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Review Article

Renal calcium and magnesium handling in Gitelman syndrome

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Abstract: Gitelman syndrome (GS) is an autosomal recessive salt-losing tubulopathy caused by biallelic inactivating mutations in the SLC12A3 gene. This gene encodes the thiazide-sensitive sodium-chloride cotransporter (NCC) which is exclusively expressed in the distal convoluted tubules (DCT). GS patients classically present with hypokalemic metabolic alkalosis with hypocalciuria and hypomagnesemia. While hypokalemia and metabolic alkalosis are easily explained by effects of the genotypic defect in GS, the mechanisms by which hypomagnesemia and hypocalciuria develop in GS are poorly understood. In this review, we aim to achieve three major objectives. First, present a concise discussion about current understanding on physiologic calcium and magnesium handling in the DCT. Second, integrate expression data from studies on calciotropic and magnesiotropic proteins relevant to the GS disease state. Lastly, provide insights into the possible mechanisms of calcium-magnesium crosstalk relating to the co-occurrence of hypocalciuria and hypomagnesemia in GS models. Our analyses highlight specific areas of study that are valuable in elucidating possible molecular pathways of hypocalciuria and hypomagnesemia in GS.

Keywords: Gitelman syndrome, distal convoluted tubule, calcium transport, magnesium transport, hypocalciuria, hypomagnesemia

Introduction

In the kidney, the bulk of filtered calcium (~85%) and magnesium (~90%) are reabsorbed in the earlier segments of the nephron, namely the proximal convoluted (PCT) and the thick ascending limb (TAL), through passive paracellular transport mechanisms [1]. Once the filtrate reaches the distal convoluted tubule (DCT), what remains of these divalent cations will be reabsorbed through a transcellular transport mechanism, making the DCT an important site that regulates calcium and magnesium reabsorption. This tightly controlled step dictates the amount of calcium and magnesium excreted into the urine. A number of kidney diseases including Gitelman syndrome (GS) are known to affect calcium and magnesium homeostasis.

GS is an autosomal recessive salt-losing tubulopathy caused by biallelic inactivating mutations in the encoding for the thiazide-sensitive sodium-chloride cotransporter (NCC) exclusively expressed in the DCT. The genetic defect of *SLC12A3* gene, which causes GS, produces NCC that are either properly inserted in the cell membrane with sub-optimal performance or are misfolded and immediately tagged for endoplasmic reticulum degradation [2-4]. The low level or absent expression of the NCC causes decreased sodium reabsorption in the DCT which then results in increased sodium delivery to the more distal, aldosterone-sensitive portions of the nephron [5]. In order to reabsorb the excess sodium in the lumen, the collecting tubules respond with an aldosterone-mediated increase in the expression of sodium pumps that exchange for potassium and hydrogen ions that are extruded from principal cells and α-intercalated cells, respectively. This compensatory potassium and hydrogen ion wasting leads to characteristic GS phenotype of hypokalemic metabolic alkalosis. Interestingly, hypomagnesemia and hypocalciuria are also diagnostic points commonly seen in GS patients [6]. While hypokalemia and metabolic alkalosis can be easily explained by the genotypic derange-

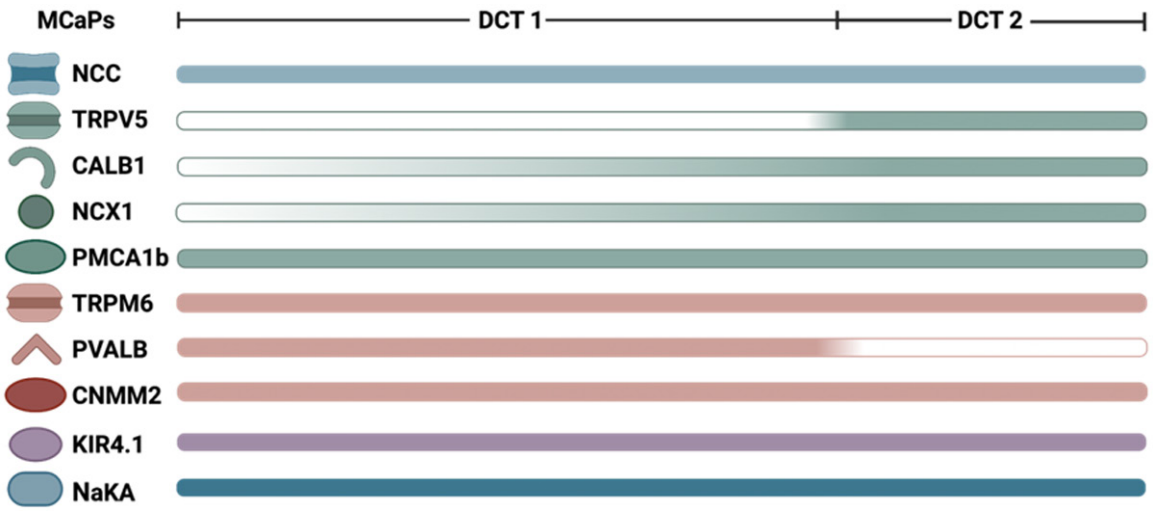

Figure 1. Differential expression of magnesium and calcium handling and related proteins in the distal convoluted tubule. NCC is expressed in both early (DCT1) and late (DCT2) segments of the DCT. Generally, calcium transport proteins (green) are exclusively or more abundantly expressed in DCT2 while magnesium transport proteins (red) are expressed throughout the whole stretch of the DCT. Expression data comparison is mainly based on the most recent single cell RNA sequencing study by the team of Chen [15]. Data on KIR4.1 and Na-K-ATPase (abbreviated as NaKA only in this figure) were from the work of McCormick and Ellison [1].

ment in GS, the mechanisms by which hypomagnesemia and hypocalciuria develop remain to be elucidated [1, 7, 8].

The DCT is subdivided into two segments, early (DCT1) and late (DCT2) segments, with the latter serving as a transition region as it closely resembles the downstream connecting tubules [9, 10]. The sodium-chloride cotransporter (NCC) is present in both segments and is the gold standard marker for the DCT [1, 11, 12]. In humans and mice, parvalbumin (PVALB) is highly abundant in the DCT1 and serves as its exclusive marker [11, 13]. On the other hand, the amiloride-sensitive epithelial sodium channel (ENaC) is expressed only in the DCT2 and later nephron segments and is used as a marker for the late DCT when it colocalizes with NCC [14]. This delineation is important because calciotropic proteins (CaPs) and magnesiotropic proteins (MaPs) are differentially expressed in these segments as presented in **Figure 1**. Generally, transcriptomic and proteomic data suggest that magnesium reabsorption involves the whole stretch of DCT while calcium reabsorption is restricted in the DCT2 and possibly the more distal nephron segments [15]. High-throughput analyses of nephrons have already identified CaPs and MaPs that are exclusively expressed in the DCT [15-17]. These proteins are potentially involved in GS-related hypocalciuria and hypomagnesemia. While these proteins have been identified and characterized in normal subjects, detailed analysis and differential expression patterns in NCC-deficient subjects, in essence GS models, are still under investigation.

This tightly controlled step is particularly important since it dictates certain amount of calcium and magnesium to excrete into the urine. Here we present and integrate evidence to drive hypothesis generation towards further experimental research and better understanding of renal calcium and magnesium handling and their involvement in Gitelman syndrome and possibly other disease processes.

Calcium handling in the DCT

CaPs expressed in the DCT have been well characterized. A simplistic depiction of the calcium handling involving the known CaPs is presented in **Figure 2**. Calcium ions enter the DCT cell via the apical transient receptor potential vanilloid subfamily member 5 (TRPV5) channel [19, 20]. It is noteworthy that there is a large calcium concentration gradient across the cell membrane with the intracellular calcium level maintained at 0.12 mmol/L compared to both

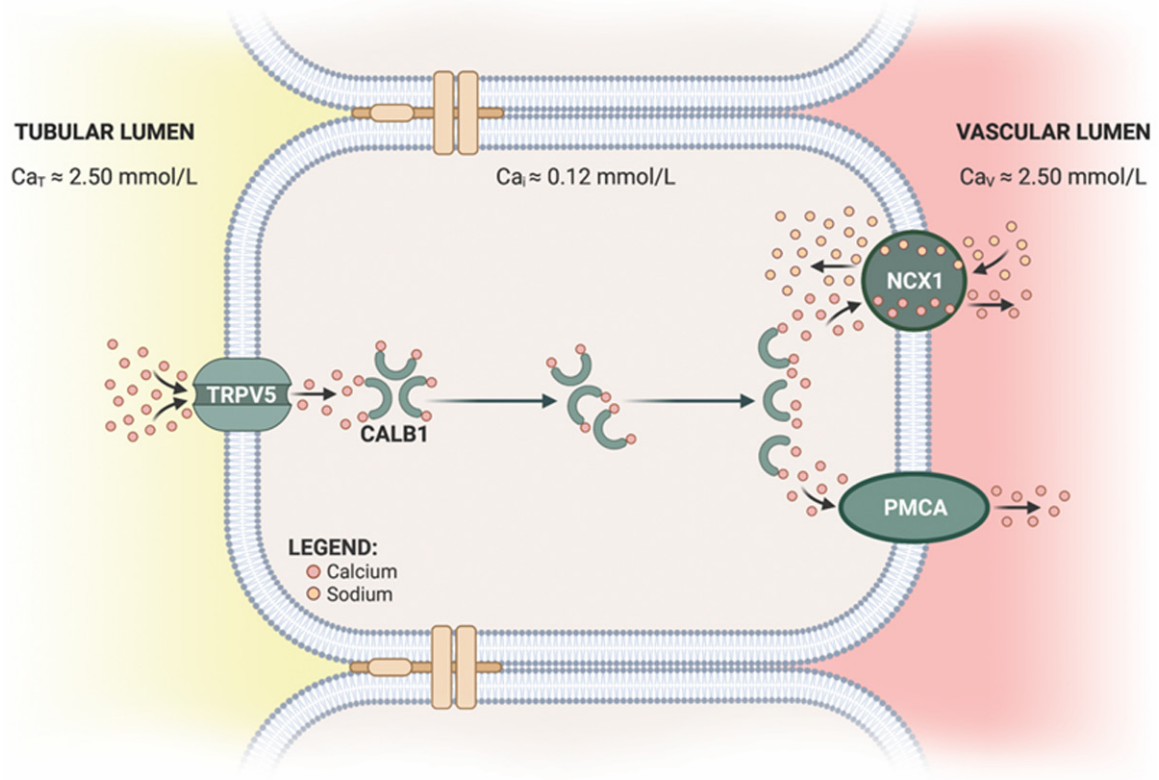

Figure 2. Calcium handling in the distal convoluted tubule. Calcium ions from the tubular lumen enter the apical side through the transient receptor potential vanilloid subfamily member 5 (TRPV5) channel. To maintain the low intracellular calcium concentration (0.12 mmol/L), calbindin 1 (CALB1) binds the calcium ions and shuttles them to the basolateral side where the sodium-calcium exchanger (NCX1) expels 70% of them in exchange for sodium ions in a 3:1 stoichiometric ratio while the remaining 30% is handled by the plasma membrane calcium ATPases 1 or 4 (PMCA1/4). Abbreviations: Ca_T, Tubular lumen calcium concentration; Ca_i, Intracellular lumen calcium concentration; Ca_V, Vascular lumen calcium concentration.

the luminal and vascular calcium levels of around 2.5 mmol/L. This provides a net inward driving force for calcium movement and is important for cellular metabolism to proceed. To maintain this physiologic calcium gradient, a cytosolic calcium carrier, calbindin 1 (CALB1), serves as an intracellular level sensor [20]. When intracellular levels are abnormally low, nearby CALB1 associates with TRPV5 and favors calcium influx [21]. As TRPV5 is constitutively active, free CALB1 dynamically buffers the increasing calcium concentration by shuttling these ions to the basolateral side for extrusion. The exit of calcium through the basolateral membrane is facilitated by two transport proteins, the Na/Ca exchanger-1 (NCX1) and the plasma membrane calcium ATPases (PMCA). Calcium extrusion into the blood stream is predominantly handled by NCX1 as it is responsible for 70% of calcium movement while the remaining 30% is handled by PMCA [22, 23]. Comparing DCT segment differential expressions, TRPV5 seems to be exclusively expressed in the DCT2 while CALB1 and NCX1 are strongly expressed in DCT2 with minimal DCT1 expression [15, 24] PMCAs have a relatively uniform expression all throughout the DCT stretch [15]. These expression data support the idea that calcium transport starts in the DCT2 and continues in the closely resembling connecting tubules.

Dysregulation of calcium handling may happen at different levels [25, 26]. First, gene expression level changes which are usually hormonal responses and is the predominant point of control. Second, modifications in the activity of functional proteins which are frequently affected by perturbations in factors like pH and ion concentrations. Third, derangements in cellular trafficking of assembled proteins. Lastly, interactions of entry channels with both intralu-

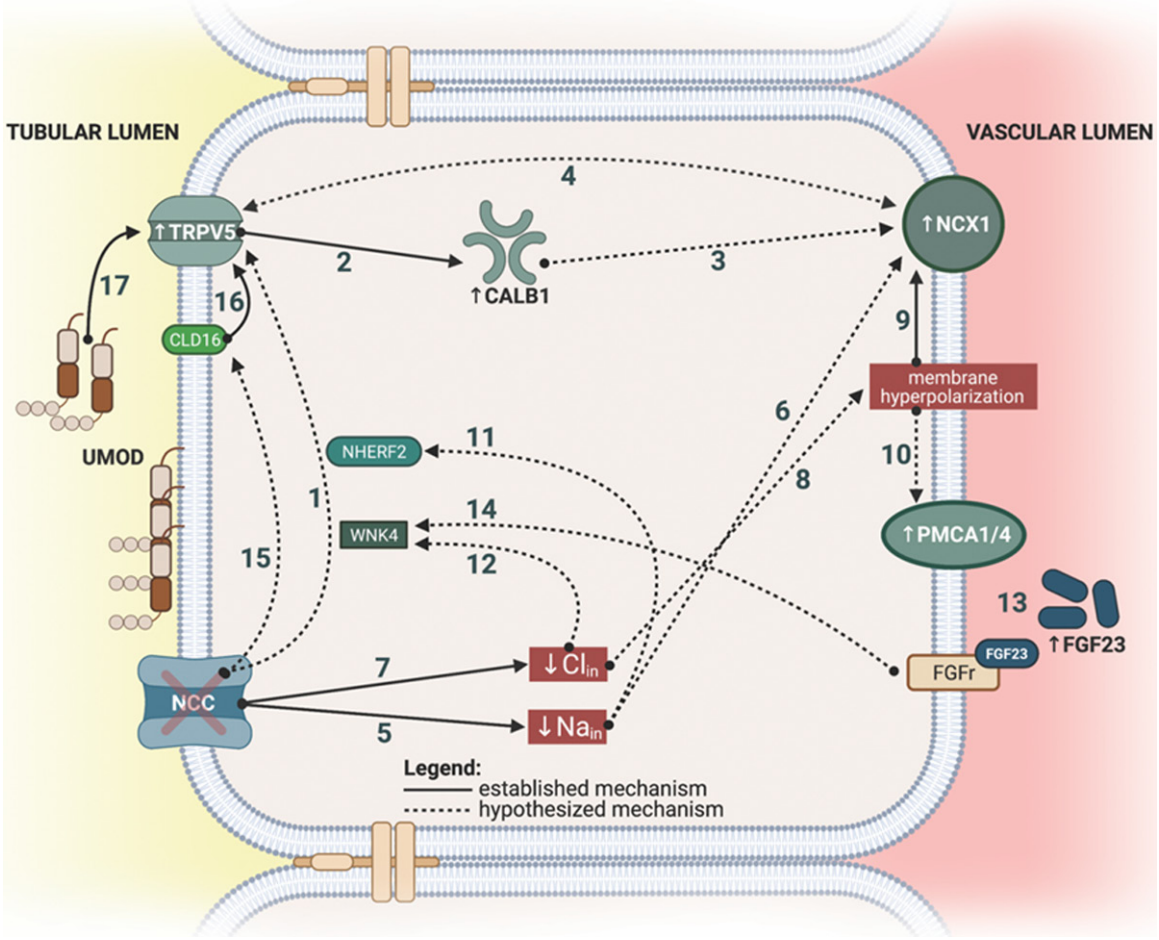

Figure 3. Mechanism of hypocalciuria in Gitelman syndrome. Expression of TRPV5, CALB1, NCX1 and possibly PMCA1/4 is increased in the setting of a defective sodium-chloride cotransporter (NCC), as in Gitelman syndrome. (1) Increase in TRPV5 expression may be mediated by regulators common to TRPV5 and NCC like WNK4, and NHERF2. (2) The increased expression of TRPV5 influences the expression of the closely associated CALB1. (3) When CALB1 drops off the calcium cargo on the basolateral side it is possible that it interacts with NCX1. (4) Since NCC is defective, the intracellular sodium and chloride concentration drops. The (5) decrease in sodium ions (6) affects the highly electrogenic NCX1 directly while the (7) change in chloride concentration (8, 9) indirectly through membrane hyperpolarization. (10) Changes in the membrane potential might also have an effect to the less electrogenic PMCA1/4. (11) NHERF2 is known to be sodium sensitive while (12) WNK4 is chloride sensitive. (13) Levels of circulating FGF23 have been shown to increase in NCC knockouts which could also (14) affect WNK4 involved in the downstream signaling of the FGF receptor (FGFr). (15) NCC may influence the phosphorylated form of claudin-16 (CLD16) which is a (16) known regulator of TRPV5. Lastly, (17) secreted uromodulin (UMOD) is known to upregulate TRPV5.

minal and extraluminal associated proteins. This section focuses on the known and proposed calciotropic proteins at different levels of control that could be potentially involved in the development of hypocalciuria in GS.

Hypocalciuria in GS

Hypocalciuria as a prime symptom of GS was first reported by the team of Rodríguez-Soriano in 1987 [27]. In 1992, Bettinelli and colleagues verified this claim when they used calcium excretion values to differentiate Gitelman syndrome from the Bartter Syndrome which presents with hypercalciuria instead [28]. In patients clinically suspected with GS, hypocalciuria is defined using spot calcium-creatinine ratio with a cutoff value of <0.2 mmol/mmol or <0.07 mg/mg in adults and an age-specific range of values for the pediatric population as they typically have lower creatinine excretion rates [6]. The mechanism by which these clinical parameters develop is poorly understood. **Figure 3** integrates established and hypothe-

sized mechanisms that explain hypocalciuria about the background of a defective NCC, the main problem in GS.

TRPV5

The apical calcium channel TRPV5 is one of the better characterized CaPs in both normal and pathophysiologic states including GS. Loss of TRPV5 function in mouse models result in hypercalciuria and disruption of bone mineralization making it the gatekeeper of calcium reabsorption [18]. In GS states, expression of TRPV5 significantly increases and has consistently been demonstrated in mouse models using thiazide-induced NCC suppression, NCC knockouts, mutation knock-ins and inactivation of upstream NCC activity modulators [29, 30]. Moreover, renal tissue biopsies of GS patients also show increased TRPV5 expression on immunofluorescent examination [30].

There are several regulatory proteins common to NCC and TRPV5 which could possibly explain the communication mechanism between the two. The molecule with-no-lysine/lysine deficient protein kinase 4 (WNK4) has been shown to modulate both NCC and TRPV5 activity in several model studies [31-35]. WNK4 is a chloride-responsive molecule [36]. The team of Hoover proposes that the decrease in intracellular chloride secondary to NCC activity loss increases WNK4 activity [37]. This could explain the increased TRPV5 expression as WNK4 is known to upregulate TRPV5 plasma membrane expression. Another possible crosstalk mechanism through the WNK4 pathway is the bone-derived fibroblast growth factor 23 (FGF23). FGF23 is known to increase TRPV5 expression through a signaling pathway involving WNK4 [38]. More recently, it has been shown that knockout of NCC in mice promotes an aldosterone-mediated upregulation of circulating FGF23 highlighting the relevance of this signaling pathway in the development of hypocalciuria in GS [39]. Another protein closely associated with WNK4 is the Na+/H+ exchanger regulating factor 2 (NHERF2) which has been shown to be a coregulator of TRPV5 surface expression [40]. Its putative involvement lies on the possible effects of sodium concentration changes following NCC derangements to NHERF2 activity, but this remains to be experimentally confirmed.

Additionally, TRPV5 deficient mice showed concomitant decrease in expression of other calciotropic proteins like CALB1 and NCX1 [18]. This means that TRPV5 or the Ca influx through TRPV5 controls the expression of the other Ca transport genes like CALB1 and possibly NCX1 which will be discussed in below sections.

CALB1

CALB1 expression levels have also been examined in several studies. When hypovolemia is prevented by salt supplementation, thiazide treatment leads to increased CALB1 transcripts and protein expression in mice DCT [28]. Whether or not salt supplementation causes exclusively or contributes to the upregulation of CALB1 was not verified. This was clarified by observations from the Ser707X knock-in GS mouse model and immunostained kidney sections from GS patients showing upregulation of CALB1 [29]. CALB1 downregulation was also observed in a murine model of idiopathic hypercalciuria which further highlights its role in calcium handling derangements [41]. Moreover, studies on CALB1-knockout mice showed abolishment of upregulation of calcium transport molecule in the DCT secondary to thiazide treatment which implies that CALB1 plays an important role in calciotropic gene expression [42].

NCX1

While altered expression of NCX1 in response to NCC deficiency have not yet been documented in hypocalciuric models, inverse interpretation could be made from its observed downregulation in a murine model of idiopathic hypercalciuria [41]. This implies that upregulation of NCX1 causes hypocalciuria. Possible mechanisms have been proposed explaining the involvement of this calcium extrusion pump. As NCC function is absent, there will be decreased intracellular sodium, one way by which the cell might theoretically compensate for this through upregulation of NCX1 expression and activity [1, 24, 43]. The exchanger will bring in sodium in exchange for calcium leaving the cell. As previously mentioned, intracellular calcium concentration is maintained within a strict range. As a response, the cell will take in more calcium through the apical TRPV5, another possible mechanism about its observed upregulation.

Additionally, intracellular chloride concentrations also fall, along with sodium concentration drop, which have been shown to cause hyperpolarization of the apical membrane which is another possible mechanism for the established TRPV5 upregulation [24, 44]. Gesek and Friedman proposed that a concomitant basolateral hyperpolarization could also occur, but this remains to be verified in DCT cells more so in the setting of NCC-deficiency [44]. The highly electrogenic NCX1 may also be upregulated and thus stimulate calcium extrusion across the basolateral membrane. Similarly, the expected cellular response would apically replenish calcium again through TRPV5. However, the parallel hyperpolarization of both the apical and basolateral membranes is more likely compared to a transcellular voltage difference, this dilemma can be solved if there are specific crosstalk mechanisms between the apical and basolateral calcium handlers, existence of which are yet to be discovered [24]. Common regulatory proteins of TRPV5 and NCX1 are good candidates to facilitate the crosstalk. Both transporters have PKC substrates which may or may not be acted upon by regulatory mechanism or at least connected with a common molecular pathway [1].

It is also important to consider possible interactions between CALB1 and the basolateral calcium extruding proteins as the closely related calmodulins have been shown to interact with NCX1 when co-expressed in human embryonic kidney cells [45]. As mentioned earlier, aside from the picking up of available calcium coming from TRPV5, CALB1 also plays a regulatory role in the intake pump. Current models describe CALB1 as a mere delivery system that drops off its parcel on the basolateral side, but it is also possible that it has regulatory interactions with the basolateral proteins. Moreover, another protein that plays this role may yet to be discovered.

PMCA1/4

Plasma membrane calcium ATPases have long been accepted to take charge of 30% of the calcium extrusion workload and the rest handled by NCX1. However, the specific isoform is still debatable until today. PMCA1 and PMCA4 are known to be expressed in the distal convoluted tubule [23, 46]. PMCA1 has long been identified as the predominant ATPase in this nephron segment and has been well studied in terms of its responses to calciotropic hormones and calcium handling experiments [1, 47]. Researches in the recent years have also presented some data on the role of PMCA4. The team of Alexander confirmed that it had highest expression in the distal convolution with minimal response to calcium level perturbations [48]. In a study on TRPV5-knockout mice, high expression level of PMCA4 was shown, but not PMCA1 [49]. This is particularly important as expression levels of NCX1 and CALB1 are known to be affected by TRPV5 knockout [18]. These findings suggest that both PMCA1 and PMCA4 might be involved in calcium handling in the DCT. Changes in PMCA levels on the background of NCC dysregulation have not been documented to date but it should be cautious to consider that their expression and activity might be deranged in GS states. Players in possible crosstalk areas are yet to be isolated. Like NCX1, although PMCA is not electrogenic, membrane hyperpolarization could potentially cause changes in its activity or expression as its structural domains and regulatory mechanisms have not yet been fully characterized.

Claudin-16

Claudin-16 (CLD16), previously known as paracellin-1, was initially thought to be exclusively expressed in the TAL where it plays a major role in paracellular magnesium transport [50]. Recent evidence showed that the phosphorylated form, untargeted by then available staining antibodies, is localized in the DCT [51]. Hou A et al established the role of CLD16, specifically the phosphorylated form, as an apical membrane player in transcellular calcium pathway in the DCT [please add reference]. Their results showed that CLD16 increases TRPV5 membrane abundance and channel conductance. Though CLD16 expression levels in the context of a defective NCC have not been documented, associations can be derived from observations of a cisplatin-treated mouse model. Cisplatins are DNA alkylating agents known to cause the so-called Gitelman-like syndrome by disrupting DCT integrity and NCC expression [52, 53]. Interestingly, cisplatin-treated mice also show CLD16 upregulation. Since the phosphorylated form of CLD16 localizing in the DCT is a relatively new information,

its functional role of in the DCT is largely unknown. So, whether claudin function and expression is directly affected by cisplatin or indirectly through an NCC-related pathway needs further investigation.

Uromodulin

UMOD, also known as Tamm-Horsfall Protein, is the most abundant secretory protein in the kidney. It has been shown to increase TRPV5 cell-surface abundance [54]. This upregulation is carried out from the extracellular side as uromodulin is secreted into the urine. It was initially thought that UMOD was exclusively synthesized in the TAL segment of the nephron until recently when the team of Tokonami confirmed its presence in microdissected DCT through RNA profiling, in situ hybridization, and immunofluorescence studies [55]. Their study also elucidated a regulatory role of UMOD in the NCC pathway. The triggers for UMOD release from DCT cells are currently unknown but are hypothesized to be NCC-related as well [55, 56]. The Tokonami study also induced distal salt loading through furosemide which is parallel to the high concentration of sodium accumulating in the DCT in GS states due to the defective NCC. It is possible that the response to increased urinary sodium content in this nephron segment is uromodulin release which intended to promote NCC maturation as observed in the same study. However, whether the regulation is through intracellular communication or extracellular binding needs further investigation. Connecting these findings, in the setting of high luminal sodium concentration in GS states, UMOD may be released with the intention to activate NCC to increase sodium uptake. Since there is defective NCC in GS, these luminally available UMOD can preferentially bind to TRPV5, upregulate its expression and therefore increase calcium uptake. This hypothesis, however, still needs experimental evidence but is a potential mechanism for the occurrence of hypocalciuria in GS.

Lastly, the mechanism by which hypocalciuria occurs is thought to be dependent on volume status [24]. When there is low ECF volume, the proximal convoluted tubule calcium recovery system is likely to be the dominant segment inducing hypocalciuria. On the other hand, when ECF volume is normal, the distal convoluted tubule's active transcellular calcium reabsorption is thought to be the site for hypocalciuria induction. This concept is particularly important since there are equivocal findings as to whether the GS clinical picture is normovolemic, hypovolemic, or even hypervolemic [6]. While studies have proven the expected lower blood pressure in GS patients due to salt-wasting, a huge portion of patients still show normal blood pressure at the time of presentation [57, 58]. In relation to these, the latest KDIGO guidelines for suspecting a diagnosis of GS include low or normal blood pressure as one of the clinical criteria. The observed compensation might be attributed to salt compensation in the diet as majority of GS patients are known to have salt-craving behaviors [59]. It is noteworthy that a prescribed blood pressure cutoff for proposed volume-dependent dichotomy presented by Reilly & Huang has not been defined to date [24]. Furthermore, GS patients having lower blood pressure measurements compared to the general population does not mean significant ECF volume reduction nor an abnormally low blood pressure based on current definitions [60, 61].

Magnesium handling in the DCT

The molecular mechanisms involved in DCT transcellular magnesium transport has been a growing field in the past years. Ellison, Maeoka and McCormick recently presented a comprehensive update on magnesium handling in the different nephron segments including the DCT [62]. Franken and colleagues z further discussed current knowledge and questions especially on sodium and magnesium in the DCT [64]. This review, specifically the succeeding sections, aims to add on to these available data and aid in the generation and answering of more hypotheses. Here, we aimed to integrate current evidence for guidance of future studies specifically on GS-related hypomagnesemia. Briefly, the section will reiterate known physiologic pathways and then zero into magnesiotropic proteins which the authors deemed to have particular relevance to GS. Additionally, a discussion on calcium-magnesium crosstalk follows.

Figure 4 presents a proposed pathway for magnesium handling in DCT synthesized from a number of studies discussed in this section. So

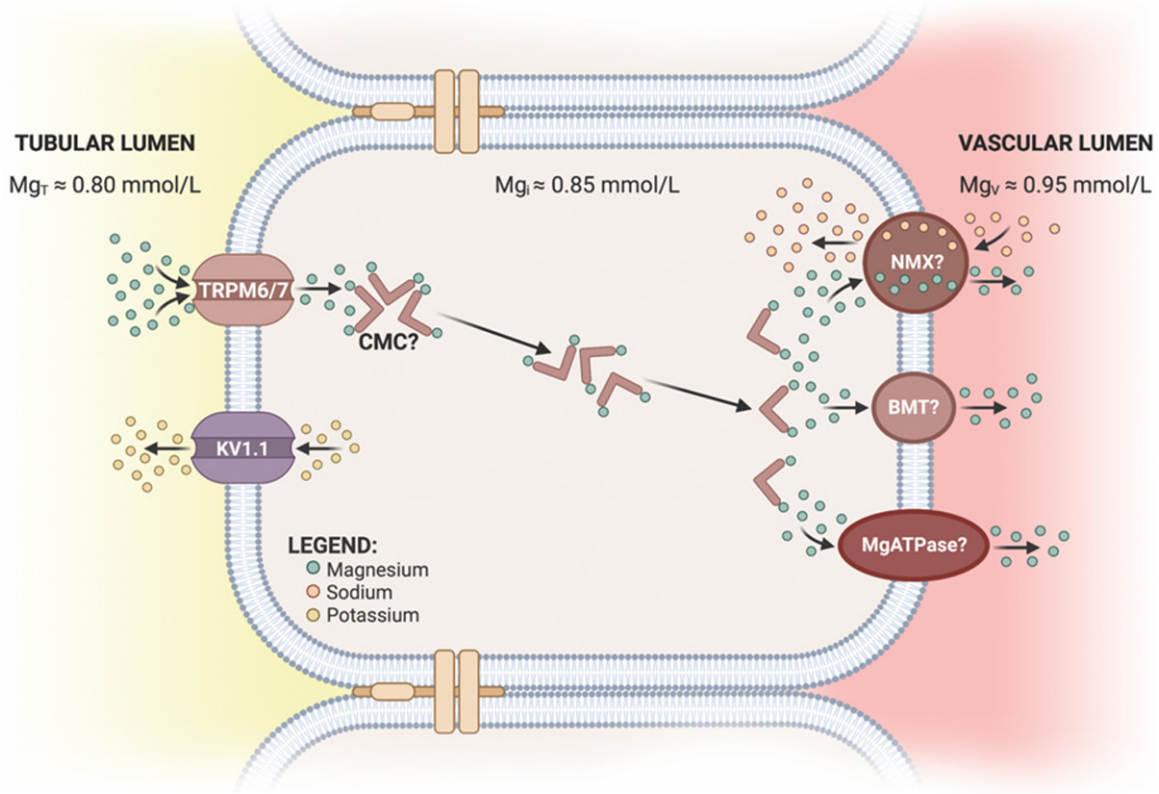

Figure 4. Magnesium handling in the distal convoluted tubule. Magnesium ions enter the apical side via the transient receptor potential melastatin subfamily member 6 (TRPM6) channel forming a tetramer with its homologue TRPM7 together functioning as the apical magnesium channel, known as the TRPM6/7 transporter complex. Since the magnesium concentration is almost uniform across the membrane, this movement of magnesium depends on the voltage gradient provided by the luminal potassium voltage-gated channel subfamily A member 1 (KV1.1). A hypothetical cytosolic magnesium carrier (CMC) moves around the magnesium from the apical to the basolateral side where putative extrusion mechanisms are carried out by a sodium-magnesium exchanger (NMX), a magnesium ATPase transporter (MgATPase), or a plain basolateral magnesium transporter (BMT). Proteins labelled with a question mark (?) have candidate proteins but have not been fully characterized for the specified function.

far, it is known that apical magnesium entry is facilitated by the transient receptor potential melastatin subfamily member 6 (TRPM6) channel which forms a tetramer with its homologue TRPM7 together functioning as the apical magnesium channel, referred to as the TRPM6/7 transporter complex hereafter [64]. Regulation of apical magnesium entry is primarily driven by the membrane voltage because unlike the thousand-fold steep calcium concentration gradient across the apical membrane, the intracellular magnesium concentration is almost similar to that of the luminal concentration [1, 65, 66]. This voltage is hypothesized to be provided by the luminal potassium voltage-gated channel subfamily A member 1 (KV1.1) [67]. Follow up studies elaborating on this relationship have not been done to date. A dedicated cytosolic carrier that transports magnesium ions from the apical to the basolateral side has not been identified to date [1, 68]. The cytosolic protein PVALB has been shown to have both calcium- and magnesium-binding domains. PVALB has higher affinity to calcium but in the resting state its cation-binding sites are predominantly occupied by magnesium [13]. This can most likely be explained by the higher intracellular magnesium concentration compared to intracellular calcium concentration. Intriguingly, the difference in intracellular concentration between magnesium and calcium and their molecular gradients with respect to the tubular lumen dampens the need for an intracellular magnesium buffering protein serving a parallel role of CALB1 for calcium represented in **Figure 2** as the hypothetical cytosolic magnesium carrier (CMC) [68]. Therefore, the need for a CMC protein is still debatable to date.

Similarly, there is no basolateral magnesium gradient which poses a mechanistic challenge for magnesium extrusion to the blood stream. Theoretically, this could be achieved if an NCX1-like magnesium counterpart, referred to a sodium-magnesium exchanger (NMX) hereafter, would function to take in sodium ions in exchange for magnesium ions [69]. The basolateral solute carrier family 41 member 1 (SLC41A1) has been shown to have NMX characteristics and is thought to be the predominant magnesium extrusion mechanism in the DCT [70]. However, more recent studies have demonstrated that its extrusion mechanism is Na-independent, therefore a basolateral DCT NMX is yet to be discovered [71]. Similar to calcium handling, an Mg-ATPase could also be a putative basolateral efflux mechanism.

The Cyclin and CBS domain divalent metal cation transport mediator-2 (CNNM2) is known to localize in the basolateral membrane of distal tubules [72, 73]. CNNM2 transcripts have also been shown to be responsive to magnesium fluctuations as shown in the DCT magnesium-sensitive transcriptomic study by the team of de Baaij [74]. It was also shown to be an important regulator of urinary magnesium reabsorption in a zebrafish model [75]. Finally, it has been shown that CNNM2-deficient mice have impaired magnesium homeostasis further substantiating its putative role in renal magnesium handling [73]. The specific mechanism by which CNNM2 carries out this role is still under debate and for further probing. It has been demonstrated to have an ATP binding site which gives the possibility of an Mg-ATPase function like the PMCA for calcium transport [72, 76]. It has also been shown to be exhibit potential magnesium sensing characteristics based on topological studies [72]. An NMX functionality has also been speculated but its genuineness as an exchanger remains to be proven by further experimental studies [77-79]. Lastly, a plain magnesium transport across the basolateral membrane is also possible without an exchange mechanism or ATPase function which could be carried out by these candidate magnesium efflux proteins or a separate basolateral magnesium transporter (BMT) yet to be discovered.

It is generally thought that transcellular magnesium reabsorption happens in both early and late DCT. The primary basis of this is the expression of TRPM6 (**Figure 1**) [79]. CNNM2 is expressed in both DCT1 and DCT2 while PVALB is exclusively expressed in DCT1 [15, 79]. The differential expression of SLC41A1 in the DCT segments has not yet been evaluated to date.

Hypomagnesemia in GS

Hypomagnesemia is one of the clinical criteria of GS during its discovery by Gitelman and colleagues in 1966. Current guidelines define hypomagnesemia as serum levels of <0.7 mmol/l or <1.70 mg/dl with inappropriate renal magnesium wasting indicated by a fractional excretion value of >4% [6]. Additionally, chronic thiazide treatment, NCC null mice, and other GS models all present with hypomagnesemia [5, 30, 80-82]. While it is known that magnesium reabsorption is affected by sodium reabsorption, the specific molecular mechanism underlying this relationship remains poorly characterized [1, 63, 81]. Magnesium derangements in Gitelman syndrome may result from membrane potential changes, differential expression of magnesium handling proteins, atrophy of the DCT or a combination of these. The succeeding sections present potential magnesiotropic proteins that may directly or indirectly play a role in the development of hypomagnesemia as a result of the NCC defect in Gitelman syndrome supplemented by a visual integration in **Figure 5**.

TRPM6

The apical magnesium channel, TRPM6 is the most characterized magnesium-handling protein to date. It has been shown that TRPM6 transcript and protein expression decreases in GS states [81, 83]. TRPM6 is expressed on both early and late DCT. Since there is atrophy and extensive early DCT remodeling in NCC-null mice, TRPM6 expression declines proportionately [5, 83]. However, an extensive signaling mechanism as a response to tissue remodeling would need more time and cannot explain the acute magnesium leakage in patients treated with thiazide diuretics [81]. Mediators for a direct NCC-TRPM6 crosstalk have not been defined to date. Players in this putative communication may be inferred from TRPM6 many interacting regions. Furthermore, Franken and colleagues speculates that common regulatory pathways involving the two ion transporters might give us an idea on how one affects the

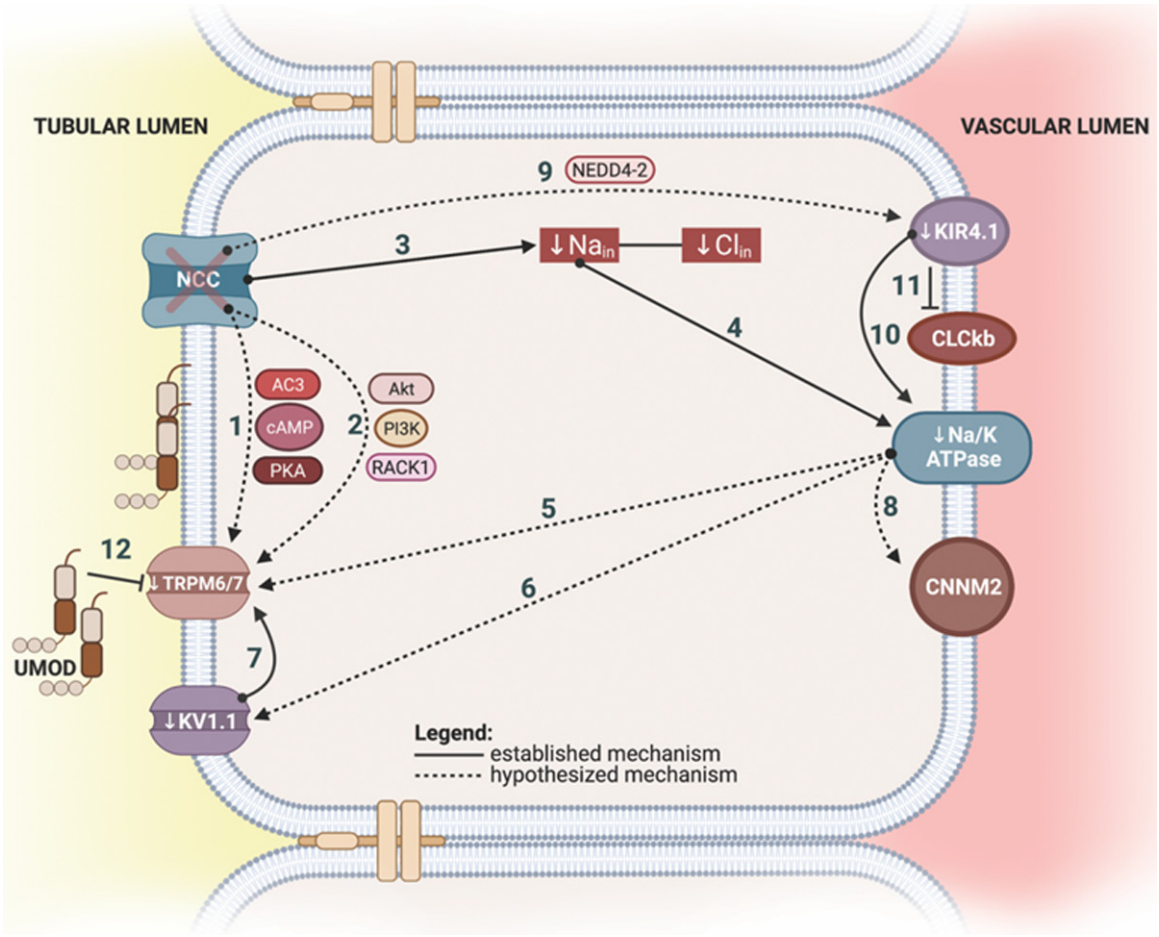

Figure 5. Mechanism of hypomagnesemia in Gitelman syndrome. The expression of TRPM6/7 decreases in NCC-defective states like Gitelman syndrome. The signaling mechanism is thought to involve two major TRPM6/7-regulating pathways, the (1) ACE-cAMP-PKA pathway and the (2) Akt-PI3K-RACK1 pathway. NCC inactivation (3, 4) downregulates Na-K ATPase activity through the resulting decrease in intracellular sodium. The decreased ATPase activity might signal (5) TRPM6 downregulation thus causing hypomagnesemia. Similarly, this may have an effect on (6, 7) the apical KV1.1 and therefore TRPM6 and/or on (8) the putative basolateral NMX protein, CNNM2. (9) A KIR4.1-NCC crosstalk facilitated by NEDD4-2 might also exist which could further have an effect on (10) Na-K ATPase as the ATPase' activity is coupled with that of KIR4.1. The proposed decrease in KIR4.1 is attributed to the attributed to a possible compensation mechanism to decreased intracellular chloride levels. As KIR4.1 provides the necessary voltage gradient for the activity of CLC-kb, lowering KIR4.1 activity will (11) reduce ClCkb function. Lastly, (12) secreted UMOD is known to affect TRPM6/7 cell surface expression by arresting the channel's endocytosis.

other one [63]. One possible pathway may AKT-P13K-RACK1 pathway which is hormonally controlled. It is proposed that aside from being an upstream regulator of TRPM6 it could also be potentially affected by NCC dysregulation due to systemic hormonal imbalances (for a more detailed review, see reference [63]). Another major control pathway of TRPM6 activation and expression is through the AC3-cAMP-PKA signaling mechanism which is hypothesized to facilitate a compromised TRPM6 activation secondary to a reduced NCC activity [84]. It would be interesting to see the expression profile of these proteins in an NCC-deficient state. Information about their sensitivity to sodium concentration changes might also give us an idea on the effects of an NCC defect and provide a more immediate response mechanism apart from hormonal alteration. However, these remain as speculation at the moment. Another way by which TRPM6 can be directly regulated is through phosphatidylinositol 4,5-bisphosphate (PIP2). PIP2 has been shown to be an activator of TRPM6 by direct binding to the channel [85]. Blockage and deletion of PIP2 causes TRPM6 inactivation and abolishes api-

cal magnesium influx. Furthermore, upregulation of phospholipase C which hydrolyses PIP2 into diacylglycerol and inositol 1,4,5-trisphosphate effectively inactivates TRPM6 in electrophysiology studies. Whether changes in PIP2 or phospholipase C happens occur as a result of NCC abrogation is also an interesting pathway to look at.

Earlier it was discussed that unlike the thousand-fold calcium gradient driving its inward rectification, apical membrane potential difference is the sole driver of magnesium entry into the DCT. Theoretically, the observed change in the apical membrane potential as a result of the chloride concentration drop secondary to an NCC defect can also cause TRPM6 downregulation in the same way that TRPV5 upregulation happens. Demonstration of this has not yet been documented in any studies at the moment.

Na/K-ATPase

Thiazide treatment causes reduction in the activity of this basolateral sodium-potassium ATPase (Na/K-ATPase) pump [86]. Furthermore, pharmacologic inhibition and inactivating mutations of the Na/K-ATPase have been shown to cause hypomagnesemia [63]. In the context of decreased NCC expression, the decrease in intracellular sodium concentration is expected to affect the Na/K-ATPase. How this induces hypomagnesemia has not yet been defined but may involve a direct or indirect signaling pathway to TRPM6. One explanation is that the reduced ATPase activity decreases intracellular potassium which could affect the apical KV1.1 which provides the voltage gradient for TRPM6-mediated magnesium entry. Apical membrane depolarization secondary to the ATPase deactivation has also been suggested in previous reports [1, 63]. Furthermore, the activity of the Na/K-ATPase pump is coupled with and enhanced by the potassium inward rectifying channel 4.1 (KIR4.1) [63, 68]. Briefly, KIR4.1 is closely associated with NCC. Several studies have identified KIR4.1-mediated NCC modulation [87, 88]. Whether the NCC defect in GS reduces KIR4.1 expression or activity has not yet been demonstrated in studies but maybe a plausible explanation to the observed reduction in Na/K-ATPase activity. The role of KIR4.1 will be discussed in detail in the succeeding section.

KIR4.1

Mutations in KCNJ10 which codes for the basolateral potassium (K+) inward rectifying channel KIR4.1 cause EAST syndrome which presents with epilepsy, ataxia, and sensorineural deafness, on top of a salt-wasting tubulopathy resembling the GS phenotype [89, 90]. Total and phosphorylated NCC expressions are markedly decreased in a kidney-selective, doxycycline-dependent CRE-recombinase KIR4.1-knockout mouse model [91]. Similar effects on NCC were also demonstrated in a kidney-specific KIR4.1 knockdown model [92]. As expected, these model mice exhibited hypokalemic metabolic alkalosis with hypomagnesemia and hypocalciuria. Although KIR4.1 expression levels in other NCC-deficient systems have not been characterized, it is theoretically possible that KIR4.1 is also functionally obliterated as a compensation mechanism to decreased intracellular chloride levels. Blockade of KIR4.1 using barium has been shown to increase chloride concentration in DCT cells through its regulatory role on CLC-kb which facilitates basolateral chloride exit [93]. KIR4.1 provides the necessary voltage gradient for the activity of CLC-kb. A direct crosstalk might also be speculated from common regulatory proteins like Nedd4-2. It is a ubiquitin ligase that has been shown to regulate both NCC and KIR4.1 [94-96]. However, these hypotheses remain to be proven until now.

Uromodulin

Aside from the previously discussed roles of UMOD in sodium and calcium reabsorption, it has also been shown to affect magnesium handling in the DCT. In a transcriptomic study searching for magnesium-sensitive genes, UMOD yielded one of the highest reads implying a regulatory role for the divalent cation [74]. Secreted UMOD in the urine physically interacts with TRPM6 through another urinary protein galectin-1 [97]. This leads to impaired endocytosis, therefore, increased TRPM6 cell surface abundance. Similar to the dilemma in UMOD's calciotropic role, the trigger for its release that could possibly affect TRPM6 is yet to be elucidated. Following the train of thought in the calcium handling hypothesis in the previous sections, high urine sodium concentration in GS states could trigger release of UMOD with the intention to activate NCC and increase sodium

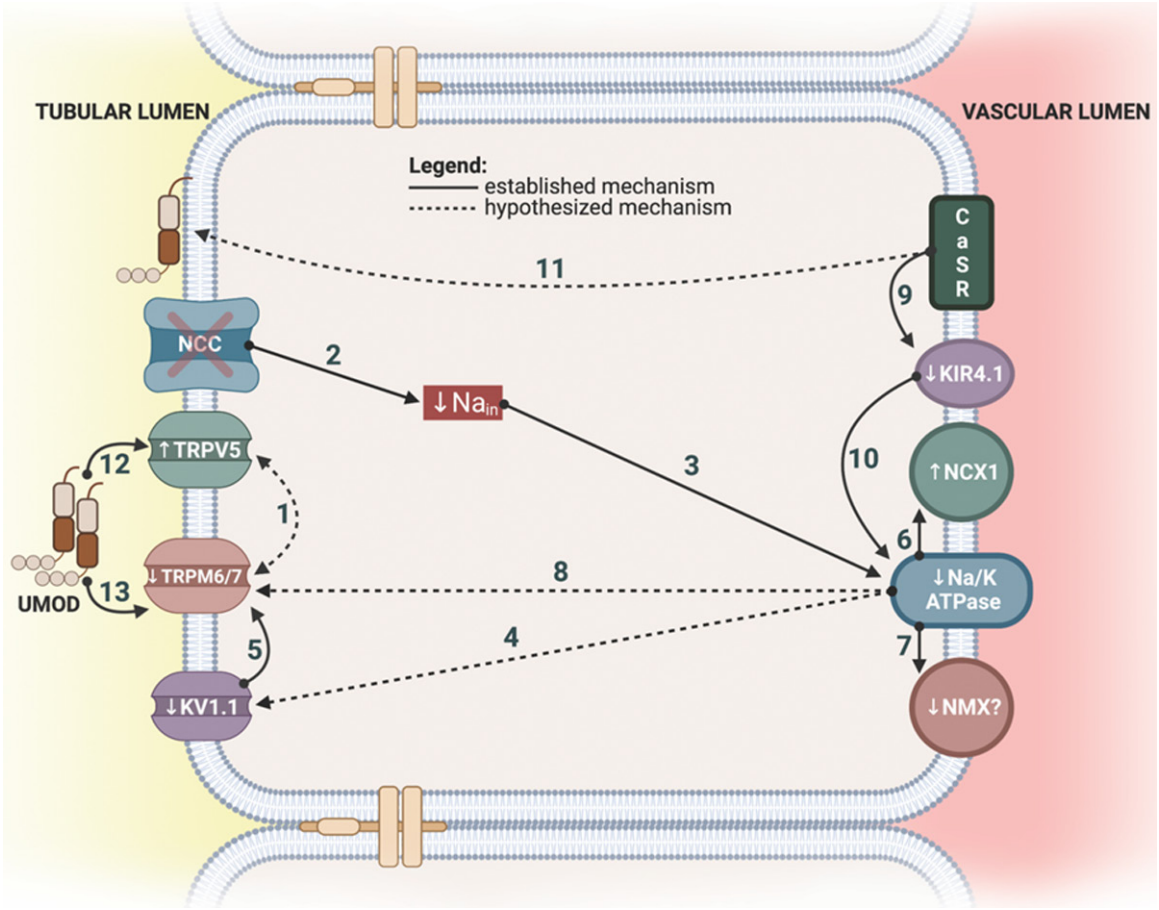

Figure 6. Calcium and magnesium crosstalk mechanism. (1) A direct crosstalk between TRPV5 and TRPM6 is proposed. However, putative players are yet to be identified. The (2) decrease in intracellular sodium secondary to a defective NCC (3) alters the activity of the basolateral Na/K-ATPase. This activity change affects both magnesiotropic and calciotropic proteins (4) KV1.1 and therefore indirectly the (5) TRPM6/7 complex, as well as the sodium exchangers, (6) NCX1 and the (7) putative NMX proteins. (8) A direct ATPase-TRPM6/7 is also possible. The (9) calcium-sensing receptor (CaSR) is known to modulate KIR4.1 activity which in turn (10) affect the activity of Na/K-ATPase. Furthermore, (11) CaSR is also proposed to regulate UMOD release in the DCT similar to their relationship in thick ascending limb cells. Lastly, secreted UMOD may paradoxically (12) increase TRPV5 activity and (13) decrease TRPM6 activity in the setting of a defective NCC.

uptake and affect TRPM6 as well. The paradoxical increased calcium uptake but decreased magnesium uptake is discussed in the crosstalk section of this paper.

Calcium and magnesium crosstalk

The conundrum on GS-related hypocalciuria and hypomagnesemia may not just be a clinical coexistence but may also imply a mechanistic crosstalk between calcium and magnesium pathways in normal and pathophysiologic states. Lee and colleagues were able to demonstrate TRPV5 upregulation and increased calcium uptake in the apical side as a result of decreased intracellular magnesium concentration and vice versa [98]. Furthermore, changes in available dietary magnesium have been shown to alter the transcriptomic profile of calciotropic genes [74].

There are several mechanistic hypotheses explaining the crosstalk mechanism integrated in **Figure 6**. TRPV5 has a selectivity filter at the aspartate-542 residue which happens to be a magnesium binding site. During high magnesium states, the selectivity filter site is occupied by magnesium effectively occluding the channel pore preventing calcium entry in the cell [98]. The second mechanism happens with slow reversibility in that magnesium unbinding from the site does not immediately cause influx

of calcium ions but instead a 30- to 40-second lag time is observed. In states of magnesium wasting as in GS, this mechanism of TRPV5 regulation is hampered thereby increasing calcium influx thus the characteristic hypocalciuria coexisting with hypomagnesemia. The mechanism might also be a feasible explanation to the rather later presentation of hypocalciuria compared to hypomagnesemia in GS patients. This, however, is yet to be verified in future studies. Aside from innate properties of the major apical calcium and magnesium transporters, other DCT proteins may also play a role in the calcium-magnesium signaling.

Na/K-ATPase

The primacy of the role of sodium in the DCT precludes the central role of the Na/K-ATPase in this nephron segment. Although its part in the reabsorption of divalent cations has only been widely accepted for the purpose of magnesium handling, it could potentially affect calcium as well. While the effect of Na/K-ATPase on magnesium possibly lies on the maintenance of the membrane potential driving the reabsorption through KV1.1, and possibly the candidate NMX proteins as well, its effect on calcium may be related to NCX1 as calcium exit is determined by sodium concentration. This theoretical physiologic response further supports the proposed crosstalk.

CaSR

The calcium sensing receptor expressed on the basolateral side of the DCT is not only sensitive to calcium but is also responsive to magnesium therefore changes in magnesium concentration trigger calcium regulatory cascades [74]. Moreover, CaSR has been shown to modulate KIR4.1 by altering both its cell surface expression and its activity [99, 100]. In this regard, a fluctuation in calcium concentration may be able to affect magnesium handling as KIR4.1 plays an important role in its homeostasis. Aside from physiologic sensing of calcium levels known to be abrogated in GS, the effect of a defective NCC to CaSR protein expression is also interesting to look at.

Uromodulin

As presented in this review, UMOD is a putative player in both calcium and magnesium homeostasis. It has been shown to interact with TRPV5 and TRPM6, the major calcium and magnesium channel, respectively, from the urinary lumen [54, 97]. Continuing on the proposed interactions of UMOD with these ion channels mentioned in the previous sections, the paradoxical increase in TRPV5 activity and decrease in TRPM6 as a possible response to a deactivated NCC remains a question. This poses the need for structural analysis of uromodulin's binding sites facilitating such interactions. This can provide us with information on possible preferential binding which could explain why it favors upregulation of one channel over the other one. The idea of uromodulin polymerization producing a macromolecular structure in the DCT has also been proposed. It is possible that varying lattice formations, involvement of different binding domains, or protein cleavage at different sites could provide permutations of many interactions with transporters expressed in the apical surface [54, 97]. Additionally, the effect of non-secreted UMOD to TRPV5 and TRPM6 packaging and cellular trafficking has not been defined to date. Interestingly, the team of Tokonami also observed that the release of UMOD in the thick ascending limb is modulated by CaSR [101]. Since UMOD and CaSR are also present in the DCT, this interaction is also potentially present. Therefore, it is interesting to observe uromodulin concentrations, both the transcript and protein level, in response to a defective NCC as in GS.

Conclusions and perspectives

The general flow of calcium transport in the DCT is relatively established and is fueled mainly by the maintenance of a low intracellular level of the ion. They key players in the apical and basolateral sides as well as the cytosolic carriers have been identified. However, the specific interactions between them are incomplete and unstudied. Further characterization of the binding domains of each CaP could facilitate establishing the connection points. For instance, calbindins and other possible similarly functioning molecules may communicate with extrusion proteins on the basolateral side through putative binding sites similar to its sensing and regulatory association with TRPV5. To connect these dots, pathway players have to be identified as well and may be inferred from common regulators. The arrows depicted in

Figure 6 are potential pathways waiting to be expanded and populated with intermediate molecules facilitating the cross talks amongst CaPs and related proteins. As calcium is voltage dependent, studies on the effect of changes in membrane potential secondary to decreased sodium and chloride concentration is also important as the basolateral CaPs are potentially electrogenic. Signaling cascades in response to membrane polarity changes are equally valuable.

A lot of work is yet to done for magnesium handling. Characterization of structural domains of not only MaPs but also of transporters of related ions providing the voltage gradient needed for magnesium movement are lacking to date. The importance of potassium handling proteins like the Na-K ATPase and KIR4.1 in relation to magnesium transport is a growing field. Cross talks between them and MaPs, them and NCC, and them and other potassium handlers are pathways that need to be characterized. While the necessity of having a magnesium cargo carrier across the cell is still questionable, identifying a dedicated cytosolic protein performing such function would be a game changer. This will also open up potential signaling mechanisms between apical and basolateral MaPs. The direct and indirect effects of membrane potential changes to MaPs are also largely unknown.

Efforts have been made to characterize development of calcium and magnesium derangements separately but not a lot of work has considered the possibility of communications or common regulatory pathways existing between their key players. Characterizing CaPs and MaPs and their potential interactions and integrations into common regulatory pathways is equally important. Upstream, they might have domains controlled by common proteins while downstream, they might have both calcium and magnesium domains as frequently identified in cytosolic ion carrier proteins.

On a cellular level, studies employing separation of DCT1 and DCT2 have only been done in silico using single cell RNA sequencing. Though challenging, in vitro separation will revolutionize modelling of not only physiologic calcium and magnesium handling but of the pathophysiology behind derangements in these electrolytes as well. Methods like immunofluorescence-guided microdissection and fluorescence-associated cell sorting are developed for studies in normal physiology. However, protocols for studies involving pathologic states like GS are yet to be optimized as expression of canonical markers for the DCT are greatly affected. Lastly, most of the expression studies used in this review involved immunohistochemistry, Western blots, and PCR-based detections. High throughput sequencing studies have focused on normal subjects, but none has been involved in pathologic subjects. Differential expression studies on GS models using next generation transcriptomic and proteomic technologies will make available a wide array of CaPs and MaPs which can be good candidates in the molecular pathways for explanation of hypercalciuria and hypomagnesemia in GS. This review has, in part, provided a shortlist of possible proteins that are calciotropic or magnesiotropic or both which worth further investigation.

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Disclosure of conflict of interest

None.

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