text{D}$  is then released into the interstitial fluid where it is complexed by free DBP molecules [modified from reference<sup>40</sup>]

## 6 | IN VIVO CLC-5 KO MODELS: IMPACT ON PHOSPHATE AND CALCIUM TRANSPORT MECHANISMS

### 6.1 | Phosphate transport

Studies using two independent CLC-5 KO mice strains, the so-called Jentsch<sup>46,47</sup> and Guggino models,<sup>18,48-50</sup> provided critical insights into the mechanisms of PT dysfunction in DD1. These two strains recapitulate the major features of DD1 including LMWP. Studies in the two models demonstrated that CLC-5 inactivation is associated with severe impairment of both fluid-phase and receptor-mediated endocytosis, and a trafficking defect with the loss of megalin and cubilin at the PT brush border (Table 1). However, targeted disruption of CLC-5 in the Jentsch model did not lead to hypercalciuria, kidney stones or nephrocalcinosis, while a similar disruption in the Guggino model did.<sup>48</sup> Mice in the Jentsch model produced slightly more acidic urines. Urinary phosphate excretion was increased in both models by about 50%. Hyperphosphaturia in the Jentsch model was associated with decreased apical NaPi2a expression, although

principally at the brush border of the S1 PT segment and at subapical vesicles of other PT segments. This was surprising because defective NaPi2a endocytosis due to loss of CLC-5 function might be expected to result in increased plasma membrane presence. Indeed, observed changes in NaPi2a surface expression in these KO mice appeared CLC-5-independent since apical NaPi2a was not decreased in any PTs of chimeric female mice, while it was in all PTs of -/y male mice.

Depriving CLC-5 -/y mice of dietary Pi enhances plasma membrane NaPi2a expression to normal levels. When these KO mice were given PTH, NaPi2a was internalized but at a slower rate compared with WT mice. The reduced internalization of NaPi2a in response to PTH suggests that altered PTH delivery might lead to vesicular localization of NaPi2a in the S3 PT segment of KO mice. Indeed, whereas serum PTH is normal in KO mice, urinary PTH is increased by about 1.7-fold. Because megalin is down-regulated in these mice, luminal PTH levels are increased. The increased stimulation of apical PTH receptor in turn enhances internalization of NaPi2a in more distal segments of the PT.

In conclusion, the phosphaturia in KO animals may be a consequence of reduced endocytosis of filtered PTH.

**TABLE 1** Comparing phenotypes and molecular players in the Jentsch and Guggino mouse models

	Jentsch model	Guggino model
CLC-5 KO method	Targeting part of exon 5 and 6; C57BL/6 strain	Targeting exon 6 C57BL/6 strain
Renal phenotype		
Low molecular weight proteinuria	Vitamin D-binding protein, retinol-binding protein	Vitamin D-binding protein, Clara cell protein of 16 kDa, transferrin
Defective receptor-mediated endocytosis	$\beta$ 2-microglobulin, lactoglobulin	$\beta$ 2-microglobulin
Megalyn	Not tested	Defective trafficking
Cubilin	Not tested	Defective trafficking
Defective fluid-phase endocytosis	Fluorescein isothiocyanate-dextran, horseradish peroxidase	Fluorescein isothiocyanate-dextran, horseradish peroxidase
Polyuria	Present	Present
Sodiuria	Not tested	Increased
NHE3	Decreased apical exposure	Decreased protein abundance
Phosphaturia	Present (decrease apical exposure of NaPi2a)	Present
Hypercalciuria	Absent	Present
Glycosuria	Not reported	Present
Amino-aciduria	Not reported	Present
Bone and mineral phenotype		
Nephrocalcinosis	Absent	Intrarenal calcium deposits von Kossa positive
Serum 1,25(OH)2D	Reduced	Increased
Serum 25(OH)D	Reduced	Not tested
Serum PTH	Normal	Normal
Urine 1,25(OH)2D	Increased	Not tested
Urine 25(OH)D	Increased	Not tested
Urine PTH	Increased	Not tested
TRPV5	Up-regulated	Not tested
Bone turnover markers	Not tested	Increased

## 6.2 | Calcium transport

The possibility that CIC-5 may participate in renal calcium transport was suggested by the finding that CIC-5 expression in the renal cortex is under PTH regulation and that renal CIC-5 expression inversely correlated with urinary calcium excretion.<sup>46</sup> The hypothesis that hypercalciuria in DD1 is intrinsic to kidney-mediated mechanisms is supported by the observation that hypercalciuria is absent after kidney transplantation.<sup>3,51</sup> However, other reports have raised the possibility that CIC-5 may be linked to gastrointestinal calcium absorption and that hypercalciuria observed in DD1 may represent a secondary event.<sup>4</sup>

The most striking difference between the Jentsch and Guggino models is the absence of hypercalciuria, nephrocalcinosis and nephrolithiasis in the Jentsch model. However, the Jentsch model strongly supports a role for endocytosis in renal PT calcium handling. Increased tubular PTH concentrations due to a lack of CIC-5-related endocytosis should stimulate the PTH receptor and induce up-regulation of 1- $\alpha$ -hydroxylase. The amount of 1- $\alpha$ -hydroxylase mRNA is in fact increased in CIC-5 KO mice. Assuming that 25(OH)D is unchanged, elevated 1,25(OH)<sub>2</sub>D levels would be expected, as is observed in CIC-5 KO mice and patients with DD1. Increased 1,25(OH)<sub>2</sub>D should enhance intestinal calcium absorption leading to hypercalciuria and kidney stones. However, uptake of 25(OH)D via apical endocytosis is also defective in CIC-5 KO mice. In fact, a great quantity of the 25(OH)D appears in the final urine. Thus, a delicate balance between reduced 25(OH)D supply and stimulation of the 1- $\alpha$ -hydroxylase, both of which result from a lack of CIC-5 dependent endocytosis, will determine the presence or absence of hypercalciuria. The ultimate outcome may depend on nutritional or genetic factors.

The hypothesis that hypercalciuria in mice with defective CIC-5 function is in fact due to intestinal hyperabsorption of calcium rather than a renal leak was supported by another CIC-5 KO mouse model.<sup>52</sup> By targeting CIC-5 expression with antisense ribozyme, the authors demonstrated that hypercalciuria was abolished by calcium deprivation, suggesting that intestinal calcium hyperabsorption was important.

The Jentsch group studied vitamin D metabolism in their CIC-5 KO mouse model in depth using expression profiling, qRT-PCR and hormone measurements.<sup>53</sup> They demonstrated a large increase in 1- $\alpha$ -hydroxylase in parallel with a decrease in 24-hydroxylase. Although the up-regulation of 1- $\alpha$ -hydroxylase and down-regulation of 24-hydroxylase would be predicted to increase formation of 1,25(OH)<sub>2</sub>D, the concentration of this active metabolite was instead reduced in the serum of CIC-5 KO mice. However, target genes of 1,25(OH)<sub>2</sub>D were up-regulated in KO kidneys. Expression analysis of intestine and bone revealed that the up-regulation of 1,25(OH)<sub>2</sub>D target genes was not systemic but kidney intrinsic. In spite of reduced serum levels of 1,25(OH)<sub>2</sub>D in CIC-5 KO mice, 1,25(OH)<sub>2</sub>D was increased in later nephron segments as a consequence of impaired PT endocytosis. These changes could be attributed to impaired endocytosis due to lack of CIC-5 function. Other target genes of 1,25(OH)<sub>2</sub>D were up-regulated in the distal nephron segments. In this model, the authors demonstrated that this up-regulation was kidney specific, that is the effect of 1,25(OH)<sub>2</sub>D on distal vs proximal tubules was

local and direct. The renal distal up-regulation of the calbindins and TRPV5 through VDR is predicted to increase renal calcium reabsorption, consistent with the lack of hypercalciuria in their mouse strain.

The results obtained from studies using the Guggino model<sup>49</sup> are in marked contrast to the results from the Jentsch model. In the Guggino model, a small number of mice had deformities of the dorsal spin and backward growth of teeth but not osteopenia or rickets. Moreover, Guggino mice had more than doubled urine calcium excretions and developed nephrocalcinosis at the corticomedullary junction. In this model, hypercalciuria on low- and high-calcium diets is of bone and renal origin, and is not caused by increased intestinal calcium absorption, despite the elevated level of serum calcitriol.

The discrepancy between the two mouse models regarding mechanisms of hypercalciuria in DD1 might suggest another possibility, that is it might be caused by defective NHE3 expression at the apical PT. A link between sodium and calcium reabsorption is well known. Thiazide diuretics that block the Na<sup>+</sup>-Cl<sup>-</sup> cotransporter (NCC) in the distal convoluted tubule have a hypocalciuric effect stimulating sodium and calcium reabsorption in the PT.<sup>54,55</sup> The effect of diuretics on renal tubular calcium transport has been recently reviewed by Alexander and Dimke,<sup>56</sup> and studies suggest the PT as the major site of calcium reabsorption strictly linked to sodium reabsorption.<sup>57</sup>

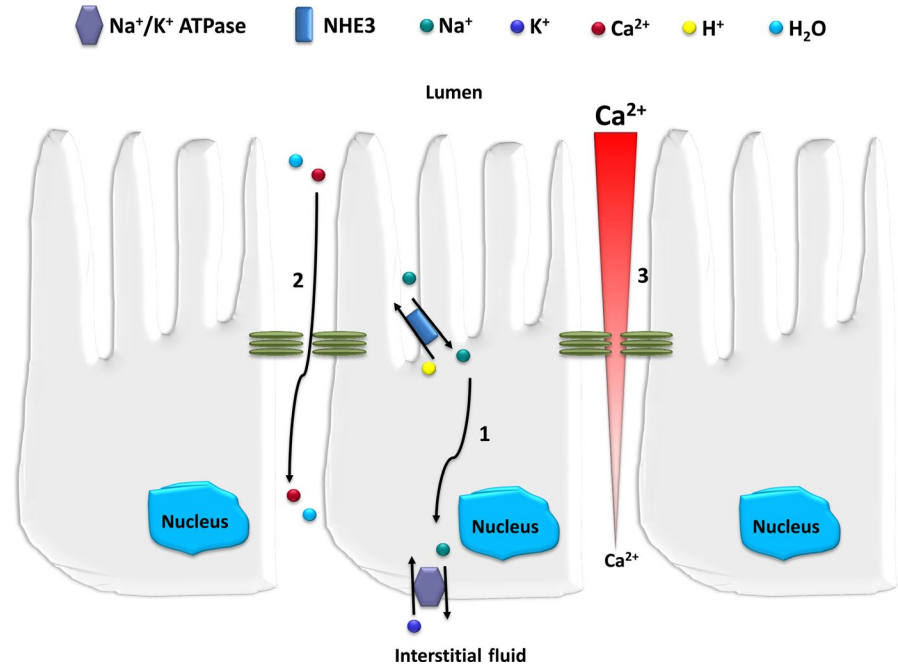
## 7 | THE NA<sup>+</sup>/H<sup>+</sup> EXCHANGER NHE3 AND CALCIUM REGULATION AT THE PROXIMAL TUBULE

The Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) protein family contains at least nine isoforms divided into plasma membrane isoforms (NHE1-NHE5) and endomembrane isoforms (NHE6-NHE9).<sup>58</sup> The plasma membrane isoforms function to exchange extracellular Na<sup>+</sup> for intracellular H<sup>+</sup>. In adult kidney, PT NHE3 is the predominant brush border Na<sup>+</sup>/H<sup>+</sup> exchanger (Figure 7).

Recent studies highlight the importance of PT NHE3 for calcium reabsorption. NHE3<sup>-/-</sup> KO mice manifest significant urinary calcium wasting.<sup>59</sup> Plasma Ca<sup>2+</sup> was not different between WT and NHE3<sup>-/-</sup> KO mice as was plasma PTH. However, 1,25(OH)<sub>2</sub>D was dramatically elevated in these mice, and despite this, intestinal Ca<sup>2+</sup> absorption was reduced. The reduced intestinal Ca<sup>2+</sup> absorption and increased urinary calcium excretion occurred at the expense of bone health. In fact, NHE3<sup>-/-</sup> KO mice demonstrated decreased cortical bone mineral density and trabecular bone mass. When NHE3 was overexpressed in opossum kidney cells, the paracellular calcium flux across confluent monolayers was increased. The authors proposed that the predominant mechanism mediating these observations was the absence of a driving force generated by NHE3 that reduced intestinal and proximal tubular paracellular calcium flux.

The SLC9A3 gene encodes human NHE3, and SLC9A3 homozygous or compound heterozygous disease-causing mutations have recently been reported in 9 patients from 8 families with congenital secretory sodium diarrhoea (MIM#616868).<sup>60,61</sup> Variants in the SLC9A3 gene influence susceptibility to bacterial infections and severity of lung disease by interacting with the CFTR gene, providing

**FIGURE 7** Role of NHE3 in paracellular  $\text{Ca}^{2+}$  reabsorption in the proximal tubule. NHE3 is responsible for the majority of  $\text{Na}^+$  and water reabsorption from the proximal tubule. In exchange for a luminal  $\text{H}^+$ , NHE3 mediates the influx of  $\text{Na}^+$  into the proximal tubular cell. This exchange is driven by an inward electrochemical gradient for  $\text{Na}^+$ , driven by basolateral  $\text{Na}^+/\text{K}^+$ ATPase (1). This active transcellular  $\text{Na}^+$  flux creates the osmotic driving force for water reabsorption, which, in turn, drives paracellular  $\text{Ca}^{2+}$  reabsorption (2) either by convection/solvent drag or by creating a concentration gradient for  $\text{Ca}^{2+}$  (3) [modified from reference<sup>57</sup>]



evidence for a potential modulatory effect in Cystic Fibrosis.<sup>62,63</sup> No association has been found to date between *SLC9A3* variants and renal proximal tubulopathies.

Sodium-hydrogen antiporter 3 is a highly regulated protein that is directly or indirectly influenced by a variety of factors.<sup>64,65</sup> PTH is known to regulate  $\text{Na}^+$  transport in PT. Animal and cell culture studies have shown that PTH inhibits NHE3 activity and expression, acutely and chronically, by reducing total and apical protein levels, as well as *NHE3* mRNA abundance.<sup>66,67</sup> Thus, besides exerting a hypercalciuric effect by stimulating  $1,25(\text{OH})_2\text{D}$  synthesis, PTH might indirectly favour a renal calcium leak by inhibiting NHE3. Perhaps counterbalancing these effects, PTH also stimulates calcium transcellular reabsorption in distal convolute tubules via the epithelial calcium channels TRPV5 and TRPV6, independent of  $1,25(\text{OH})_2\text{D}$ .<sup>68</sup>

Very recently, Edwards and Bonny<sup>69</sup> examined PT  $\text{Ca}^{2+}$  reabsorption in relation to  $\text{Na}^+$  and water influx and PTH by expanding a previously published mathematical model of water and solute transport in the rat PT. Their results indicated that PT  $\text{Ca}^{2+}$  reabsorption is principally driven by a transepithelial  $\text{Ca}^{2+}$  concentration gradient that stems from water reabsorption which is itself coupled to  $\text{Na}^+$  reabsorption. The model predicts that PTH-mediated inhibition of the apical NHE3 reduces  $\text{Na}^+$  and  $\text{Ca}^{2+}$  transport to a similar extent. Furthermore, PTH is predicted to exert an indirect impact on  $\text{PO}_4$  reabsorption via its inhibitory action on NHE3.

From these studies, the importance of PT NHE3 regulation clearly emerges, not only on calcium but also on phosphate handling.

## 8 | CLC-5 KO MODELS: WHAT CAN THEY TELL US ON NHE3 REGULATION?

Sodium-hydrogen antiporter 3 is dysregulated in CLC-5 KO mice. In the Jentsch model,<sup>46</sup> PT NHE3 expression was reduced to a similar

degree as NaPi2a, and however, dietary phosphate deprivation resulted in increased apical expression. PTH-induced endocytosis of NHE3 was also markedly reduced in the KO mice. Thus, vesicular localization of NHE3 appeared mediated by CIC-5-dependent defective PTH endocytosis. The decreased activity of NHE3 may be the cause of polyuria observed in these mice.

Decreased PT NHE3 protein abundance was also observed in the Guggino CLC-5 KO model. Using transport activity measurement assessed via two-photon microscopy, they demonstrated reduced basal activity of NHE3.<sup>70</sup> Moreover, CIC-5 inhibition by shRNAi in opossum kidney cells resulted in reduced expression of both megalin and NHE3. However, it is not currently possible to assess direct effects of CIC-5 on NHE3 trafficking. In fact, NHE3 is a part of a macromolecular complex required for LMW protein receptor-mediated endocytosis.<sup>16,17,71</sup> Likewise, NHE3 appears to play a crucial role in PT receptor-mediated endocytosis.<sup>72</sup> In these *in vitro* studies, NHE3 inhibition dramatically reduced PT endocytosis and alkalinized the early endosome. Results were further confirmed in NHE3 KO mice to demonstrate that urinary protein excretion was significantly higher in mutant compared with WT mice. The urinary protein patterns resembled that of megalin or CIC-5-deficient mice, that is typical models of tubular proteinuria. Thus, PT deficiency of NHE3 leads to reduced protein reabsorption.

Blockade of NHE3 activity by an NHE3 inhibitor in CLC-5-depleted opossum kidney cells reduced endocytosis to 46% of control levels.<sup>17</sup> In CLC-5 KO cells, the inhibitor still effectively decreased endocytosis to 56% of that in the untreated KO cells. This study further confirms the contribution of NHE3 to endocytosis and to acidification of the early endosomal compartment.

In CLC-5 KO opossum kidney cells, surface NHE3 expression was reduced despite an unchanged total protein level.<sup>70</sup> PTH decreased NHE3 surface expression, but the extent of the decrease and the rate



of endocytosis were not different from control cells. Conversely, the rates of basal and dexamethasone-stimulated exocytosis of NHE3 were diminished in CIC-5 KO cells. The authors concluded that CIC-5 is necessary for NHE3 exocytosis and offers an alternative hypothesis regarding reduced NHE3 expression in CIC-5 KO mice.

Collectively, these studies highlight not only the crucial role of NHE3 in renal calcium handling and the importance of CIC-5 in regulation of NHE3 trafficking, but also the role of NHE3 in PT receptor-mediated endocytosis.

## 9 | SODIUM AND WATER WASTING IN DENT DISEASE PATIENTS

Besides reduced PT NHE3 expression in CIC-5 KO mice, CIC-5 impairment could be hypothesized to cause defects in salt reabsorption not only in PT but also in more distal segments. A defect in mTAL, where CIC-5 is expressed, could impact tubulo-glomerular feedback and potentiate the salt reabsorption defect. A Bartter-like syndrome has also been occasionally reported in patients with DD1.<sup>73-76</sup> Bartter-Gitelman and hyperprostaglandin E syndromes, inherited disorders that manifest hypokalemic metabolic alkalosis, are caused by malfunction of renal tubular electrolyte transporters or ion channels including CIC-K-type chloride channels.<sup>77</sup> All variants share several clinical characteristics, including renal salt wasting, hypokalemic metabolic alkalosis and hyper-reninemic hyperaldosteronism.

Wojciechowski et al<sup>78</sup> very recently reported that barttin, the protein impaired in Bartter syndrome type 4, is an essential subunit for the CIC-Ka and CIC-Kb chloride channels and appears to regulate subcellular localization and post-translational modification of CIC-5. The functional characterizations of the CIC-5 mutant G261E that has been associated with Bartter-like symptoms demonstrated the increased abundance in the ER that could reduce the CIC-K trafficking to the surface membrane and produce the atypical symptoms observed in the affected patients. Based on these results, they hypothesized that abnormal Barttin-CIC-5 interactions in the TAL or CD could contribute to the Bartter phenotype observed in some patients with DD1.

Blanchart et al<sup>5</sup> found that about 50% of patients with DD1 older than 18 years of age presented with hypokalemia without changes in serum bicarbonate. Renal potassium loss was explored in six hypokalemic patients with DD1. All had inappropriate kaliuresis and low-range blood pressure, with a high renin concentration. Plasma aldosterone concentration was high in some patients and normal in others owing to a combination of the stimulatory effect of renin and the inhibitory effect of hypokalemia on aldosterone production. Four patients had marked polyuria. These data reveal that hypokalemia is an important trait of DD1 phenotype, at least in adult patients.

## 10 | CONCLUSIONS

Observations in patients with DD1 and model systems highlight the importance of receptor-mediated endocytosis for PT function.

The PT plays a pivotal role in renal phosphate and calcium handling, exerted mainly through the regulation of CIC-5-dependent (PTH) endocytosis. In fact, reduced expression of megalin, by mediating the uptake of PTH, antagonizes the effect of increased PTH availability on the luminal PTH receptor. Thus, the delicate balance between PTH reabsorption and PTH receptor activation influences both NaPi2-mediated proximal tubular phosphate reabsorption and the highly regulated proximal tubular synthesis of vitamin D.

CIC-5-dependent PTH endocytosis and CIC-5-dependent exocytosis may regulate NHE3-mediated proximal tubular sodium reabsorption and consequently paracellular calcium reabsorption. The importance of NHE3 dysfunction in determining DD1 phenotype was not investigated in patients with DD1. However, it could be hypothesized that the high rate of hypokalemia among adults patients with DD1 might be due to the increased Na<sup>+</sup> delivery into the distal tubules and the activation of the renin-angiotensin system secondary to hypovolaemia may result in metabolic alkalosis, with hypokalemia as a secondary phenomenon. Thus, NHE3 appears to play a crucial role in PT receptor-mediated endocytosis, suggesting that CIC-5-dependent NHE3 dysfunction might be an important factor in the cascade of events leading to the common DD1 phenotype that includes LMWP, hypercalciuria and hypophosphatemia. These observations relate to recent *in vivo* studies in hypercalciuric humans that implicate the PT as the site of abnormal calcium reabsorption. Thus, the insights from the rare disease DD1 may have implications for the more common phenotype of hypercalciuria and idiopathic calcium stones.

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## CONFLICTS OF INTEREST

The authors confirm that there are no conflicts of interest.

## AUTHOR CONTRIBUTIONS

AF, LBL and JL wrote the manuscript, LG conceived and designed figures and contributed to the discussion on the review topics.

## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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