Calcium and magnesium handling: beyond ion transport

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by

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General introduction

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Body Ca^{2+} and Mg^{2+} balance

Ca^{2+} and Mg^{2+} are essential for many physiological processes such as intracellular signalling, enzyme activation, neural excitability, muscle contraction and bone formation. In healthy individuals, the majority of total bodily Ca^{2+} resides in the skeleton, whereas a minor amount is distributed in soft tissues and extracellular fluids. After Ca^{2+}, K$^+$ and Na$^+$, Mg^{2+} is the fourth most abundant cation in the human body and is distributed as following: 65% in bone, 35% in soft tissue, and less than 1% in plasma. Disturbed blood ionized Ca^{2+} and Mg^{2+} levels are associated with severe clinical symptoms mainly related to instability of the neurological and cardiac systems, and perturbations in bone formation. Therefore, the extracellular Ca^{2+} and Mg^{2+} concentrations need to be maintained within a narrow range by the concerted action of intestinal absorption, exchange with bone and renal reabsorption. Noteworthy, the kidneys determine the final excretion of Ca^{2+} and Mg^{2+} in the urine and thus accomplish an important role in the homeostatic control of these divalent cations.[1]

Renal Ca^{2+} reabsorption

Plasma Ca^{2+} levels are maintained around 2.20-2.65 mmol/L.[2] Approximately 50-60% of the total plasma Ca^{2+} is filtered by the glomeruli, either in its ionized form or in complexes with other ions. In the proximal tubule (PT), the Na$^+$-driven water reabsorption increases the intraluminal concentration of Ca^{2+} leading to passive diffusion of 60-70% of the filtered Ca^{2+} through the tight junctions towards the renal interstitium (**Figure 1**).[3, 4] In the thick ascending limb of Henle (TAL), a lumen-positive transepithelial voltage is the main force for the paracellular reabsorption of 20-25% of the filtered Ca^{2+}. The generation of this electric gradient relies on the Na$^+$, K$^+$ and Cl$^-$ uptake by the apical Na$^+$-K$^+$-2Cl$^-$ cotransporter (NKCC2) coupled with the subsequent recycling of K$^+$ across the apical membrane via the renal outer medullary K$^+$ channel (ROMK; **Figure 1**).[3] At the basolateral membrane, the Cl$^-$ channel CLC-Kb is responsible for Cl$^-$ extrusion, whereas the Na$^+$-K$^+$-ATPase generates the primary inward driving force for Na$^+$ transport via NKCC2. Along the distal convolutions of the nephron, Ca^{2+} reabsorption relies essentially on transcellular transport.[5] Approximately 10% of the filtered Ca^{2+} is reabsorbed in the late distal convoluted tubule (DCT2) and the connecting tubule (CNT) via the epithelial Ca^{2+} channel, transient receptor potential vanilloid 5 (TRPV5; **Figure 1**).[6] This channel co-localizes with the Ca^{2+}-binding protein calbindin-D$_{28K}$, an intracellular protein that buffers free Ca^{2+} and facilitates transport of bound Ca^{2+} towards the basolateral membrane. Ca^{2+} is finally extruded in the renal interstitium to re-enter the bloodstream by the Na$^+$-Ca^{2+} exchanger (NCX1) and the plasma membrane Ca^{2+}-ATPase (PMCA1b).[3] The relative contribution of the collecting duct (CD) to overall Ca^{2+} reabsorption is probably negligible. In conclusion, only 1-2% of the filtered Ca^{2+} is excreted by the kidney into the urine.[7]

Renal Mg^{2+} reabsorption

Plasma Mg^{2+} levels are maintained around 0.7-1.1 mmol/L.[8] Approximately 70% of the total plasma Mg^{2+} is filtered through the glomeruli. Of this amount, 10 to 20% is reabsorbed by the PT, whereas the majority (65-70%) is taken up in the cortical TAL. Mg^{2+} reabsorption in PT and TAL occurs via the paracellular route, as reported for Ca^{2+}, and is driven by similar electrochemical forces (**Figure 1**). Nevertheless, PT and TAL show opposite reabsorptive capacity for Ca^{2+} and Mg^{2+}. The reason why the bulk of filtered Ca^{2+} is reabsorbed in the PT, whereas filtered Mg^{2+} is mainly taken up in the TAL is not entirely clear. Probably the paracellular barrier of these two segments displays distinct permeabilities for divalent cations. Of the filtered Mg^{2+}, 10-15 % reaches the early DCT (DCT1), where 70 to 80% is reabsorbed. Mg^{2+} transport in DCT1 is an active and transcellular process, which is critically influenced by cellular energy metabolism.[9] DCT1 determines the final urinary Mg^{2+} concentration by reabsorbing Mg^{2+} via the epithelial Mg^{2+} channel, transient receptor potential melastatin 6 (TRPM6; **Figure 1**).[10, 11] The maintenance of a negative membrane potential by the voltage-gated K$^+$ channel, Kv1.1, provides the driving force for Mg^{2+} transport via TRPM6.[12] The Na$^+$-K$^+$-ATPase, situated in the basolateral membrane of DCT1 cells, generates a local negative membrane potential and provides a Na$^+$ gradient for the thiazide-sensitive Na$^+$-Cl$^-$-cotransporter (NCC) to facilitate transport of Na$^+$ from the lumen into the cytoplasm.[13] K$^+$ is supplied to the Na$^+$-K$^+$-ATPase through the K$^+$ channel, Kir4.1.[14] Finally, the transcription factor HNF1B affects the expression of the regulatory protein γ-subunit that binds and modulates the activity of Na$^+$-K$^+$-ATPase.[15] At the basolateral compartment, cyclin M2 (CNNM2) probably senses intracellular Mg^{2+} concentrations and regulates other Mg^{2+} transporters involved in the extrusion of Mg^{2+} towards the blood.[16] As no evidence exists for Mg^{2+} being extruded from the cell via a Mg^{2+}-ATPase, other driving forces, such as a Na$^+$ gradient, might be used to transport this cation by secondary active transport mechanisms. Up to now the gene encoding for the Mg^{2+}-extrusion protein in the kidney remains unknown. No significant Mg^{2+} reabsorption occurs in the more distal nephron segments. Overall, less than 5% of the filtered Mg^{2+} normally appears in the urine.

Renal TRP channels

The transient receptor potential (TRP) superfamily consists of cation-selective ion channels with similar molecular structures, but diverse tissue expression and large functional heterogeneity, ranging from ion homeostasis to mechanosensation.[17] TRP channels mediate Ca^{2+} influx in many cell types. Ca^{2+} ions in turn act as second messenger and can, therefore, affect various intracellular pathways, resulting in a wide scale of physiological consequences.
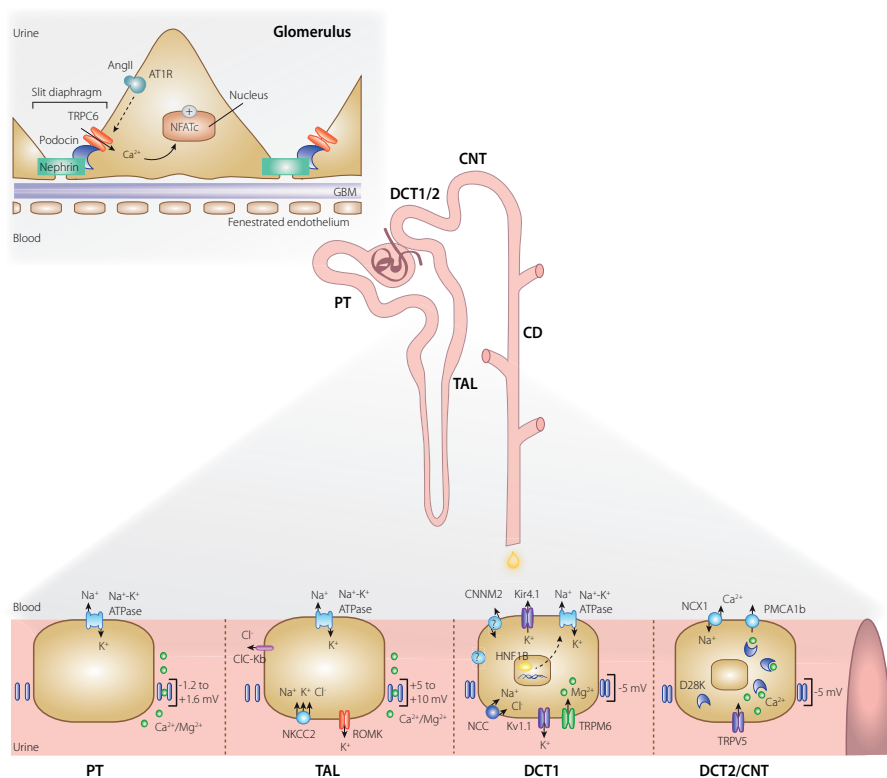
Figure 1 Cartoon depicting the molecular players involved in the glomerular and tubular handling of Ca^{2+} and Mg^{2+}.

PT: proximal tubule; TAL: thick ascending limb of Henle; DCT1/2: early and late distal convoluted tubule, respectively; CNT: connecting tubule; CD: collecting duct; GBM: glomerular basement membrane; TRPC6: transient receptor potential channel subfamily C member 6; AngII: angiotensin II; AT1R: angiotensin type I receptor; NFAT: nuclear factor of activated T-cells; NKCC: Na^+-K^+-$2Cl^-$ cotransporter; ROMK: renal outer medullary K^+ channel; ClC-Kb: Cl^- channel Kb; NCC: Na^+-Cl^--cotransporter; Kv1.1: potassium voltage-gated channel subfamily A member 1; TRPM6: transient receptor potential cation channel subfamily M member 6; CNNM2: cyclin-M2; Kir4.1: ATP-sensitive inward rectifier potassium channel 10; TRPV5: transient receptor potential cation channel subfamily V member 5; D_{28K}: calbindin-D_{28K}; NCX1: Na^+-Ca^{2+} exchanger; PMCA1b: Ca^{2+}-ATPase.

So far, 28 mammalian members have been identified, which are divided into 7 subfamilies on the basis of sequence homology. In the human kidney, TRP channels are expressed in various segments of the nephron and have been implicated in the pathogenesis of several kidney diseases.[18, 19] Renal TRP channels include: canonical TRP proteins (TRPC), melastatin-related TRP proteins (TRPM), vanilloid-receptor-related TRP proteins (TRPV) and polycystin (TRPP). The characterization of renal TRP channels provided

new insights into renal (patho)physiology and, in particular, into the regulation of Ca^{2+} and Mg^{2+} homeostasis.[20]

TRPV5 and TRPV6

TRPV5 and TRPV6 are so far the only known highly Ca^{2+}-selective channels in the TRP superfamily.[21] TRPV5 was originally cloned from primary cultures of rabbit CNT cells, whereas TRPV6 was identified from rat duodenum.[6, 22] Further investigations revealed that both TRPV5 and TRPV6 are mainly expressed in the Ca^{2+}-transporting tissues, i.e. kidney, intestine and bone, making these channels the molecular gatekeepers of Ca^{2+} homeostasis.[3]

Particularly, TRPV5 plays an important role in renal Ca^{2+} handling by fine-tuning the final amount of Ca^{2+} excreted via the urine. *TRPV5* knockout mice (*TRPV5$^{-/-}$*) displayed several phenotypic features related to a diminished active Ca^{2+} reabsorption, like hypercalciuria, reduced trabecular and cortical bone thickness due to disturbed bone morphology, hypervitaminosis D and increased intestinal Ca^{2+} absorption in response to the impaired renal Ca^{2+} reabsorption.[23] So far, human monogenic have not been associated with mutations in *TRPV5*.

TRPV6 is mainly involved in transepithelial small intestinal Ca^{2+} transport. *TRPV6* knockout mice (*TRPV6$^{-/-}$*) displayed disturbed Ca^{2+} homeostasis, including defective intestinal Ca^{2+} absorption, increased urinary Ca^{2+} excretion, decreased bone mineral density and deficient weight gain.[24] More recently, a *TRPV6* knock-in mouse model expressing functionally inactive TRPV6 channels have been described.[25] These animals exhibit decreased duodenal Ca^{2+} uptake and increased intestinal TRPV5 gene expression compared to wild-type mice probably as compensatory mechanism for the loss of TRPV6 activity.[25] In humans, gain-of-function polymorphisms of the *TRPV6* gene have been found in renal Ca^{2+} stone patients, suggesting hyperactivation of intestinal Ca^{2+} absorption followed by absorptive hypercalciuria.[26]

TRPM6

Within the TRPM subfamily, TRPM6 shares with TRPM7 the unique feature of an ion channel coupled to a α-kinase activity, from which the nickname "chanzyme". Functional analysis identified TRPM6 as a Mg^{2+}- and Ca^{2+}-permeable channel, although the affinity for the latter ion is five times lower.[27] By controlling active Mg^{2+} reabsorption in the kidney, TRPM6 is the final determinant of plasma Mg^{2+} concentration. Two independent research groups identified *TRPM6* as the causative gene in the autosomal recessive disorder named hypomagnesemia with secondary hypocalcemia (HSH [MIM 602014]).[10, 11] HSH patients develop severe hypomagnesemia (blood Mg^{2+} concentrions of 0.1–0.4 mmol/L), secondary hypocalcemia, disturbed neuromuscular excitability, muscle spasms, tetany and convulsions. By use of immunohistochemistry, the TRPM6 protein was shown to localize at the apical membrane of DCT1 in the kidney and at the brush border membrane

of absorptive epithelial cells of the ileum and colon.[10, 27] Accordingly, the identified homozygous and compound heterozygous mutations in HSH patients lead to impaired intestinal and renal Mg^{2+} absorption. During the past decade, many proteins mutated in inherited forms of hypomagnesemia have been found to directly affect TRPM6 or to alter the driving force for Mg^{2+} influx in DCT1 (**Figure 1**).[28] Further insight into the regulation of TRPM6 activity came from the investigation of regulatory factors affecting TRPM6 α-kinase domain.[29] The latter can act as an indirect player involved in Mg^{2+} homeostasis by its feedback action on the TRPM6-mediated Mg^{2+} influx. Interestingly, it was recently showed that dissociation of the TRPM7 kinase from the ion-conducting pore upon cleavage by intracellular proteases elicits two distinct functional proteins (channel and kinase) that can differentially localize and regulate important cellular processes, like death and differentiation.[30] Possibly a similar regulatory mechanism may also concern TRPM6.

TRPC6

The TRPC subfamily is a group of Ca^{2+}-permeable channels that are important in the regulation of the intracellular Ca^{2+} concentrations, thus modulation of various signalling processes. In contrast to the above renal TRP channels, TRPC6 is not involved in systemic homeostatic responses. The renal expression of TRPC6 received significant interest when gain-of-function mutations in the gene encoding for this protein were linked to familial focal segmental glomerulosclerosis (FSGS [MIM 603965])[31, 32] and TRPC6 was shown to associate with the glomerular slit diaphragm (**Figure 1**).[31] This protein complex connects podocyte foot processes and plays a crucial role in regulating glomerular filtration and preventing the leakage of proteins into the urine (**Figure 1**). Increased TRPC6 activity enhances Ca^{2+} influx into the podocyte, and thereby contributes to podocyte injury, causing proteinuria, glomerulosclerosis and progressive kidney failure.[33] In addition to *TRPC6* gain-of-function mutations leading to hereditary FSGS, TRPC6 expression correlates with severity of acquired podocyte injury and glomerular disease [31, 32, 34, 35] Noteworthy, known anti-proteinuric therapies downregulate TRPC6 expression, accompanied by improved podocyte outcome and amelioration of proteinuria.[33, 36, 37] , Altogether, the study of TRPC6 regulation in the kidney improved the mechanistic understanding of TRP channel function and emphasizes their role as new putative therapeutic targets for the treatment of kidney diseases. Specifically, these findings open the possibility to directly target TRPC6 as treatment strategy in proteinuric glomerular diseases.

Regulatory mechanisms of ion channel expression

The total capacity of a cell to mediate transport of a specific ion across the plasma membrane is determined by the amount of ions passing a selective channel and the number of channels at the cell surface. Many intracellular events that occur at multiple levels regulate the abundance of ion channels at the plasma membrane (**Figure 2**).
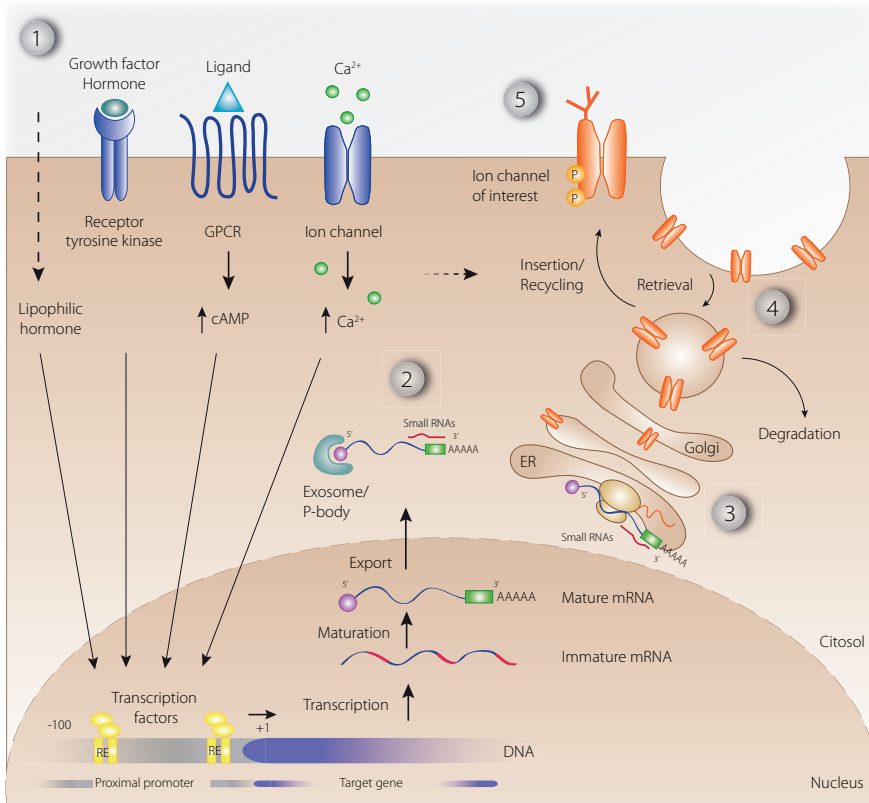
Figure 2 A model for the molecular mechanisms involved in the regulation of ion channel expression at the plasma membrane.

Control of cell surface expression of ion channels occurs at several levels: 1) Transcription; 2) Post-transcription; 3) Translation; 4); Trafficking; 5) Post-translation. GPCR: G protein-coupled receptor; cAMP: cyclic adenosine monophosphate; -100/+1: base pairs that define the proximal promote, counting from the transcription initiation site; RE: responsive elements; ER: endoplasmic reticulum; P: phosphorylation site.

The vesicular insertion and retrieval of channels at the cell surface represents a rapid regulatory mechanism. Upon retrieval, channels are targeted for degradation, while others are capable of re-entering the plasma membrane. Post-translational modification, like phosphorylation or glycosylation, can affect the cell-surface dynamics of ion channels.[38, 39] Moreover, intracellular signalling cascades initiated by the binding of growth factors or hormones to their receptors can modulate the vesicular trafficking towards the plasma membrane.[38, 40]

On the other hand, transcriptional regulation of genes encoding for ion channels accounts for a long-term control of their abundance at the cell surface. Activation of G protein-coupled receptors, receptor tyrosine kinases and Ca^{2+}-channels at the plasma membrane, or diffusion of lipophilic hormones into the intracellular compartment initiate signalling cascades that stimulate diverse families of transcription factors, either ubiquitous or tissue-specific.[33, 41-43] Binding of the transcription factors to their responsive DNA elements within the proximal promoter of target genes modulates the recruitment of the transcriptional machinery to the transcription initiation site. Moreover, the presence of more distal regulatory elements (enhancer or silencer) and the level of chromatin packing (epigenetic regulation) also affect gene transcription. Finally, at post-transcriptional level, splicing, 5'-methyl capping, polyadenylation and cleavage by small RNAs, determine whether the mature mRNA will undergo degradation, either in exosomes or in P-bodies, or translation in ribosomes. Small RNAs may also silence target mRNAs through translational repression, therefore affecting the rate of protein synthesis and the number of ion channels that will reach the plasma membrane.

The magnesiotropic transcription factor HNF1B

A novel molecular player in Mg^{2+} homeostasis is the hepatocyte nuclear factor 1 homeobox B (HNF1B).[15] HNF1B belongs to a family of homeodomain-containing transcription factors. HNF1B binding to the DNA is mediated by a POU specific (Pit-1, OCT1/2, UNC-86; POU_S) and an atypical POU homeodomain (POU_H) that recognize the consensus sequence 5'-RGTTAATNATTAAC-3' (R: purine, N: unspecified nucleotide; **Figure 3A**). The dimerization domain located at the N-terminal of the protein favors the formation of HNF1B homo- or heterodimers with the structurally related HNF1A in a complex that also includes two molecules of the dimerization cofactor of HNF1, named PCBD1 (**Figure 3A** and **3B**). Finally, the transactivation domain at the C-terminal of the protein is responsible for the initiation of transcription (**Figure 3A**).

In many eukaryotic transcription factor families, dimerization represents an additional control point or even a switch in a regulatory pathway.[44] So far, the functional differences and regulation of the HNF1B homo- or heterodimers are not well defined. It is known that the dimerization cofactor PCBD1 is able to bind HNF1 proteins in the nucleus and favor the formation of more stable HNF1 dimers, without direct binding to the DNA or interaction with other proteins belonging to the transcriptional machinery.[45] Interestingly, PCBD1 also localizes in the cytosol where it acts as pterin-4-alpha-carbinolamine dehydratase. The enzymatic activity of cytosolic PCBD1 allows the regeneration of tetrahydrobiopterin (BH4), an essential cofactor in the degradation of the amino acid phenylalanine.[46] Mutations in *PCBD1* have been reported as the underlying defects of a rare form of hyper-phenylalaninemia with primapterinuria (HPABH4D [MIM 264070], **Figure 3B**).[47-50] It was

A **HNF1B protein**
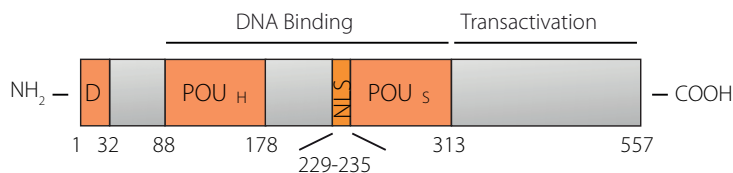
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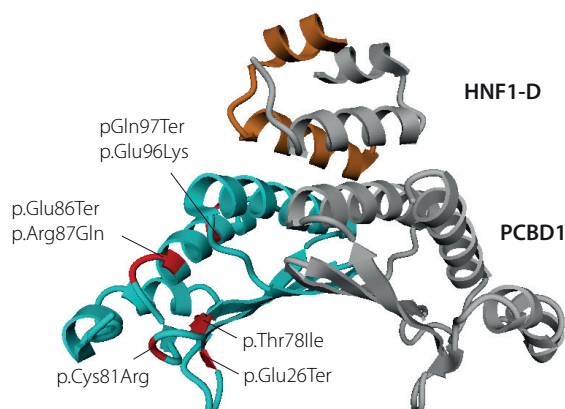
Figure 3 The magnesiotropic transcription factor HNF1B.

(A) Organization of the HNF1B protein domains. D: dimerization domain; POU$_H$: atypical POU homeodomain; POU$_S$: POU specific domain; NLS: nuclear localization signal. **(B)** Structure of the PCBD1/HNF1-D complex. The PCBD1 dimer (dark blue and gray) binds two HNF1 dimerization domains (HNF1-D, light blue and gray; Protein Data Bank file reference: 1F93). Residues in the PCBD1 protein that were found mutated in patients affected by hyperphenylalaninemia are depicted in red.

demonstrated that the enzymatic function of PCBD1 is not dependent on its function as dimerization cofactor, and viceversa.[51]

During early embryogenesis, HNF1B is involved in the development of tubular structures in kidney, pancreas, liver and the genital tract,[52] where its expression is postnatally restricted to epithelial cells. Recent studies reported that common variants mapping at the HNF1B locus associate with prostate and endometrial cancer risk,[53, 54] but the functional consequences of these polymorphisms remain subject for future research. With respect to the adult kidney, HNF1B is expressed throughout the entire nephron, whereas the expression of HNF1A is restricted to the PT. Heterezygous mutations in the *HNF1B* gene are responsible for dominant renal cysts and diabetes syndrome (RCAD [MIM 137920]) that includes a broad range of phenotypes with both renal and extrarenal

manifestations.[55] Reported defects in the *HNF1B* gene are whole-gene deletions in about 50% of the patients, whereas point mutations are detected in most of the remaining cases, along the entire coding sequence. In a large cohort of patients, no correlation was found between the type of mutation and the type and severity of the disease.[56] Finally, *de novo* mutations in the *HNF1B* gene are encountered in up to 30–50% of new cases.

Renal phenotype

HNF1B is a renal developmental gene whose mutations represent one of the genetic causes of familial congenital abnormalities of kidney and urinary tract (CAKUT).[57] Prenatally, the most frequent presentation consists of bilateral hyperechogenic kidneys with or without cortical cysts, whereas bilateral renal hypodysplasia with few or multiple cysts is prevalent in early childhood. The cause of cysts formation in the context of HNF1B mutations resides in the impaired transcriptional activation by HNF1B of renal cystic genes, i.e. genes responsible for distinct cystic kidney syndromes, like Pkhd1, Pkd2, UMOD.[58] Other targets of HNF1B in the kidney include the organic anion transporters URAT1[59] and OAT3[60], but the pathophysiological consequences of an altered transcription of these genes are unknown. So far, the best characterized tubular transport abnormalities in *HNF1B* patients involves a defect in the Mg^{2+} reabsorptive ability of DCT, that leads to renal Mg^{2+} wasting with hypocalciuria in up to 50% of the patients. Screening for HNF1B binding sites in genes known to affect renal Mg^{2+} transport revealed a role of HNF1B in the transcriptional regulation of the *FXYD2* gene encoding for the γ-subunit of the Na^+-K^+-ATPase.[15] Furthermore, Faguer *et al.* described that HNF1B nephropathy in adulthood is frequently accompanied by hypokalemia.[61]

Extra-renal phenotype

HNF1B mutations can cause maturity-onset diabetes of the young type 5 (MODY5), exocrine pancreas dysfunction, liver abnormalities, genital malformations and gout. Autosomal dominant mutations in genes encoding for various transcription factors are associated with different MODY subtypes: HNF4A (MODY1), HNF1A, (MODY3), insulin promoter factor (IPF1/PDX1; MODY4), HNF1B (MODY5) and NeuroD1 (MODY6).[62] HNF4A and HNF1A are primarily involved in glucose sensing and insulin secretion in pancreatic β-cells. During early development, HNF1B regulates the expression of HNF4A and HNF1A in embryoid bodies, but its role in mature β-cells is not clear.[62] We know that subjects with HNF1B mutations have an impaired insulin secretory response to glucose and sulphonylureas suggesting that insulin secretion defects and concomitant decline in β-cell mass are possibly involved. Nevertheless, the extent of the extra-renal HNF1B-related phenotype is highly variable and occasionally patients can reach end stage renal disease without developing diabetes. The evidence that HNF1B mutations can have a causal role in the development of new-onset diabetes after transplantation (NODAT) further supports its involvement in glucose metabolism and insulin sensitivity,[63] even if it cannot be

excluded that the HNF1B-mediated hypomagnesemia may contribute to the onset of diabetes.[64] NODAT is a frequent complication of kidney transplantation, partially as a consequence of the immunosuppressive regimens. As HNF1B mutations represent an additional risk factor for NODAT, the choice of immunosuppressive treatments in HNF1B patients should be tailored accordingly.

Hormonal regulation of Ca^{2+} and Mg^{2+} homeostasis

In the human body, Ca^{2+} and Mg^{2+} homeostatic systems rely on three components: *i)* tissues (re)absorbing or storing Ca^{2+} and Mg^{2+}; *ii)* sensors responding to fluctuations in extracellular Ca^{2+} and Mg^{2+} concentrations; and *iii)* hormones that modulate the transport and mobilization of these minerals. While the hormonal regulation governing Ca^{2+} reabsorption and mobilization from tissues has been largely investigated,[65] a comprehensive regulatory mechanism describing systemic Mg^{2+} handling is still missing. So far, the epidermal growth factor (EGF)[66], insulin[38] and estrogens[67] have been suggested as magnesiotropic hormones directly affecting plasma Mg^{2+} levels by increasing renal reabsorption via TRPM6 (**Figure 4**). Interestingly, Mg^{2+} can bind and influence the activity of the Ca^{2+}-sensing receptor (CaSR), an important molecular player in the hormonal coordination of Ca^{2+} homeostasis.[68] The cloning and characterization of the extracellular CaSR has demonstrated that Ca^{2+} ions can participate actively in their own regulation through receptor-mediated interactions. CaSR belongs to the superfamily of G-protein-coupled receptors (GPCRs) and is expressed in a broad range of tissues. Most importantly, in the parathyroid glands, CaSR closely monitors blood Ca^{2+} levels and controls the handling of this cation in kidney, bone and intestine via release into the circulation of the parathyroid hormone (PTH; **Figure 4**). In other tissues, local CaSR activation triggers adaptive responses to micro-environmental variations in Ca^{2+} and Mg^{2+} levels.

PTH and $1,25(OH)_2D_3$
In the parathyroid glands, under hypocalcemic conditions, reduced binding of Ca^{2+} to CaSR induces rapid PTH release from the secretory granules, delays PTH degradation and eventually results in parathyroid hyperplasia. Increased levels of circulating PTH restore normal plasma Ca^{2+} values through: *i)* resorption of Ca^{2+} from bone; *ii)* directly increasing Ca^{2+} reabsorption in the DCT2/CNT segments of the nephron via TRPV5[69]; *iii)* stimulating activation of vitamin D ($1,25(OH)_2D_3$) synthesis in the PT cells of the nephron. Increased $1,25(OH)_2D_3$ levels activate vitamin D receptor (VDR)-mediated gene expression, resulting in the enhanced transcription of Ca^{2+} transport proteins.[41] This regulatory network promotes absorption of dietary Ca^{2+} in the small intestine, Ca^{2+} reabsorption in the kidney and release of Ca^{2+} mineralized in bones in a PTH-independent manner.[41, 70, 71] As negative feedback, $1,25(OH)_2D_3$ represses the transcription of PTH[72] and may have an indirect effect
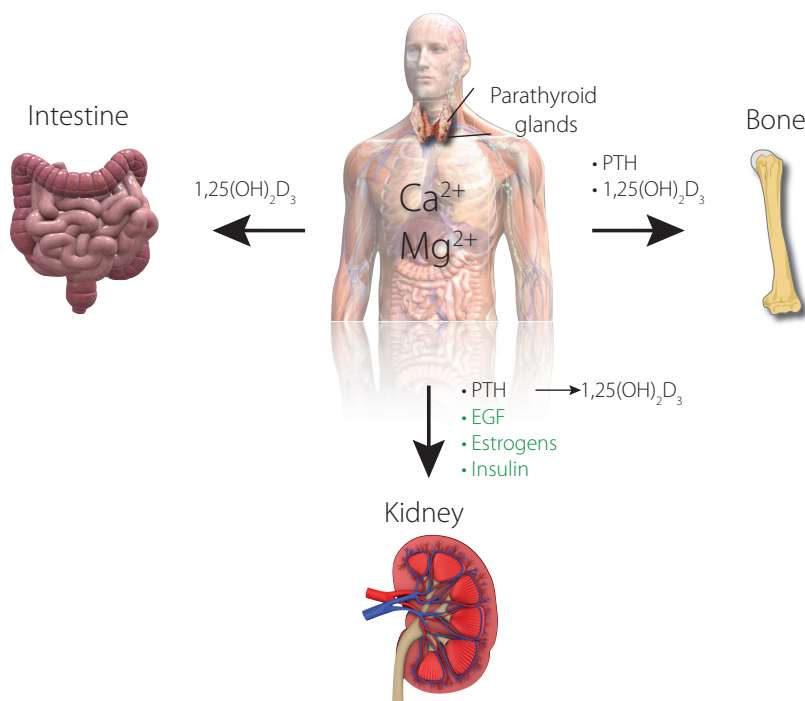
Figure 4 Hormonal regulation of Ca^{2+} and Mg^{2+} homeostasis.

Maintenance of plasma Ca^{2+} and Mg^{2+} concentrations within a narrow range relies on the coordinated actions of the kidney, intestine and bone under the sensing control of the parathyroid glands. Regulation of these processes occurs by a number of hormones, including PTH, $1,25(OH)_2D_3$, EGF, estrogens and insulin. The hormones that specifically modulate renal Mg^{2+} reabsorption are depicted in green. PTH: parathyroid hormone; $1,25(OH)_2D_3$: active form of vitamin D; EGF: epidermal growth factor.

on PTH release by increasing the expression of CaSR.[73] In a hypercalcemic situation, CaSR inhibits PTH release and stimulates the secretion of calcitonin (CT) by the thyroid C cells. CT secretion represents a robust defence against hypercalcemia via decreases osteoclast-mediated bone resorption.[74] Generally, Mg^{2+} binds and activates the CaSR *in vitro*, albeit with a much lower affinity compared to Ca^{2+},[75] and therefore may influence PTH release by the parathyroid glands. However, hypomagnesemia is mostly associated with a paradoxical block of PTH secretion and a resulting hypoparathyroidism.[76-78] The differences between the effects of Ca^{2+} and Mg^{2+} on the CaSR-mediated secretory responses probably reside in the differential involvement of these cations in the cell signalling cascade initiated by the CaSR.[68, 79]

1,25(OH)$_2$D$_3$: beyond Ca^{2+} homeostasis

In addition to primarily affecting Ca^{2+} homeostasis, 1,25(OH)$_2$D$_3$ has potent anti-proliferative, immunosuppressive and immunomodulatory activities.[80] This is achieved via the broad expression of VDR that is not restricted to Ca^{2+}-transporting tissues. More recently, clinical and pre-clinical studies demonstrated that treatment with 1,25(OH)$_2$D$_3$ analogues has a protective effect against podocyte damage in the course of various proteinuric diseases.[81-83] As previously described, podocyte foot processes are interconnected by protein complexes to form the slit diaphragms that can be defined as the functional units of the glomerular filter (**Figure 1**). Proteins located in the slit diaphragms, e.g. nephrin, podocin, TRPC6, provide physical linkage, but also behave as signalling molecules able to affect the podocyte cell behaviour. Upon injury to the podocytes or mutations in one of the proteins of the slit diaphragm, marked proteinuria occurs, which may give rise to progressive renal disease. Angiotensin II (AngII) is a key contributor to the pathogenesis of glomerular disease, through a mechanism that possibly includes activation and regulation of TRPC6.[33, 84] Briefly, binding of AngII to the angiotensin type I receptor (AT1R) results in increased intracellular Ca^{2+} concentrations due to the opening of TRPC6 channels at the plasma membrane. Ca^{2+}-dependent activation of the phosphatase calcineurin leads to translocation of its substrate, the nuclear factor of activated T cells (NFAT), to the nucleus where it enhances the transcription of NFAT-responsive genes, such as TRPC6 (**Figure 1** and **5**).[33] This could give rise to a positive feedback loop that maintains and/or worsens the proteinuria. Indeed, Eckel *et al.* demonstrated that AngII-induced proteinuria was ameliorated in *TRPC6* knockout mice (*TRPC6$^{-/-}$*). In accordance with the central role of AngII, the mainstay of current anti-proteinuric treatment consists of angiotensin-converting enzyme (ACE) inhibition (ACEi) and/or AT1R blockade (ARBs)[85, 86]. In addition, calcineurin inhibitors are used to reduce proteinuria in some glomerular diseases.[33, 87] Importantly, ACEi, ARBs and calcineurin inhibitors all reduce TRPC6 expression in *in vitro* podocyte injury and *in vivo* glomerular disease models.[33] In addition to its known effect on AngII biosynthesis,[88] 1,25(OH)$_2$D$_3$ directly affects the expression of the structural slit diaphragm proteins podocin and nephrin.[89, 90] Since other TRP channel family members, such as TRPV5 and TRPV6, are regulated by 1,25(OH)$_2$D$_3$, the above data gives rise to the hypothesis that TRPC6 regulation by 1,25(OH)$_2$D$_3$, either directly or through AngII, could contribute to the anti-proteinuric effect of 1,25(OH)$_2$D$_3$.

Acquired disturbances in Ca^{2+} and Mg^{2+} homeostasis

Mutations in key regulators of Ca^{2+} and Mg^{2+} homeostasis can be causative for genetic forms of disturbances in these cations.[28] Moreover, perturbations of mineral homeostasis can represent important side effects of certain therapies, e.g. immunosuppressive therapy,[91-93] diuretic treatment,[94, 95] anticancer therapy by use of the EGF receptor blocking antibody
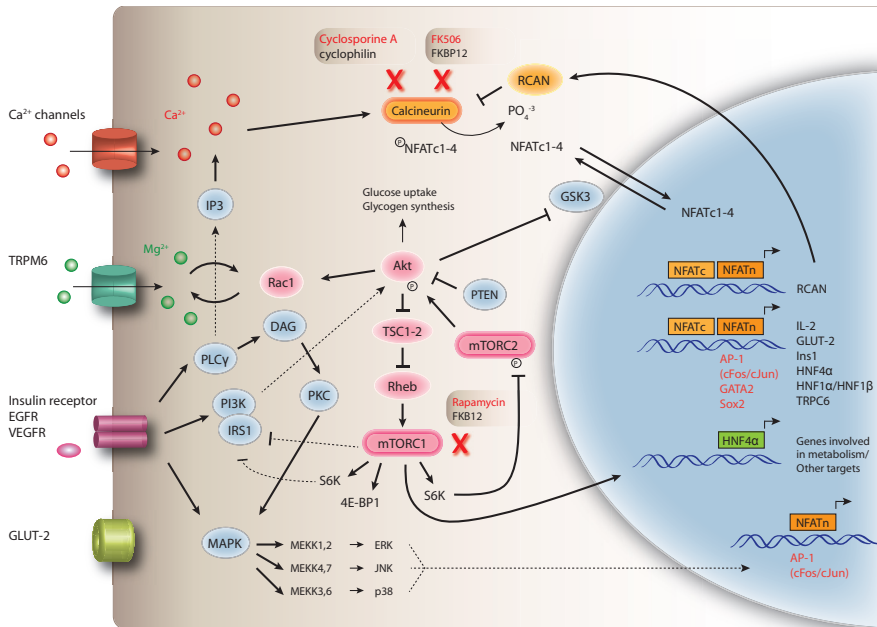
Figure 5 Calcineurin/NFAT and mTOR pathways in a representative cell model and their inhibition by immunosuppressive treatments.

The binding of the immunosuppressant cyclosporine A or tacrolimus (FK506) to cyclophilin or FKBP12, respectively, inhibits calcineurin and in turn the translocation of NFATc to the nucleus, where transcription of tissue-specific genes by NFATn/NFATc complexes cannot take place. On the other hand, the immunosuppressant rapamycin inhibits the mTOR pathway known to be involved in the cellular response to nutrients availability and growth signals. TRPM6: transient receptor potential melastatin 6; EGFR: epidermal growth factor receptor; VEGFR: vascular endothelial growth factor receptor; GLUT-2: glucose transporter type 2; NFATc: nuclear factor of activated T-cells, cytoplasmic; NFATn: nuclear factor of activated T-cells, nuclear; mTOR: mammalian target of rapamycin; Ins1: insulin 1; IL-2: interleukin-2; HNF: hepatocyte nuclear factor; TRPC6: transient receptor potential channel 6.

cetuximab or by use of cisplatin,[66, 96] or can be secondary to other medical conditions like chronic metabolic acidosis[97, 98] and diabetes mellitus type II.[99] The underlying pharmacological and (patho)physiological mechanisms of these disturbances might involve the epithelial Ca^{2+} and Mg^{2+} channels. The class of immunosuppressants known as calcineurin-inhibitors (CNI) includes the active compounds tacrolimus (also known as FK506) and cyclosporine A. FK506 and cyclosporine A bind the cytosolic proteins FKBP12 and cyclophillin, respectively, causing the inhibition of the phosphatase calcineurin (**Figure 5**). The calcineurin/NFAT pathway is a broadly expressed intracellular Ca^{2+}-responsive pathway that, under physiological conditions, regulates the transcription of multiple factors involved

in cell specific functions in various tissues.[100, 101] Thus, inhibition of the calcineurin/NFAT signalling results in a wide range of side effects. In particular, treatment with FK506 and cyclosporine A associate with adverse renal consequences and impaired mineral homeostasis, including hypercalciuria and hypomagnesemia.[91-93] *In vitro* and *in vivo* studies demonstrated that CNI administration causes a primary defect of Ca^{2+} and Mg^{2+} reabsorption by specifically downregulating the expression of proteins involved in active transport of these cations, among others TRPV5 and TRPM6.[102-106] The treatment with another immunosuppressive compound, rapamycin, also associates with renal Mg^{2+} wasting in mice, whereas Ca^{2+} homeostasis does not seem to be affected.[92, 107] Rapamycin binds the cytosolic protein FKBP12, similarly to FK506, and inhibits the mammalian target of rapamycin (mTOR; **Figure 5**). mTOR is a serine/ threonine kinase that exists in two separate complexes, mTORC1 and mTORC2. mTORC1 is involved in nutrient sensing and growth factors signalling, whereas mTORC2, primarily regulates cytoskeleton dynamics via a network of small GTP-binding proteins.[108, 109] Initially identified as mTORC1- specific inhibitor, rapamycin has been recently shown to also affect signalling through mTORC2.[110] Overall, inhibition of the mTOR pathway by rapamycin in a variety of animal models revealed beneficial effects on the progression of several renal disorders like, for example, chronic kidney disease and polycystic kidney disease.[111, 112] Nevertheless, many molecular mechanisms at the basis of these protective effects remain elusive. So far, contrasting *in vivo* and *in vitro* data did not elucidate whether impaired passive[107] or active[113] Mg^{2+} reabsorption along the nephron of the kidney account for the rapamycin-induced renal Mg^{2+} wasting.

Aim and outline of this thesis

The general aim of this thesis was to disclose new regulatory pathways of epithelial Ca^{2+} and Mg^{2+} transport, mainly with respect to renal function, in order to extend our knowledge in the (patho)physiology of disturbances involving these divalent cations. In particular, primary interest was devoted to the study of novel transcriptional networks that control the gene expression of ion channels, transporters and hormones relevant to the glomerular or tubular handling of Ca^{2+} and Mg^{2+} in the kidney. **Chapter 2** elucidates the role of the transcription factor HNF1B on the alternative transcription of the *FXYD2* gene, encoding the two isoforms of the γ subunit of the Na^+-K^+-ATPase. The functional consequences of four HNF1B mutations associated with hypomagnesemia on the iso-form-specific promoter activity were tested using two different reporter assays. The molecular mechanisms of the HNF1B-mediated transcription are further elucidated in **Chapter 3**. Based on the evidence that mutations in the *PCBD1* gene, encoding the dimerization cofactor of HNF1, associates in patients with hypomagnesemia and renal Mg^{2+} loss, *in vitro* studies were performed to test the ability of PCBD1 mutants to

co-activate the transcription of *FXYD2* by HNF1B. **Chapter 4** describes a cohort of patients with HNF1B mutations and high PTH levels that seem unrelated to the severity of the kidney failure. The involvement of HNF1B in the direct transcriptional regulation of the *PTH* gene was studied *in vitro* by luciferase-reporter assay and serially deleted promoter analysis. Moreover, HNF1B expression in human parathyroid tissue and in a rat parathyroid cell line (PT-r) was evaluated via RT-PCR and immunohistological stainings. The study presented in **Chapter 5** proposes a molecular mechanism for the rapamycin-induced renal Mg^{2+} wasting. Extracellular signals, that are able to stimulate the mTOR pathway, have been linked to the regulation of TRP channels. The role of the mTOR-inhibitor rapamycin on TRPM6 expression and activity was, therefore, investigated. This was accomplished in mice treated with rapamycin and by electrophysiological techniques using HEK293 cells expressing TRPM6. In **Chapter 6,** it was studied whether the anti-proteinuric effect of $1,25(OH)_2D_3$ may reside, at least in part, in the regulation of the podocyte TRPC6 expression. To this end, the transcriptional regulation of TRPC6 by $1,25(OH)_2D_3$ was investigated in injured podocytes *in vitro,* in a rat adriamycin-induced nephropathy model for FSGS as well as in $1,25(OH)_2D_3$-deficient 25-hydroxy-1α-hydroxylase knockout mice. Finally, a general discussion and future perspectives are presented in **Chapter 7.**

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HNF1B specifically regulates the transcription of the γa-subunit of the Na+-K+-ATPase

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2

Abstract

Hepatocyte nuclear factor 1 homeobox B (HNF1B) is a transcription factor involved in embryonic development and tissue-specific gene expression in several organs, including the kidney. Recently heterozygous mutations in the *HNF1B* gene have been identified in patients with hypomagnesemia due to renal Mg^{2+} wasting. Interestingly, ChIP-chip data revealed HNF1B binding sites in the *FXYD2* gene, encoding the γ-subunit of the Na^+-K^+-ATPase. The γ-subunit has been described as one of the molecular players in the renal Mg^{2+} reabsorption in the distal convoluted tubule (DCT). Of note, the *FXYD2* gene can be alternatively transcribed into two main variants, namely γa and γb.

In the present study, we demonstrated via two different reporter gene assays that HNF1B specifically acts as an activator of the γa-subunit, whereas the γb-subunit expression was not affected. Moreover, the HNF1B mutations p.His69fsdelAC, p.Lys156Glu, p.His324Ser325fsdelCA and p.Tyr352fsinsA, previously identified in patients with hypo-magnesemia, prevented transcription activation of γa-subunit via a dominant negative effect on wild-type HNF1B. By immunohistochemistry, it was shown that the γa- and γb-subunits colocalize at the basolateral membrane of the DCT segment of mouse kidney. On the basis of these data, we suggest that abnormalities involving the *HNF1B* gene may impair the relative abundance of γa and γb, thus affecting the transcellular Mg^{2+} reabsorption in the DCT.

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Introduction

HNF1B is a transcription factor that is critically involved in the early vertebrate development and embryonic survival**.** Although it was first identified in the liver,[1] it is also highly expressed in the pancreas, kidney, lung, ovary, testis and gastrointestinal tract.[2] In kidney, it is expressed exclusively in the tubular epithelial cells along all nephron segments.[3] In the past decades, candidate gene strategies have led to the identification of several targets that are regulated by HNF1B, such as the cystic disease genes *PKHD1*, *PKD2*, *UMOD*, and *SOCS-3*.[4-6]

HNF1B belongs to a family of homeodomain-containing transcription factors. It consists of a POU specific (Pit-1, OCT1/2, UNC-86; POU_S) and an atypical POU homeodomain (POU_H) that mediate DNA binding to the consensus sequence 5'-RGTTAATNATTAAC-3'. HNF1B forms homo- or heterodimers with the structurally related HNF1A in a stable complex that includes the dimerization cofactor PCBD1. In humans, heterozygous mutations of *HNF1B* result in several congenital kidney and urinary tract abnormalities, as well as a variety of extrarenal phenotypes.[7, 8] Since deletion of the entire *HNF1B* gene is frequently found in human patients, it seems likely that a gene dosage effect is involved. However, some mutated factors behave as dominant negative proteins that may possibly inactivate the wild-type protein.[9] Recently, novel mutations in the *HNF1B* gene, both *de novo* and inherited, have been described.[10, 11]

Hypomagnesemia, although not deeply investigated, is often reported in patients carrying *HNF1B* defects.[11, 12] Interestingly, Adalat *et al.* described five cases of *HNF1B* whole-gene deletions, two splice site mutations and one frame-shift mutation being associated with renal malformations and hypomagnesemia (< 0.65 mmol/L) due to a specific renal defect in the transcellular Mg^{2+} transport in the distal convoluted tubules (DCT). The DCT plays an important role in fine-tuning the plasma Mg^{2+} levels. Here, Mg^{2+} is actively reabsorbed from the pro-urine into the cell via the transient receptor potential channel melastatin subtype 6 (TRPM6)[13] and subsequently extruded to the blood via an unknown mechanism. In the recent years, several new proteins have been linked to active renal Mg^{2+} handling by directly affecting TRPM6 or by altering the driving force for Mg^{2+} influx via the channel.[14]

Of note, bioinformatics prediction tools in combination with functional genomic approaches confirmed the presence of HNF1B binding sites in the *FXYD2* gene, encoding the regulatory γ-subunit of the Na^+-K^+-ATPase.[15] Interestingly, the missense mutation p. Gly41Arg in the same protein was previously identified as the underlying defect of isolated dominant hypomagnesemia associated with hypocalciuria (IDH [MIM 154020]).[16] The γ-subunit (FXYD2) is a type I transmembrane protein, mostly expressed in the kidney.[17] Its gene can be alternatively transcribed into two main variants, namely γa and γb, which differ only at their extracellular N-termini.[18] The two γ-subunit isoforms modulate the Na^+-K^+-ATPase affinities for its major physiological ligands.[19] In particular, this pump is

responsible for maintaining the normal transmembrane gradients of Na^+ and K^+, which in the kidney drive the trans-epithelial salt reabsorption.

In the present study, we investigated the role of wild-type HNF1B and HNF1B p.His69fsdelAC, p.Lys156Glu, p.His324Ser325fsdelCA, p.Tyr352fsinsA on the alternative transcription of the two human γ-subunit variants. Via two different approaches based on reporter genes, we were able to show that wild-type HNF1B specifically induces the γa-subunit transcription whereas all HNF1B mutants partially prevented it, probably due to a dominant negative effect on the wild-type transcription factor.

Materials and Methods

Parvalbumin-EGFP mice
The transgenic parvalbumin-EGFP mice were obtained from the University of Heidelberg, Germany.[20] Parvalbumin is a Ca^{2+}-binding protein predominantly distributed in the early distal convoluted tubule (DCT) of the kidney [21]. Briefly, the enhanced green fluorescent protein (EGFP) was expressed under the control of the parvalbumin gene promoter by bacterial artificial chromosome (BAC) transgene, resulting in the specific EGFP-labeling of the parvalbumin-expressing DCTs.

DNA constructs
The human FXYD2 region from -1300 bp upstream the FXYD2b exon, till exon 2 was obtained by amplification of genomic DNA, using a high fidelity DNA polymerase (Phusion, Finnzymes), and the PCR product was cloned into a pGEM-T Easy vector (Promega, Fitchburg, USA). Subsequently, an EGFP SV40 polyA terminator product was amplified from a previous construct and cloned downstream of the FXYD2 genomic sequence. HNF1B full-length cDNA was amplified by PCR from HNF1B pCMV-SPORT6 (clone IRATp970A0421D, ImaGenes), subcloned into the bicistronic expression vector pCINeo IRES GFP and HA-tagged at the N-terminus. HNF1B p.His69fsdelAC, p.Lys156Glu, p.His324Ser325fsdelCA and p.Tyr352fsinsA were obtained by site-directed mutagenesis according to the manufacturer's guidelines (Stratagene, La Jolla, USA). For co-transfection experiments with FXYD2-EGFP pGEM T-Easy, the IRES GFP was removed from the bicistronic HNF1B constructs. Firefly luciferase constructs were obtained by amplification of the promoter regions of interest using FXYD2-EGFP pGEM T-Easy as a template and subcloned into pGL3-Basic vector (Promega, Fitchburg, USA). Primer sequences used for cloning or mutagenesis PCR are reported in **Supplemental Table 1.** All constructs were verified by sequence analysis.

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Cell culture and transfection

Human Embryonic Kidney cells (HEK293) were grown in Dulbecco's modified Eagle's medium (Bio Whittaker-Europe, Verviers, Belgium) containing 10% (v/v) fetal calf serum, 2 mM L-glutamine and 10 ug/ml ciproxin at 37°C in a humidity-controlled incubator with 5% (v/v) CO_2. The cells were transiently transfected with the respective constructs using poly-ethylenimine (Polysciences Inc.) cationic polymer and analyze for protein or mRNA expression at 48 hours post-transfection.

Immunohistochemistry

Immunohistochemistry was performed as previously described.[22] In short, staining of serial sections was performed on 7-μm cryosections of periodate-lysine-paraformalde-hyde–fixed kidney samples from parvalbumin-EGFP mice. The mouse kidney cryosections were incubated overnight at 4°C with the following primary antibodies: rabbit anti-γb (1:2,000) raised against the N-terminus peptide MDRWYLGG of the mouse γb isoform conjugated to tetanus toxoid as a carrier,[17] and rabbit anti-γa (1:50) raised against the N-terminus peptide MAGEISDLSANS of the mouse γa isoform (kind gift from Prof.dr. Gerald Kidder, The University of Western Ontario). Sections were then incubated with Alexa Fluor 594 conjugated secondary antibodies. Photographs of the entire cortex were taken with the microscope Zeiss Axio Imager 1 microscope (Oberkochen, Germany) equipped with the fluorescence lamp HXP120 Kubler Codix.

Immunocytochemistry

HEK293 cells, which lack endogenous HNF1B,[23] were transiently transfected with wild-type and mutant HA-HNF1B pCINeo IRES GFP. 48 hours after transfection the cells were seeded on glass and fixed with 4% w/v paraformaldehyde (PFA) for 30 minutes at room temperature, followed by permeabilization for 15 minutes with 0.2% v/v Triton X-100 in PBS supplemented with 0.1% w/v BSA. After incubation overnight with a 1:100 dilution of a mouse anti-HA antibody (6E2, Cell Signalling), cells were washed three sites with PBS-CM (1 mM $MgCl_2$, 0.1 mM $CaCl_2$) and incubated with a secondary Alexa Fluor 594 coupled goat anti-mouse antibody (1:100) for 45 minutes at room temperature. Cells were washed three times with PBS-CM prior mounting with DAPI–Vectashield (Vector Laboratories, Burlingame, CA).

Immunoblotting

Mouse kidney protein lysate was prepared as previously described.[24] HEK293 cells were lysed in 1x Laemmli sample buffer containing 100 mM dithiothreitol and protein inhibitors, subsequently denatured for 30 minutes at 37°C, and then subjected to SDS-PAGE. Immunoblots were incubated with the following antibodies: mouse anti-HA (1:500), rabbit anti-γb (1:5,000), rabbit anti-γa (1:500) or rabbit anti-γ (1:500) raised against the C-terminus of the γ subunit (kind gift from Prof.dr. Steven J.D. Karlish, Rehovot, Israel).[25] Subsequently,

blots were incubated with sheep horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Sigma, MO, USA) and then visualized using the enhanced chemiluminescence system (ECL, Pierce).

Luciferase reporter assay

In a 12-well plate, 700 ng of the promoter firefly luciferase plasmids and 100 ng of the HNF1B pCINeo plasmid were transfected into HEK293 cells. For standardization of the transfection efficiency, 20 ng of Renilla luciferase plasmid CMV-pRL was used as a reference. Firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay (Promega, Fitchburg, USA).

Quantitative Real-Time Polymerase Chain Reaction

HEK293 cells were transiently cotransfected in a 6-well with 1.75 μg DNA of FXYD2-EGFP and 30 ng of either wild-type or mutant HNF1B. The potential dominant negative effect was investigated by co-transfection of 15 ng of wild-type HNF1B and 15 ng of mutant HNF1B. The total amount of DNA was kept equal using the pCINeo empty vector. Total RNA isolation and reverse transcription were performed, as described previously.[26] The cDNA was used to determine mRNA expression levels by CFX96 Real-Time PCR detection system (Bio-Rad) both of the target genes of interest and of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT), as an endogenous control. Real-time RT-PCR primers (**Table 1**) were designed using the program Primer3 (v.0.4.0).

Table 1 Oligonucleotide sequences used for Real-time RT-PCR analysis.

Target	Forward (5'-3')	Reverse (5'-3')
FXYD2a-EGFP	GTCGATGGACGGTGGCGGCAG	GTCCAGCTCGACCAGGATGG
FXYF2b-EGFP	CAGGTGGTACCTGGGCGGCAG	GCTGAACTTGTGGCCGTTTA
HPRT	CATTATGCTGAGGATTTGGAAAG	GCTTTGATGTAATCCAGCAGGTC

FXYD2a: γ-subunit of the Na^+-K^+-ATPase, isoform a; FXYD2b: γ-subunit of the Na^+-K^+-ATPase, isoform b; EGFP: enhanced green fluorescent protein; HPRT: hypoxanthine-guanine phosphoribosyl transferase.

Data analysis

All results presented are based on a minimum of three different experiments. Values are expressed as means ± SEM. Statistical significance ($p < 0.05$) was determined using one-tailed Students t-test or one-way ANOVA with Bonferroni's procedure.

2

Results and discussion

HNF1B enhances FXYD2a but not FXYD2b transcription

FXYD2 gene (Gene ID: 486) maps on chromosome 11q23 and consists of seven exons spanning 9.2 kb of genomic DNA.[15] Three transcripts are associated to this gene, with *FXYD2a* (NM001680) and *FXYD2b* (NM021603) being the main ones. Promoter elements were identified around the alternative start sites for exon-b and exon-a, that encode different N-termini of the γ–subunit protein (**Figure 1A**). The highly conserved HNF1B binding sites localize within the first intron of the human *FXYD2* gene (**Figure 1B**), at positions +1092 and +3256 from the transcription initiation site of *FXYD2b* and act as enhancer elements.[12] Nevertheless, it is unknown whether HNF1B sites may act as upstream regulatory elements for γa and/or downstream enhancer elements for γb. In order to elucidate the HNF1B transactivation activity on the alternative transcription of *FXYD2b*, the first intron of the human *FXYD2* gene was cloned upstream to the proximal (-100) and distal (-1269) promoter of the *FXYD2b* isoform and linked to a luciferase reporter gene. HEK293 cells were transfected with the promoter-reported plasmid and luciferase activity was measured after HNF1B stimulation. The enhancer moderately affected γb promoter activity at a proximal but not at a distal position (**Figure 1C**). Noteworthy, γa promoter activity was substantially enhanced by HNF1B (**Figure 1C**). We conclude that HNF1B specifically acts as an activator of the γa-subunit.

Characterization of HNF1B mutants

In this study, three frameshift mutations (p.His69fsdelAC, p.His324Ser325fsdelCA, p. Tyr352fsinsA), of which one associates with hypomagnesemia, and one missense mutation (p.Lys156Glu) were tested for its transactivation activity on the alternative transcription of the *FXYD2* gene. Noteworthy, HNF1B p.Lys156Glu, p.His324Ser325fsdelCA and p.Tyr352fsinsA have an intact nuclear localization sequence and, therefore, localized to the nucleus whereas HNF1B p.His69fsdelAC was mainly retained in the cytosol (**Figure 2A**).[27] Importantly, all mutants were checked for equal expression as shown in **Figure 2B**. HNF1B p.His69fsdelAC was not detectable at the expected molecular weight of 10 kD, probably due to the faster protein turnover or the different exposure of the HA epitope compared to the other mutants.

HNF1B mutants have a dominant negative effect on wild-type HNF1B transcription of FXYD2a

The human genomic region spanning from -1269 to +5393 downstream the *FXYD2b* exon was cloned in front and in frame with the EGFP reporter gene (**Figure 3A**, left panel). Upon stimulation with exogenous HNF1B in HEK293 cells, alternative transcription takes place eventually translating two EGFP fusion transcripts into two proteins that differ in their N-terminus. Dose-response assays with increasing amounts of wild-type HNF1B
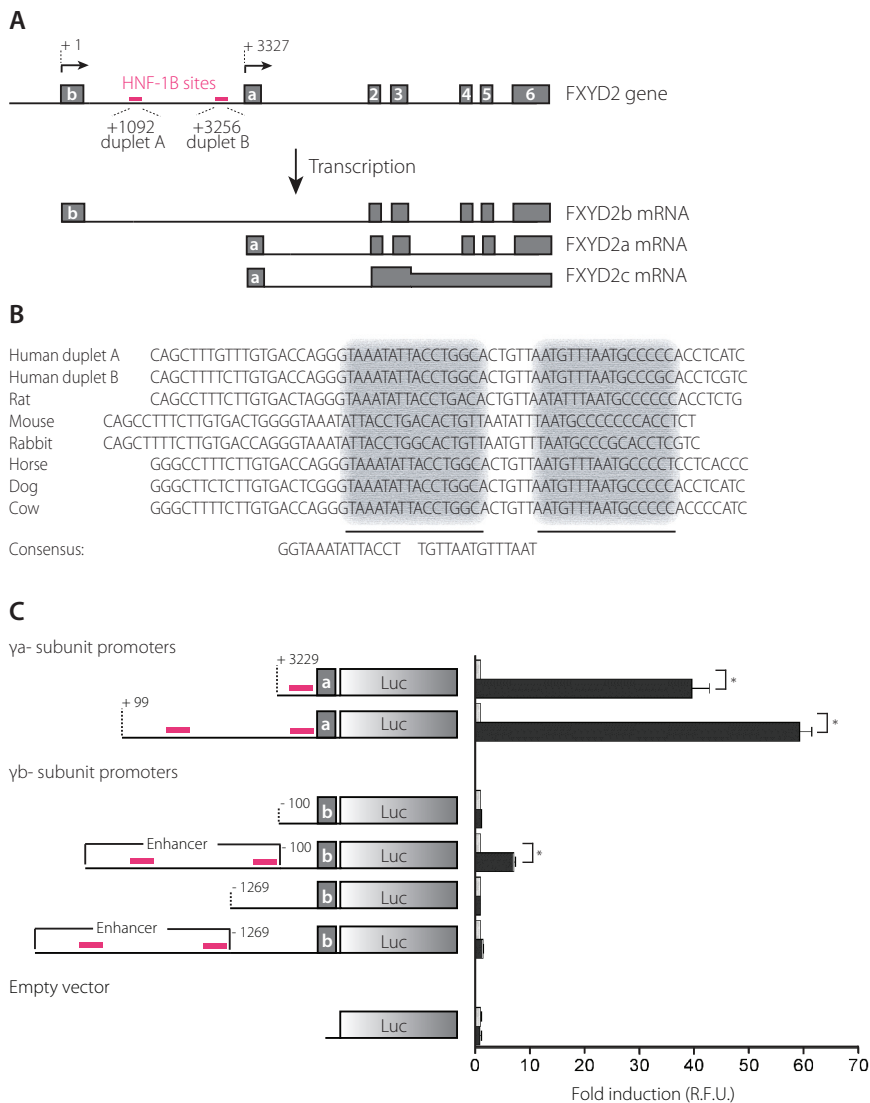
Figure 1 HNF1B promotes the γa-subunit transcription initiation.

(A) Organization of the human *FXYD2* gene and its alternative transcripts. Each exon is indicated with its length. Numbering of the nucleotides starts from the transcription initiation site of FXYD2b. Alternative transcription can be initiated at exon-b (+1) or exon-a (+3327). HNF1B sites are arranged as duplets at positions +1092 and +3256. ➥ Transcription initiation site.

(B) Alignment of the promoter region -1000 from the start site of exon-a of different species revealed highly conserved HNF1B binding sites arrange as a duplet. For the human *FXYD2* gene, both duplet A (+1092) and duplet B (+3327) are shown. **(C)** Luciferase assay. HEK293 cells were transiently transfected with luciferase constructs carrying the γa- and γb- subunit regulatory elements. The promoter activity was tested with (black bars) or without (grey bars) HNF1B stimulation.

R.F.U.: Renilla Firefly Units. Error bars indicate SEM (n=3). *, p<0.05, compared with non-stimulated condition.
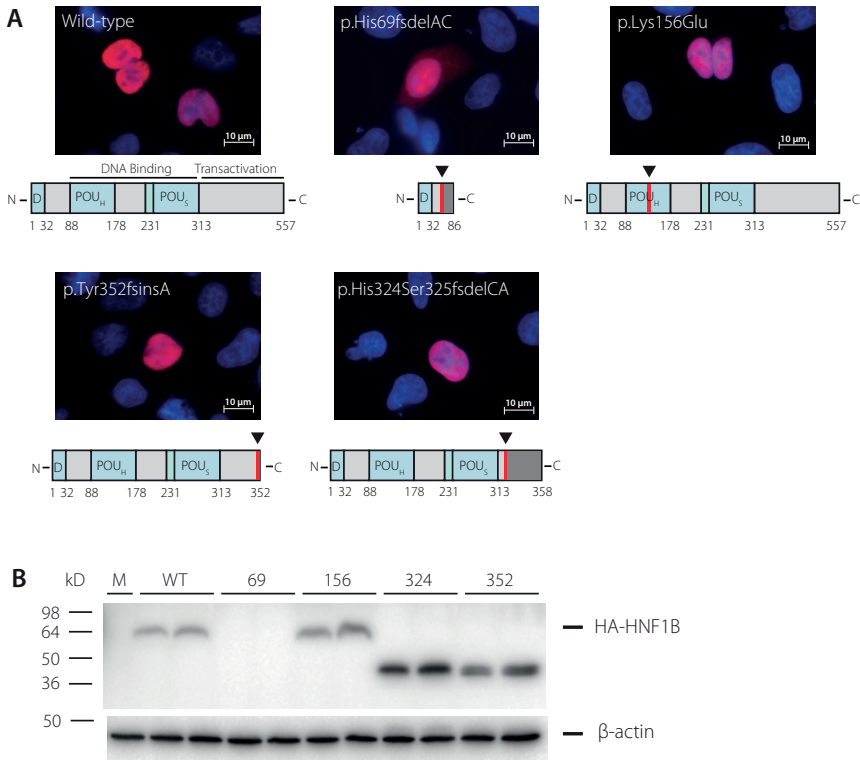
Figure 2 Characterization of the human HNF1B mutants.

(A) Intracellular localization of wild-type HNF1B and HNF1B mutants in transiently transfected HEK293 cells. Red: HA tag; Blue: DAPI (nuclear marker); D: Dimerization; POU$_H$: POU homeodomain; POU$_S$: POU specific. Scale bars: 10 μm. **(B)** HA-HNF1B WT (65.5 kD) and its mutants p.His69fsdelAC (69; 9.47 kD), p.Lys156Glu (156; 65.5 kD), p.His324Ser325fsdelCA (324; 41.5 kD) and p.Tyr352fsinsA (352, 39.4 kD) were transiently transfected in HEK293 cells and checked for expression via immunoblotting. Mock (M) plasmid was transfected in HEK293 cells as negative control. β-actin was included to check for equal loading.
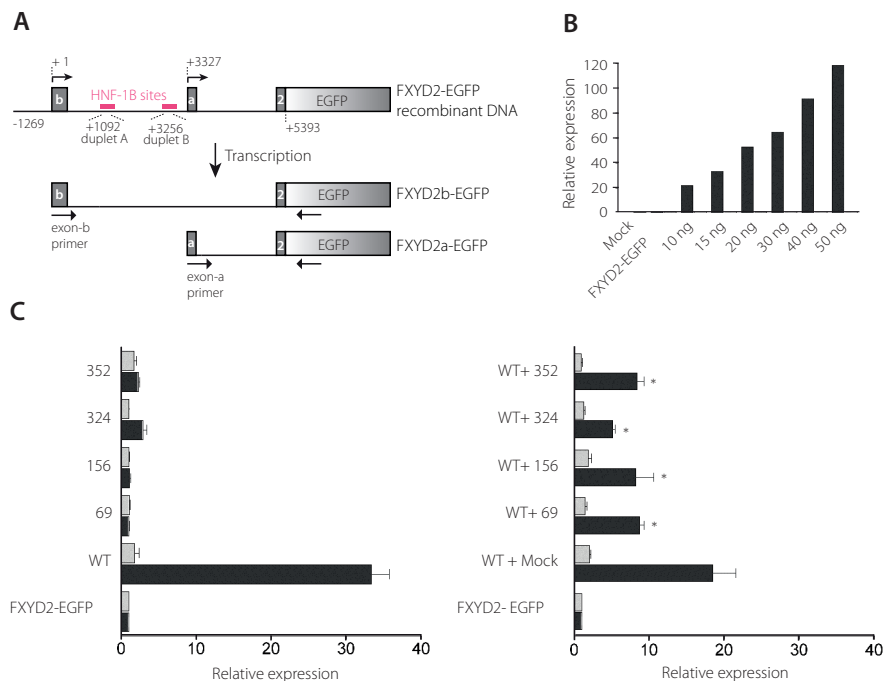
Figure 3 Study of the alternative transcription of the human *FXYD2* gene by wild-type HNF1B and its mutants.

(A) Scheme of the human FXYD2-EGFP construct. FXYD2a- and FXYD2b-EGFP transcripts were detected by use of an isoform-specific forward primer, spanning the exon-exon junction, and an EGFP reverse primer. **(B)** FXYD2a-EGFP (black bars) and FXYD2b-EGFP (grey bars) mRNA levels after co-transfection with increasing DNA quantities of wild-type HNF1B. **(C)** (Left panel) Wild-type HNF1B (WT) stimulates FXYD2a-EGFP (black bars) but not FXYD2b-EGFP (grey bars) mRNA synthesis. (Right panel) FXYD2a-EGFP mRNA expression is significantly reduced by a dominant negative effect of HNF1B p.His69fsdelAC (69), p.Lys156Glu (156), p.His324Ser325fsdelCA (324) and p.Tyr352fsinsA (352) on the transactivation activity of wild-type HNF1B (WT). Error bars indicate SEM (n=3). *, p<0.05, compared to FXYD2a-EGFP mRNA stimulation by wild-type HNF1B.

indicated 30 ng as the amount of DNA that generates the half maximal induction of the FXYD2a-EGFP transcription (**Figure 3B**).

To determine the effect of the HNF1B mutants on the transcription of *FXYD2a* and *FXYD2b*, the FXYD2-EGFP plasmid was transiently transfected into HEK293 cells and alternative transcripts levels were determined by use of isoform-specific primers after HNF1B induction. As expected, FXYD2a-EGFP mRNA was stimulated by wild-type HNF1B whereas the mutant transcription factors lost their transactivation properties. FXYD2b-EGFP mRNA was in all cases not affected (**Figure 3C**, left panel).

Considering the autosomal dominant inheritance of HNF1B mutations, a potential dominant effect was tested by co-transfection of equal amounts of plasmid DNA encoding wild-type HNF1B, HNF1B mutants or mock in HEK293 cells. The induction of *FXYD2a* transcription in HEK293 cells coexpressing wild-type HNF1B and each of the HNF1B mutants was significantly reduced in comparison with cells coexpressing wild-type HNF1B and mock plasmid (**Figure 3C**, right panel). To date, HNF1B p.Lys156Glu has not been associated with hypomagnesemia, but plasma Mg^{2+} concentrations in *HNF1B* mut+ patients remain to be extensively investigated.

γa- and γb-subunits colocalize in the DCT of the kidney

In order to investigate γa and γb expression in the DCT, parvalbumin-EGFP mouse kidney sections were stained with antibodies raised against the specific N-terminus of each γ isoform. Both γ-subunits are expressed at the basolateral membrane (**Figure 4A**), where the Na^+-K^+-ATPase is also localized along the nephron.[28] The staining of γb was clearly restricted to the EGFP-positive DCTs, whereas γa-associated immunofluorescence showed staining of several tubular segments in the kidney cortex, in agreement with previous localization studies.[18] Noteworthy, using serial kidney sections, we demonstrated that EGFP-positive tubules show overlap with the immunopositive staining of both γ variants (**Figure 4A**).

The specificity of the γa and γb antibodies was further investigated via immunoblot on a mouse kidney lysate. Due to postranslational modifications, γ-subunit migrates on SDS-PAGE gels as a doublet with γb migrating faster than γa.[29] As expected, staining for the C-terminus of the protein showed two bands (**Figure 4B**). Nevertheless, in our experimental conditions both variants were detected at a much higher molecular weight compared to the predicted size (7.18 kD γa, 7.34 kD γb).[29] Probably other still unknown factors that influence gel migration are involved, as anti-γa and anti-γb antibodies clearly recognized the upper and the lower band of the doublet, respectively (**Figure 4B**).

In conclusion, our data indicate that mutations in the *HNF1B* gene affect the expression of γa-subunit. We speculate that reduced amount of γa at the basolateral membrane of the DCT may influence Mg^{2+} reabsorption via three putative mechanisms: *i*) changes in the equilibrium between γa and γb expression can impair the Na^+-K^+-ATPase activity and cause an imbalance in salt reabsorption. Recently, it has been shown that dysfunction of the K^+ channel Kir4.1 in the basolateral membrane of the DCT inhibits the Na^+-K^+-ATPase via loss of recycling, renders the membrane potential less negative, and thereby reduces Mg^{2+} reabsorption through TRPM6;[30, 31] *ii*) γ-subunit variants can oligomerize and behave as an inward-rectifying cation channel that can mediate extrusion of Mg^{2+} to the blood.[32] Nevertheless, this hypothesis seems unlikely due to the unfavorable electrochemical gradient across the basolateral membrane; *iii*) γa and γb may interact with a still unknown Mg^{2+} extrusion mechanism, involving a primary or secondary active transporter. In the future, functional studies may validate one of these hypotheses.

A

EGFP	γa	Merge

EGFP	γb	Merge

B

kD   γa   γb   C-t

17 —

→ γa
→ γb

N-t   C-t

Mouse γa   MAGEISDLSANSGGSAKGTEN   21
Mouse γb   ------MDRWYLGGSAKGTEN   15
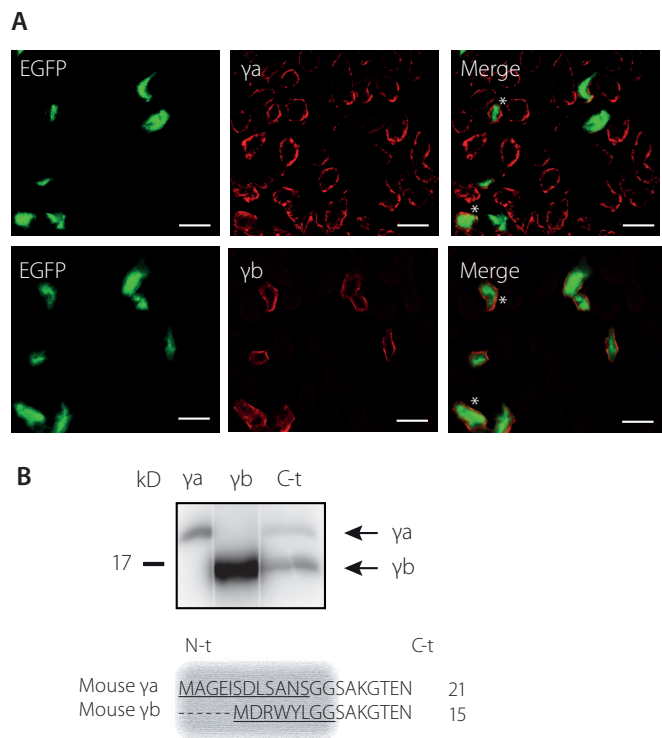
Figure 4 Immunohistochemical detection of γa- and γb-subunit in the DCT of the kidney.

(A) Staining for γa- and γb-subunit (red; upper and lower panel, respectively) of serial kidney sections from parvalbumin–EGFP mice. In green, the EGFP positive DCTs. The asterisks in the merged panels indicate representative overlapping distal tubules on serial sections intensively stained for γa and γb. Scale bars: 50 μm. **(B)** A protein lysate from mouse kidney was resolved in a wide lane of a 16% SDS-PAGE gel. The blot was cut into three pieces and stained with anti-γb, anti-γa or anti-γ C-terminus antibodies (C-t). The faster-migrating band was identified as the γb-subunit. The epitopes recognized by the isoform-specific antibodies are underlined in the alignment of the mouse γa- and γb-subunits. In grey are indicated the amino acids that differ between the two isoforms at the N-terminus (N-t) of the proteins.

Acknowledgements

Supplemental data

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Supplemental Table 1 Oligonucleotide sequences used for cloning and mutagenesis PCR.

Target	Forward (5'-3')	Reverse (5'-3')
FXYD2	CACCTCTTAGACCTCCTGGTC	GACTAGTATCATAGTAGAACGGGTCCAC
EGFP SV40 polyA	GACTAGTATCGTGAGCAAGGGCGAGGAG	GTCGACTAAGATACATTGATGAGTTTGGAC
FXYD2a +99	GGGGTACCCCGTGAGTCCTCTGAGCCCC	GGAAGATCTTCCCACCGTCCATCGACAACC
FXYD2a +3229	GGGGTACCCCCCACACTGCAGCTTTTCTTG	GGAAGATCTTCCCACCGTCCATCGACAACC
FXYD2b -1269 +enhancer	GGGGTACCCCGTGAGTCCTCTGAGCCCC	GGAAGATCTTCCGGCTGCCTCCACGGGGTGG
FXYD2b -1269	GGAAGATCTTCCCACCTCTTAGACCTCCTG	CCCAAGCTTGGGACCTGTCCATGGCAGGAGCTG
FXYD2b -100 +enhancer	GGGGTACCCCGTGAGTCCTCTGAGCCCC	GGAAGATCTTCCGGCTGCCTCCACGGGGTGG
FXYD2b -100	GGAAGATCTTCCGGCTTTCACGTGCATCCC	CCCAAGCTTGGGACCTGTCCATGGCAGGAGCTG
HNF1B	GGCGCGGCCATGGTGTCCAAGCTCACGTCGC	TCTAGATCACCAGGCTTGTAGAGGACAC
HNF1B p.His69delAC	CTCTCACCAACGGCCGCCAAGGGCCGCTTG	CAAGCGGCCCTTGGCGGCCGTTGGTGAGAG
HNF1B p.Lys156Glu	CCCAGCATCTCAACGAGGGCACCCCTATG	CATAGGGGTGCCCTCGTTGAGATGCTGGG
HNF1B p.His324Ser325fsdelCA	CTCCAACCAGACTCAGCCTGAACCCTCTGC	GCCAGAGGGTTCAGGCTGAGTCTGGTTGGAG
HNF1B p.Tyr352fsinsA	GTCAGGAGTGCGCTAACAGCCAGCAGGGAAAC	GTTTCCCTGCTGGCTGTTAGCGCACTCCTGAC

FXYD2a: γ-subunit of the Na+-K+-ATPase, isoform a; FXYD2b: γ-subunit of the Na+-K+-ATPase, isoform b; EGFP: enhanced green fluorescent protein; HNF1B: hepatocyte nuclear factor homeobox B.

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Early development of hyperparathyroidism due to loss of *PTH* transcriptional repression in patients with *HNF1B* mutations

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Abstract

Parathyroid hormone (PTH) plays a key role in calcium and phosphate homeostasis. Heterozygous mutations or deletions of the transcription factor HNF1B result in a heterogeneous syndrome characterized by renal cysts and diabetes, together with a variety of other extra-renal and renal manifestations. Since we observed hyperpara-thyroidism in several of these patients, we tested the hypothesis of a direct role of HNF1B in the transcriptional regulation of the human *PTH* gene in the parathyroid gland. We assessed eleven patients, nine with heterozygous *HNF1B* whole gene deletions and two with heterozygous *HNF1B* mutations, of which eight showed hyperparathyroidism. In two of these patients the hyperparathyroidism was appropriate for the level of kidney function. PTH could be discrepant in the others, especially given the concomitant hypomagnesemia, which is known to induce a paradoxical hypoparathyroidism. We demonstrated HNF1B expression in PTH-positive cells of human parathyroid gland. Chromatin immunoprecipitation analysis showed that HNF1B directly binds responsive elements within the human *PTH* promoter. Co-transfection of a *PTH* promoter- luciferase construct with a wild-type HNF1B construct resulted in a maximal reduction of 30% of *PTH* promoter activity. Importantly, HNF1B mutants lacked this inhibitory property. Serial deletions in the *PTH* promoter construct revealed that the inhibitory effect of HNF1B resides -200/-70 bp from the transcription initiation site. Our data demonstrate that HNF1B is a novel repressor of human *PTH* gene transcription, which could explain the early development of hyperparathyroidism in patients with *HNF1B* mutations or deletions.

Introduction

The parathyroid gland has a central role in calcium (Ca^{2+}) and phosphate (PO_4^{3-}) homeostasis. Parathyroid hormone (PTH) regulates the synthesis of 1,25-dihydroxyvitamin D_3 (1,25-D_3), alters Ca^{2+} and PO_4^{3-} (re)absorption in the kidney and intestine, and modulates bone metabolism.[1-3] Serum PTH levels depend on direct secretion of PTH from the secretory granules in the parathyroid gland as well as on synthesis of new PTH molecules secondary to *PTH* gene transcription.[2] PTH expression is restricted to the parathyroid glands in humans and is under the control of specific stimuli and repressors. A low serum Ca^{2+} alters the activation of the Ca^{2+}-sensing receptor (CaSR) on the surface of parathyroid glands, leads to the rapid release of PTH from the secretory granules and stimulates *PTH* gene expression, while high Ca^{2+} inhibits PTH secretion.[4-6] Contrary to Ca^{2+}, high PO_4^{3-} leads to increased PTH levels.[6, 7] Furthermore, *PTH* transcription is repressed by binding of a complex of 1,25-D_3, the 1,25-D_3 receptor (VDR) and retinoic acid receptor (RXR), to vitamin D responsive elements (VDRE) in the promoter region of the *PTH* gene.[8, 9] Recently, it was shown that *PTH* gene transcription is also inhibited by Fibroblast Growth Factor 23 (FGF23), a novel phosphaturic hormone that acts though the FGFR1/Klotho receptor complex present in parathyroid cells.[10, 11] Finally, the *PTH* promoter activity is regulated by the concerted action of tissue specific transcription factors, such as GCMB,[4] and non-specific transcription factors, like SP1, NF-Y, CRE, GATA.[12, 13]

The hepatocyte nuclear factor 1 homeobox B (HNF1B) is a Pit-1, OCT1/2, UNC-86 (POU) domain transcription factor that participates in organogenesis during early embryonic development.[14] More specifically, it regulates tubulogenesis in the liver, pancreas, kidney, and genital tract. In the kidney and urinary tract, HNF1B is expressed in renal tubules as well as developing ureters. Heterozygous mutations or deletions in the *HNF1B* gene are responsible for a dominant syndrome characterized by highly heterogeneous renal and extrarenal phenotypes that can comprise: i) renal malformations with or without cyst formation (glomerulocystic disease, cystic renal dysplasia, calyceal abnormalities, oligome-ganephronia or solitary kidney), ii) liver and genital tract abnormalities, iii) defects in the exocrine and endocrine pancreatic functions including maturity-onset diabetes of the young type 5 (MODY5 [MIM 137920]). Furthermore, HNF1B nephropathy is distinguished by a large variability in renal tubular transport abnormalities.[15] Functional HNF1B binding sites have been identified in the promoter regions of many renal cystic genes,[16-18] as well as genes involved in tubular transport,[19, 20] such as the *FXYD2* gene encoding for the γ-subunit of the Na^+-K^+-ATPase. The impaired transcription of this gene by HNF1B is suggested to be involved in the renal Mg^{2+} wasting observed in almost half of the patients.[21]

In this study, we describe the observation that PTH levels are inappropriately high in several patients with known *HNF1B* mutations and/or deletions visiting the outpatient clinic of our institution. Since HNF1B is a tissue-specific transcription factor highly expressed in the epithelia of specialized endocrine organs and tissues with secondary

endocrine functions,[22-25] we hypothesized that HNF1B might regulate PTH expression in the parathyroid gland. Therefore, the aim of our study was to investigate whether HNF1B could act as a transcriptional regulator of the human *PTH* gene, possibly by directly affecting *PTH* promoter activity.

Materials and Methods

Cohort details

All patients were diagnosed by nephrologists and/or clinical geneticists at the Radboud University Nijmegen Medical Centre, The Netherlands. Clinical histories were collected from hospital records. Informed consent for genetic analyses was obtained from all patients. Intact PTH was measured on an Architect random access analyzer (Abbott). In a limited number of samples, intact PTH was measured by another method which gave comparable results conform the EP9 (CLSI) protocol.

DNA constructs

The 5'-promoter region of the human *PTH* gene (−1476/+25; + 1 designates the transcription start site, NM_000315.2) was obtained by amplification of genomic DNA using a high fidelity DNA polymerase (Phusion, Finnzymes; Forward: AAAAAAGGTACCCAGCTATA-AAGTCATCCCGTCTT, Reverse: GGGGGGGGAGCT CGCAGACCCCTTAAATGGTGA). To generate a Firefly luciferase reporter construct, the PCR product was cloned into a pGL3-Basic vector (Promega, Fitchburg, USA), using the restriction sites KpnI and SacI. Firefly luciferase constructs for deletion analysis were prepared with a similar cloning strategy after amplification of the promoter regions -1000/+25 (Forward: AAAAAAGGTACCAGTCAGA-CATGTGGCAGCATCATG, Reverse: GGGGG GGAGCTCGCAGACCCCTTAAATGGTGA), -630/+25 (Forward: AAAAAAGGTA CCCATGCAGTTAGTGCTTATCAAATG, Reverse: GGGGGGGGAGCTC-GCAGACC CCTTAAATGGTGA), -500/+25 (Forward: AAAAAAGGTACCCAAATTATTCTTAA CACTTCCTTTAAG, Reverse: GGGGGGGGAGCTCGCAGACCCCTTAAATGGTGA), -200/+25 (Forward: AAAAAAGGTACCGTCTTTGCATAAGCCCCTTGTC, Reverse: GGGGGGGGAGCTCG-CAGACCCCTTAAATGGTGA), -70/+25 (Forward: AAAAAAG GTACCCAGAGAATTGGGAGT-GACATC, Reverse: GGGGGGGGAGCTCGCAGA CCCCTTAAATGGTGA). The pRL-CMV vector encoding Renilla luciferase was commercially available (Promega, Fitchburg, USA) and used to correct for transfection efficiency. HA-HNF1B wild-type and HA-HNF1B H69fsdelAC, K156E, H324S325fsdelCA and Y352finsA pCINEO IRES GFP were cloned as previously described.[26] All constructs were verified by sequence analysis.

Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR) analysis

Total RNA from human parathyroid glands and PT-r cells was isolated using Trizol (Ambion, Life Technologies). Total RNA (1.5 μg) was reverse transcribed and end-point PCR reactions

performed. Part of the RNA sample was not reverse transcribed before PCR and thereby served as a negative control. Sequences of the oligonucleotides used for the RT-PCR are shown in **Table 1**.

Cell culture and transfection

Human Embryonic Kidney cells (HEK293) were grown in DMEM (Bio Whittaker-Europe, Verviers, Belgium) containing 10% (v/v) FBS (Thermo Fisher HyClone), 2 mM L-glutamine and 10 ug/ml ciproxin at 37°C in a humidity-controlled incubator with 5% (v/v) CO_2. The cells were transiently transfected with the respective constructs using polyethylenimine cationic polymer (PEI, Polysciences Inc.) and assayed 48 hours post-transfection. When performing dose-response analysis, an empty vector (including nonsense mock DNA) was used to keep the total amount of transfected DNA constant.

The immortalized PT-r cell line was previously isolated from rat parathyroid and characterized to show endogenous PTH expression, which can be inhibited by extracellular Ca^{2+}, as well as 1,25-D_3 via a VDR-mediated mechanism.[4, 27] The PT-r cells were maintained in DMEM/F12 (Gibco, Life Technologies) supplemented with 10% (v/v) FBS (Thermo Fisher HyClone) and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin; Gibco, Life Technologies) under a 5% (v/v) CO_2 atmosphere at 37°C. For immunocytochemistry experiments, PT-r cells were plated on fibronectin-coated glasses in 12-well plates at 50% confluence and assayed the following day.

Luciferase reporter assay

In a 12-well plate, 700 ng of the *PTH* promoter-luciferase constructs and 100 ng of either empty vector (mock DNA) or HA-HNF1B wild-type, HA-HNF1B H69fsdelAC, K156E, H324S-325fsdelCA, and Y352finsA pCINEO IRES GFP constructs were co-transfected into HEK293 cells. To correct for transfection efficiency, 10 ng of pRL-CMV was used as a reference. Firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay (Promega, Fitchburg, USA).

Immunohistochemistry

Staining was performed on 6-µm sections of frozen human cadaveric parathyroid samples that were fixed with 100% methanol for 10 min at -20°C. Subsequently, sections were washed three times with TN buffer (0.15 mol/L NaCl, 0.1 mol/L Tris adjusted to pH 7.6 with HCl) prior incubation in blocking buffer for 30 min. Sections were stained overnight at 4°C with a rabbit anti-human HNF1B (1:20; Santa Cruz, sc-22840). The following day, sections were washed and incubated with a goat anti-rabbit secondary antibody coupled to Alexa Fluor 488 (1:300; Invitrogen, A11008) for 1 hour at room temperature. Sections were washed and incubated for 2 hours with a mouse anti-human PTH antibody (1:50; AbD Serotec, 7170-6216). After washing, a goat anti-mouse secondary antibody conjugated to Alexa Fluor 594 (1:300; Invitrogen, A11005) was applied for 1 hr. Subsequently, sections

Table 1 Oligonucleotide sequences used for RT-PCR analysis.

Gene product	Accession	Forward (5'-3')	Reverse (5'-3')	Amplicon size
hPTH	NM_000315.2	CATTGTATGTGAAGATGATACCTGC	GCAGCATGTATTGTTGCCCT	424 bp
hHNF1B	NM_000458.2	CATACTCTCACCAACGGCCA	AAACAGCAGCTGATCCTGACT	426 bp
hFXYD2a	NM_001680.4	GTCGATGGACGGTGGCGGCAG	GAAGGCCAGTCCAGCGAAGATC	103 bp
hFXYD2b	NM_021603.3	CAGGTGGTACCTGGGCGGCAG	GAAGGCCAGTCCAGCGAAGATC	103 bp
hGAPDH	NM_002046.4	GGAGTCAACGGATTTGGTCGTA	GGCAACAATATCCACTTTACCAGAGT	78 bp
rPth	NM_017044.1	GAAGGATCCTCTCTGAGAGTC	CAGGGTCTAGAATACGTCAGC	405 bp
rPthrp	NM_012636.1	GGAGGCGCTGATTCCTACAA	TTTCTTCTTCTTCCCGGGCG	428 bp
rCasr	NM_016996.1	CTGATGACCACTATGGCAGAC	CATGTTGTTGGTGAAGTTCAGG	779 bp
rHnf1b	NM_013103.1	GAATTACTGCCGTCCCCGAA	CTCCACTAAGGCCTCCCTCT	705 bp
rGapdh	NM_017008.4	GGTGAAGGTCGGTGTGAACGG	CCATGTAGTTGAGGTCAATGAAG	122 bp

PTH: parathyroid hormone; HNF1B: hepatocyte nuclear factor B; FXYD2a: γ-subunit of the Na⁺-K⁺-ATPase, isoform a; FXYD2b: γ-subunit of the Na⁺-K⁺-ATPase, isoform b; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; Pthrp: parathyroid hormone-related peptide; Casr: calcium sensing receptor.

were washed, incubated for 30 minutes with DAPI and mounted with Mowiol. Photographs were taken using a Zeiss Axio Imager 1 microscope (Oberkochen, Germany) equipped with a HXP120 Kubler Codix fluorescence lamp and a Zeiss Axiocam MRm digital camera.

Immunocytochemistry

PT-r cells were fixed with 4% (w/v) paraformaldehyde (PFA) for 30 minutes at room temperature, followed by permeabilization for 15 minutes with 0.2% (v/v) Triton X-100 in PBS supplemented with 0.1% (w/v) BSA. After incubation overnight at 4°C with a rabbit anti-human HNF1β antibody (1:50, sc-22840, Santa Cruz), cells were washed three times with PBS supplemented with 0.1 mmol/L $CaCl_2$ and 1.0 mmol/L $MgCl_2$ (PBS-CM), and subsequently incubated with a secondary goat anti-mouse antibody (1:100), coupled to AlexaFluor 594, for 45 minutes at room temperature. Cells were washed three times with PBS-CM prior mounting with DAPI–Vectashield (Vector Laboratories, Burlingame, CA). Photographs were taken using a Zeiss Axio Imager 1 microscope (Oberkochen, Germany) equipped with a HXP120 Kubler Codix fluorescence lamp and a Zeiss Axiocam MRm digital camera.

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed using HEK293 cells co-transfected with 700 ng of the human *PTH* promoter luciferase constructs, −1476/+25 or −70/+25, and 100 ng of the HNF1B pCINEO IRES GFP construct. Cells were harvested 48 hours after transfection and a Magna ChIP A assay was performed according to the manufacturer's protocol (Merck Millipore, Billerica, USA). In short, cells were treated with formaldehyde to crosslink the chromatin. After cell lysis, samples were sonicated twice for 30 seconds on wet ice using a 22-μm amplitude with a Soniprep 150 (MSE, London, UK). Samples were incubated with 5.0 μg of rabbit polyclonal anti-human HNF1B antibody (Santa Cruz, sc-22840), rabbit IgG isotype antibodies as negative control or anti-trimethyl-histone H3 antibody as positive control, which were bound to protein A magnetic beads. Subsequently, chromatin complexes were eluted, the crosslinks were reversed, and the DNA was isolated. The presence of *PTH* promoter DNA was evaluated using real-time PCR targeting the human *PTH* promoter (5′-GCCTGGAGCAACACTCTAAG-3′ and 5′-CATCCTGGCTTCATGTCATCC-3′). Subsequently, samples were loaded on a 2% agarose gel and visualized using ProXima C16 software version 3.0 (Isogen Life Science, De Meern, The Netherlands).

Data analysis

Results are based on a minimum of three independent experiments, with each condition performed in triplicate, unless otherwise stated. Values are expressed as means ± standard error of the mean (SEM). Statistical significance ($P < 0.05$) was determined using unpaired Student t-tests.

Results

PTH levels in a cohort of HNF1B patient

In this study, we retrospectively reviewed a total number of eleven patients, five familial and six sporadic cases, with known *HNF1B* mutations or whole-gene deletions visiting the outpatient clinic of our institution (**Table 2**). Nine patients had a *HNF1B* whole gene deletion, one patient had a frameshift mutation (c.18delG, p.Ser7Argfs*7) and one patient had a missense mutation (c.883C>T, p.Arg295Cys). Serum intact PTH levels were available for ten out of eleven patients. Eight out of these ten patients had hyperparathyroidism (6.6 to 16.4 pmol/L, normal range 1.0 to 6.5 pmol/L). Eight patients had hypomagnesemia (0.41 mmol/L to 0.65 mmol/L), while their plasma PO_4^{3-} and Ca^{2+} levels were within the normal range. Five out of seven patients with increased PTH levels belong to the same family (patients I.1 through I.5), and showed concomitant hypomagnesemia. No hypomagnesemia was observed in a patient with a c.883C>T (p.Arg295Cys) *HNF1B* mutation and high PTH levels (patient V, **Table 2**). In two patients the hyperparathyroidism was initially clearly appropriate for the level of kidney function (patient I.2 and VI, **Table 2**). After renal trans-plantation, the former patient with a c.18delG (p.Ser7Argfs*7) *HNF1B* mutation showed improved renal function and normal plasma Mg^{2+} levels, while the PTH levels remained increased. One patient underwent parathyroidectomy at the age of 23 years for primary hyperparathyroidism (patient VII, **Table 2**). This patient was 36 years of age before any connection between *HNF1B* mutations and his clinical symptoms was revealed, at which time he again displayed hyperparathyroidism. Urinary calcium/creatinine ratio revealed that nine out of ten patients whose data were available displayed hypocalciuria. Tubular reabsorption of phosphate (TRP) was at our below the lower limit of normal in all patients suggested to display hyperparathyroidism. Overall, these data suggest a link between *HNF1B* mutations or whole-gene deletions and hyperparathyroidism, distinct from secondary hyperparathyroidism due to renal function decline.

HNF1B is expressed in parathyroid cells

First, we investigated HNF1B expression in human parathyroid gland tissue. HNF1B mRNA expression in human parathyroid glands was detected by end-point PCR and compared to HNF1B mRNA expression in a control human kidney sample (**Figure 1A**). The specificity of the tissue was confirmed by amplification of the PTH transcript, which was clearly present in parathyroid tissue but, as expected, not in the kidney. The histology of parathyroid glands is easily recognizable by the densely packed cells. PTH was highly expressed in the cytosol of the parathyroid cells (**Figure 1B**). Importantly, HNF1B was localized in PTH-positive cells in a nuclear pattern, as shown by the co-localization with the nuclear marker DAPI that binds to double-stranded DNA (**Figure 1B**).

Table 2 Laboratory investigations of 11 patients with *HNF1B* abnormalities.

Patient	Gender	Age	PTH	Ca^{2+}	PO$_4^{3-}$	Mg^{2+}	Cr	C$_{Cr}$ or eGFR	Urinary Ca^{2+}/Cr	TRP	Abnormality	Additional Information
I.1	M	51	13.9	2.26	0.80	0.55	114	62	< 0.1	71%	Deletion	
I.2	F	47	14.7	2.31	0.79	0.54	141	35	< 0.01	82%	Deletion	
I.3	F	42	8.8	2.38	0.98	0.54	121	83	0.18	81%	Deletion	
I.4	F	15	9.2	2.38	1.27	0.41	65	109	< 0.01	88%	Deletion	
I.5	M	14	6.6	2.52	1.01	0.51	64	>90	ND	ND	Deletion	
II	M	7	ND	2.52	1.40	0.65	41	>90	0.23	91%	Deletion	
III	M	10	5.2	2.45	1.31	0.70	37	>90	0.96	94%	Deletion	
IV	F	42	5.3	2.39	1.35	0.43	107	48	< 0.1	87%	Deletion	
V	F	39	9.5	2.34	0.88	0.79	93	87	< 0.1	ND	c.883C>T p.Arg295Cys	
VI	F	1	23.1	2.52	1.53	1.21	331	ESRD	ND	ND	c.18delG p.Ser7Argfs*7	
		9	13.4	2.57	1.36	0.77	79	69	0.29	86%		Data after kidney transplantation
VII	M	23	ND	2.96	0.52	0.47	128	64	ND	ND	Deletion	Parathyroidectomy for hyperparathyroidism at age 23
		36	16.4	2.30	0.79	0.64	150	74	< 0.06	86%		Data at eventual diagnosis

Gender (F, female; M, male); Age (years); PTH, parathyroid hormone (pmol/L; N: 1.0–6.5); Ca^{2+}, serum calcium concentration (mmol/L; N: 2.20–2.65); PO$_4^{3-}$, serum phosphate concentration (mmol/L; < age 12 years: 1.3–1.9, > age 12 years: 0.8–1.4); Mg^{2+}, serum magnesium concentration (mmol/L; N: 0.7–1.1); Cr, serum creatinine (μmol/L; N: 45–110); C$_{Cr}$, creatinine clearance (ml/min; N: 89–143); eGFR, estimated glomerular filtration rate by Modification of Diet in Renal Disease (MDRD) formula (adults) or Cockcroft-Gould (children) (ml/min per 1.73 m2); ESRD, end-stage renal disease; Ca^{2+}/Cr, calcium over creatinine ratio (mmol/mmol; N: 0.40–0.57); TRP, renal tubular reabsorption of phosphate (%; N >85%); ND: not determined.
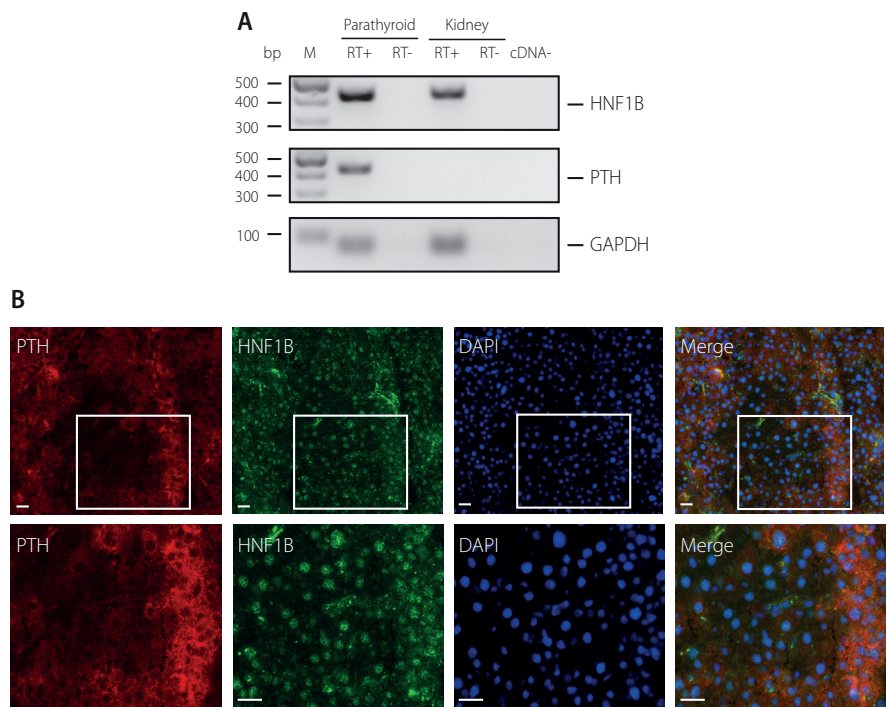
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Figure 1 HNF1B expression in human parathyroid glands.

(A) Endogenous expression of HNF1B in human parathyroid tissue was investigated using RT-PCR. PTH was included as positive control for tissue specificity and HPRT as housekeeping gene. RT+: RT sample; RT-: no RT control; cDNA-: no cDNA control. **(B)** Representative images of immunohisto-chemical analysis of HNF1B and PTH co-localization in human parathyroid tissue. Scale bars: 20 μm. HNF1B: hepatocyte nuclear factor 1 beta; PTH: parathyroid hormone; HPRT: hypoxanthine-guanine phosphoribosyl transferase; CaSR: calcium-sensing receptor; DAPI: nuclear marker.

So far, PT-r cells represent the only available immortalized cell line that retains characteristics of parathyroid cells, including Pth expression sensitive to extracellular Ca^{2+} and 1,25-dihydroxyvitamin D_3 treatment, when transiently transfected with Casr or vitamin-D receptor (VDR), respectively.[4] In our cell culture conditions, PT-r cells expressed limited amount of Pth mRNA compared to the expression levels of parathyroid hormone-related protein (Pthrp; **Figure 2**). HNF1B was expressed and localized to the nucleus of PT-r cells (**Figure 2**). Casr was not detectable, as previously described by Kawahara *et al.*[4]
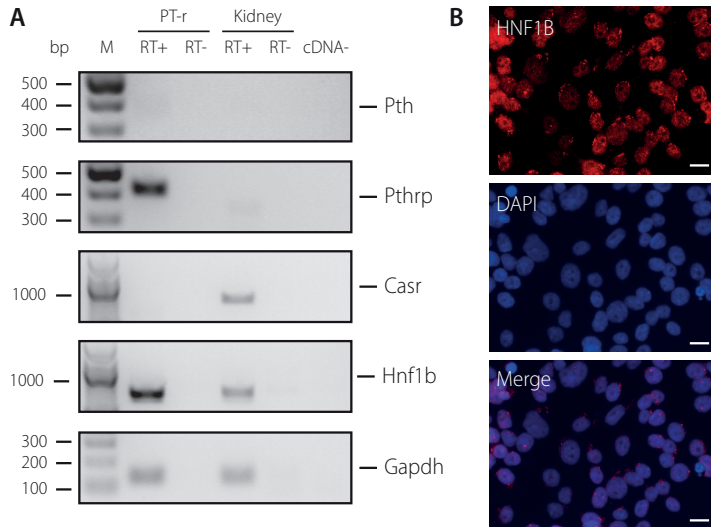
Figure 2 Rat parathyroid PT-r cells express HNF1B.

(A) Expression profile of genes relevant to parathyroid cell physiology were investigated in PT-r cells using RT-PCR. Gapdh was included in the analysis as housekeeping gene. RT+: RT sample; RT-: no RT control; cDNA-: no cDNA control. **(B)** Immunostaining for Hnf1b in PT-r cells. Scale bars: 20 µm. Pth: parathyroid hormone; Pthrp: parathyroid hormone-related peptide; Casr: calcium-sensing receptor; Hnf1b: hepatocyte nuclear factor 1 beta; Gapdh: glyceraldehyde 3-phosphate dehydrogenase; DAPI: nuclear marker.

Wild-type HNF1B binds the human *PTH* gene promoter and inhibits its activity

HNF1B affects transcription of target genes through binding of the POU (Pit-1, OCT1/2, UNC-86) domains to a DNA consensus sequence, reported in **Figure 3A**. Using the Consite program (asp.ii.uib.no:8090/cgi-bin/CONSITE/consite), prediction analysis for HNF1-binding sites in the -1476 bp region upstream of the transcription initiation site of the human *PTH* promoter identified two relatively well-conserved sites, at -1238 and -690 (**Figure 3B**). Demay *et al.* have previously suggested the presence of a poorly conserved consensus sequence of a POU transcription factor around position -101.[12] To determine whether the *PTH* promoter is bound by HNF1B, we performed a ChIP assay using a human *PTH* -1476 promoter construct transfected in HEK293 cells, which do not endogenously express HNF1B.[28] The immunoprecipitated genomic fragment bound by exogenously expressed HNF1B was quantified by real-time PCR analysis, which showed a 9-fold enrichment of the *PTH* promoter when precipitated with the anti-HNF1B antibody (**Figure 3C**) compared to the rabbit IgG isotype control (**Figure 3C**). Using a human *PTH* -70 promoter construct no significant difference was seen between both antibodies. Immunoprecipitation with

an anti-trimethyl-histone H3 antibody was performed as positive control. Two percent of the chromatin used for immunoprecipitation (input) was also included in the analysis (**Figure 3C**).
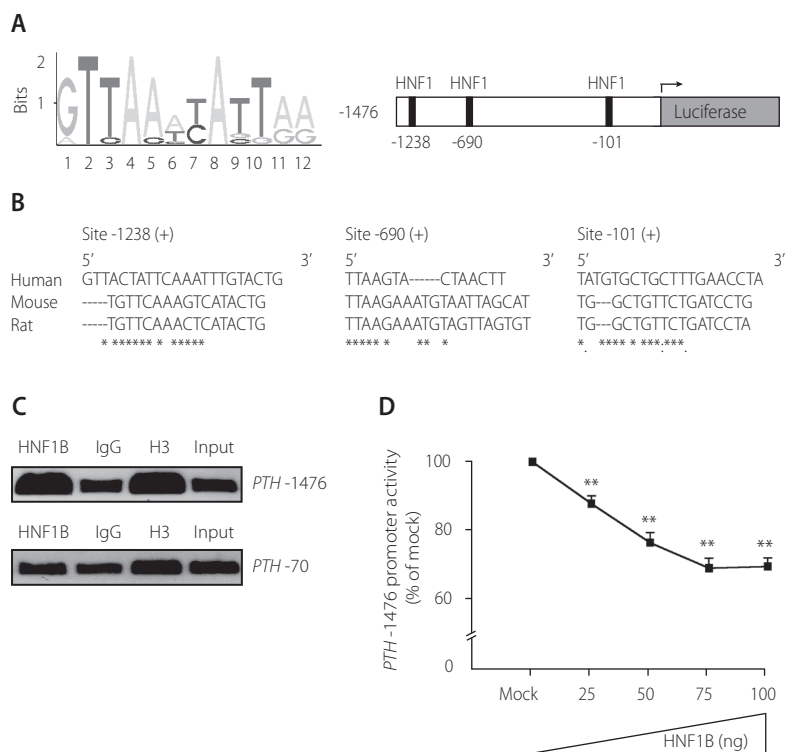

Figure 3 The human *PTH* gene as target of the HNF1B transcription factor.

(A) Sequence logo of the HNF1B motif as from Jaspar database (ID: MA0153.1). In the human *PTH* promoter region (-1476 bp from the transcription initiation site), three putative HNF1 binding sites map at −1238, −690 and −101 of the forward DNA strand (+). **(B)** Sequence alignment of the three putative HNF1 recognition sites in the PTH promoter region of different mammalian species. (C) Binding of the *PTH* promoter by HNF1B was verified by a ChIP assay in HEK293 cells co-transfected with the human *PTH* promoter luciferase constructs, −1476/+25 or −70/+25, and wild-type HNF1B. For immunoprecipitation, an anti-HNF1B antibody or a rabbit IgG isotype control antiserum was used. Immunoprecipitation with an anti-trimethyl-histone H3 antibody was included as positive control. Two percent of the chromatin used for immunoprecipitation (input) was also included as a control. **(D)** Dose-response curve of increasing amounts of wild-type HNF1B co-transfected in HEK293 cells with a luciferase construct carrying the human *PTH* gene promoter region −1476/+25 (n=9). **, $p < 0.001$, compared to mock.

To study the involvement of HNF1B in the transcriptional regulation of the human *PTH* gene, luciferase-reporter assays were performed. When wild-type HNF1B was transiently co-transfected with the human *PTH* -1476 promoter construct, a dose-dependent reduction of the promoter activity was observed, up to a 30% decrease compared to cells transfected with the mock plasmid (**Figure 3D**).

This inhibitory effect was not demonstrated for the HNF1B H69fsdelAC, K156E, H324S-325fsdelCA and Y352finsA mutants. When the latter were co-expressed with the *PTH* reporter construct, the promoter activity was comparable or higher to what was observed in mock plasmid-expressing cells (**Figure 4A**). In order to pinpoint the HNF1B-responsive region within the *PTH* promoter, serial deletions of the reporter construct were prepared (**Figure 4B**). HNF1B H324S325fsdelCA lacks a complete, functional transactivation domain, and does not downregulate *PTH* promoter activity. For the wild-type HNF1B, inhibition of *PTH* promoter activity persisted until –200 bp from the transcription initiation site. A further deletion to –70 bp no longer showed a significant difference between wild-type and mutant HNF1B (**Figure 4B**). These data demonstrate that one or more HNF1B-responsive elements reside in this proximal promoter region of the human *PTH* gene.
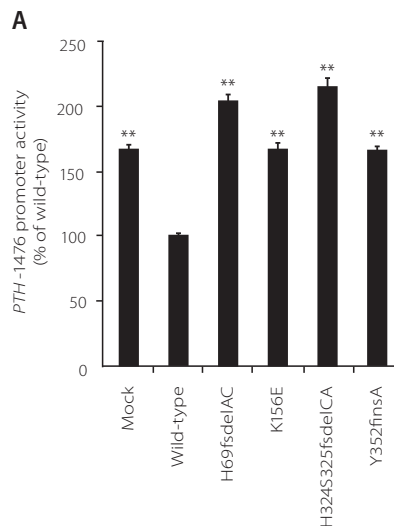

Figure 4 HNF1B mutants lack the ability to inhibit *PTH* promoter activity.

(A) Study of the human *PTH* promoter activity when co-transfected with wild-type HNF1B or HNF1B H69fsdelAC, K156E, H324S325fsdelCA and Y352finsA mutants, or the mock plasmid in HEK293 cells (n=3). ** $P< 0.001$, compared to wild-type HNF1B.
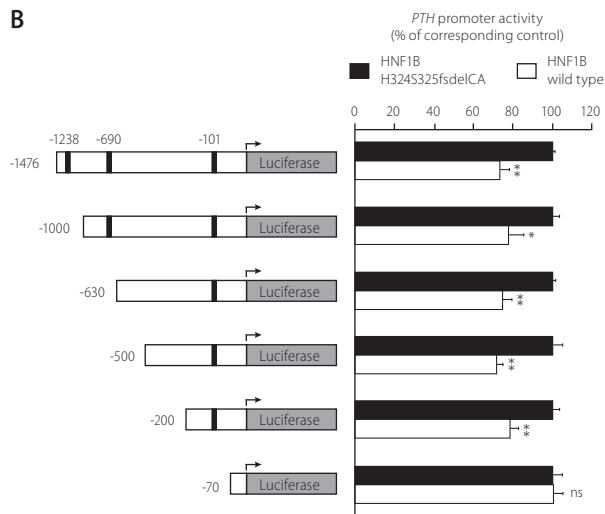
Figure 4 Continued.

(B) Serial deletion analysis in which the *PTH* promoter activity was tested in HEK293 cells in the presence of wild-type HNF1B (empty bars) or HNF1B H324S325fsdelCA mutant (black bars) (n=9). ** $p < 0.001$, compared to HNF1B H324S325fsdelCA; * $p = 0.006$, compared to HNF1B H324S325fsdelCA. ns: nonsignificant compared to HNF1B H324S325fsdelCA, $p = 0.9$.

Discussion

In the present study, we demonstrated that the nuclear transcription factor HNF1B is expressed in the parathyroid gland, where it reduces PTH expression by inhibiting *PTH* promoter activity upon direct binding to HNF1B recognition sites in this promoter region. *In vitro* luciferase-assays showed that HNF1B inhibits *PTH* promoter activity via *cis*-elements located in the proximal promoter. This inhibition is lost when *HNF1B* is mutated or absent. Altogether these findings provided explanation for our observation that PTH levels are often elevated in patients with *HNF1B* gene mutations. While further experiments are needed to determine which molecular pathways govern HNF1B activity in the parathyroid gland, our data demonstrated that HNF1B acts as a repressor of human *PTH* gene transcription.

Our hypothesis that HNF1B might be involved in parathyroid gland function was based on the observation that, in our patients with *HNF1B* mutations or whole-gene deletions, PTH levels were often relatively high compared to the degree of renal function decline, if present. This was even more striking given the fact that most of these patients were hypomagnesemic, which is usually associated with a paradoxical block of PTH

secretion and a resulting hypoparathyroidism.[29, 30] This was accompanied by reduced urinary Ca^{2+} excretion and renal tubular reabsorption of PO_4^{3-} that was in the lower range of normal or reduced. Importantly, HNF1B was known to function as a transcription factor regulating gene transcription in a number of specialized endocrine organs and tissues with secondary endocrine functions.[22-25] Previously, Adalat *et al.* reported plasma PTH levels in a cohort of patients with chronic kidney disease, in stages 1 through 3, with and without HNF1B-associated disease.[21] They mainly focused on the hypomagnesemia observed in 44% of their patients with *HNF1B* defects, but they also reported plasma PTH levels were 6.3 in patients with *HNF1B* defects versus 4.9 pmol/L in the patients with renal dysplasia in the absence of such defects (P=0.2). Possibly due to the small series of patients, these data did not reach statistical significance. Importantly, Ca^{2+} and PO_4^{3-} levels were within normal ranges, while the glomerular filtration rate (GFR) was similar in both groups. The present data clearly demonstrated that HNF1B is expressed in a nuclear pattern in the PTH-positive cells of human parathyroid gland as well as in the only representative parathyroid cell line described to date. Importantly, we demonstrated that HNF1B binds to the *PTH* promoter and that the presence of HNF1B inhibits *PTH* transcription, at least in part by a direct effect on the activity of the *PTH* promoter. This inhibitory effect was lost when known mutations in *HNF1B* were introduced. These data indicated that HNF1B acts as a functional repressor of *PTH* gene transcription. Loss of this inhibitory effect readily explains the tendency towards hyperparathyroidism in patients in which the *HNF1B* gene is deleted or mutated. So far, many regulatory pathways that control PTH secretion, both transcriptionally and post-transcriptionally, have been defined and several are currently therapeutic targets for the treatment of secondary hyperparathyroidism in the course of chronic kidney disease.[2] These include active vitamin D (1,25-dihydroxyvitamin D_3, or 1,25-D_3) analogs and calcimimetics.[31-34] The best-known *PTH* transcriptional repressor, 1,25-D_3, acts by way of the liganded 1,25-D_3 receptor-retinoic acid X receptor (VDR-RXR) complex binding to vitamin D responsive elements (VDRE) in the promoter region of the *PTH* gene. Interestingly, this VDRE was previously mapped to a region very close to the putative HNF1B-binding site in the *PTH* promoter.[35] There might be crosstalk between pathways involving 1,25-D_3 and HNF1B, but the exact signaling mechanism in which HNF1B is the final effector remains to be determined. Other possibilities include involvement in CaSR-mediated signaling or the recently identified FGF23-FGFR1/Klotho axis [36, 37]. Mitogen-activated protein kinase (MAPK) pathways presumably play a role downstream of both CaSR and FGFR1/Klotho activation in the parathyroid gland, and an interaction between FGF-induced/MAPK-mediated signaling and HNF1B has been previously suggested in other tissues.[38-42]

Although our data clearly identify HNF1B as a negative regulator of *PTH* gene expression, the clinical significance of these findings in patients with a *HNF1B* mutation or deletion has to be studied in larger patient cohorts. Apart from our observations, a single reference to a patient with hyperparathyroidism in a cohort

of 27 patients with HNF1B-associated disease, and the previously mentioned study by Adalat *et al.*, there are to our knowledge no other reports of PTH levels in patients with HNF1B-related disease.[15, 21] Of note, in our cohort of patients a concomitant hypo-magnesemia was diagnosed in the majority of individuals. It was previously reported that in the distal part of the nephron, impaired transcriptional regulation of the *FXYD2* gene, encoding for the γ-subunit of the Na^+-K^+-ATPase, could be responsible for this hypomagnesemic effect.[26] Extracellular Mg^{2+} binds and activates the CaSR *in vitro*, albeit with a much lower affinity compared to Ca^{2+},[43, 44] and therefore may influence PTH release by the parathyroid glands. However, hypomagnesemia is associated with a paradoxical block of PTH secretion resulting in a hypoparathyroidism, possibly due to enhancement of G-protein mediated signaling downstream from the CaSR.[29, 30] Interestingly, one patient in our cohort, displaying a c.883C>T (p.Arg295Cys) *HNF1B* mutation, showed normo-magnesemia and an endogenous creatinine clearance of >80mL/min on repeated measurements, but elevated PTH levels. Together, these data are in line with a putative role of *HNF1B* abnormalities in the development of primary hyperparathyroidism independent from extracellular Mg^{2+} levels.

Since the Na^+-K^+-ATPase pump is essential to generate the driving force for PTH secretion[45], a hypothesis that remains to be tested is whether in the parathyroid glands the impaired transcriptional regulation of *FXYD* family members due to HNF1B abnormalities may contribute to the onset of primary hyperparathyroidism. FXYD proteins are known to associate with the Na^+-K^+-ATPase and to modulate its kinetic properties in a tissue-specific manner.[46] However, RT-PCR experiments we performed on human parathyroid samples (**Figure 5**) and a human tissue microarray study failed to detect FXYD2 expression in the parathyroid glands.[47] So far, impaired renal FXYD2 expression is the only molecular mechanism connecting the misregulation of the Na^+-K^+-ATPase activity to a tissue-specific effect due to *HNF1B* mutations.

In conclusion, we identified HNF1B as a novel transcriptional regulator of *PTH* gene expression in the parathyroid gland. Patients with HNF1B-associated disease display a tendency towards hyperparathyroidism, possibly due to the loss of the HNF1B-mediated repression of *PTH* promoter activity in the parathyroid gland. Future studies will have to confirm the clinical significance of HNF1B affecting the synthesis of PTH in patients with *HNF1B* mutations, e.g. by evaluating PTH levels in a larger cohort of patients, preferably with no renal failure and no hypomagnesemia. Furthermore, the exact HNF1B binding site within the *PTH* promoter needs to be identified, and the role of HNF1B in the responsiveness of parathyroid cells to extracellular Ca^{2+}, 1,25-D_3 and FGF23 has to be investigated.

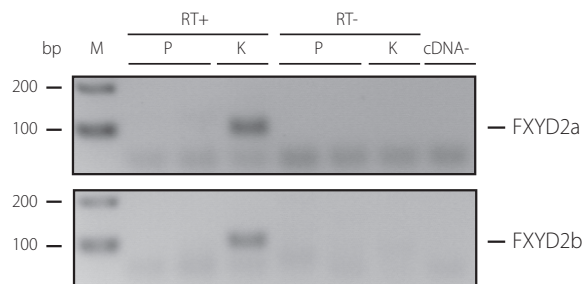**Figure 5** FXYD2 expression in human parathyroid glands.

Endogenous expression of the two main FXYD2 isoforms in human parathyroid tissue was investigated using RT-PCR. FXYD2a: γ-subunit of the Na^+-K^+-ATPase, isoform a; FXYD2b: γ-subunit of the Na^+-K^+-ATPase, isoform b; P: parathyroid samples; K: kidney sample; RT+: RT samples; RT-: no RT controls; cDNA-: no cDNA control.

Acknowledgements

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3

Mutations in *PCBD1* are associated with hypomagnesemia, renal magnesium wasting and MODY

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4

Abstract

Mutations in *PCBD1* are causative for transient neonatal hyperphenylalaninemia and primapterinuria (HPABH4D). Until now HPABH4D has been regarded as a transient and benign neonatal syndrome without complications in adulthood. In our study, two adult patients with homozygous mutations in the *PCBD1* gene were diagnosed with hypo-magnesemia and renal Mg^{2+} loss. One patient also developed diabetes with characteristics of maturity onset diabetes of the young (MODY). Our results suggest that these clinical findings are related to the function of PCBD1 as dimerization cofactor for the transcription factor HNF1B. Mutations in the *HNF1B* gene have previously been shown to cause renal malformations, hypomagnesemia and MODY. Gene expression analysis in the kidney showed that Pcbd1 is co-expressed with Hnf1b in the distal convoluted tubule (DCT) where Pcbd1 transcript levels are upregulated by a low Mg^{2+}-containing diet. Overexpression in a human kidney cell line demonstrated that wild-type PCBD1 binds HNF1B to co-stimulate the *FXYD2*-promoter, whose activity is instrumental in Mg^{2+} reabsorption in DCT. Five out of seven PCBD1 mutations previously reported in HPABH4D patients caused proteolytic instability leading to a reduced *FXYD2*-promoter activity. Furthermore, HNF1B mutations may disturb PCBD1 localization in the nucleus, since PCBD1 showed an increased cytosolic localization when co-expressed with HNF1B mutants. Overall, our findings establish PCBD1 as an important co-activator of the HNF1B-mediated transcription necessary for fine-tuning of *FXYD2* transcription in DCT. Thus, patients with HPABH4D should be monitored for previously unrecognised late complications, such as hypomagnesemia and MODY diabetes.

Introduction

Hypomagnesemia is a common clinical manifestation in patients with mutations in the transcription factor hepatocyte nuclear factor 1 homeobox B (HNF1B [MIM 189907]).[1] Haploinsufficiency for HNF1B is associated with an autosomal dominant syndrome characterized by renal malformations with or without cysts, liver and genital tract abnormalities, gout, and maturity-onset diabetes of the young type 5 (MODY5; renal cysts and diabetes syndrome [MIM 137920]).[2, 3] Hypomagnesemia (plasma Mg^{2+} levels <0.7 mmol/L) with hypermagnesuria affects up to 50% of the *HNF1B* patients.[3, 4] Patients are generally treated with magnesium (Mg^{2+}) supplements to ameliorate the symptoms of the hypomagnesemia that are related to instability of the neurological and cardiovascular systems.[3, 4]

In the adult kidney HNF1B is expressed in epithelial cells along all segments of the nephron. The role of HNF1B in renal Mg^{2+} handling was, however, pinpointed to the distal convoluted tubule (DCT), where the final urinary Mg^{2+} excretion is determined.[3, 5] In DCT, the Na^+-K^+-ATPase provides the necessary driving force for active Mg^{2+} reabsorption from the pro-urine into the blood.[5] Heterozygous mutations in the *FXYD2* gene, encoding the γ-subunit of the Na^+-K^+-ATPase, result in an autosomal dominant renal hypomagnesemia with hypocalciuria (IDH [MIM 154020]).[6] More recently, functional HNF1B binding sites were identified in the promoter region of *FXYD2* suggesting that impaired transcription of *FXYD2* by HNF1B results in renal Mg^{2+} wasting.[3, 7] Additional HNF1B target genes in kidney include renal cystic genes[8-10] as well as genes involved in tubular electrolyte transport.[11, 12]

HNF1B forms heterotetrameric complexes with the protein pterin-4 alpha-carbinolamine dehydratase / dimerization cofactor of hepatocyte nuclear factor 1 alpha (PCBD1 / DCOH [MIM 126090]).[13] PCBD1 is a protein of 12 kDa with two distinct biological functions: transcriptional co-activator of HNF1-mediated transcription within the nucleus,[13] and pterin-4α-carbinolamine dehydratase (EC 4.2.1.96) in the cell cytosol.[14] The enzymatic activity of PCBD1, together with dihydropteridine reductase, regenerates tetrahydrobiopterin (BH_4), which is the cofactor for phenylalanine hydroxylase (PAH) and other aromatic amino acid hydrolases.[15] The crystal structure of PCBD1 revealed that the protein forms a tetramer of identical subunits comprising two saddle-like shaped dimers.[16, 17] HNF1 binding sites are located at the same surface that mediates interaction of the PCBD1 homodimers, on the opposite side of the catalytic domain.[18] *PCBD1* knockout mice display hyperphenylalaninemia, predisposition to cataracts and mild glucose-intolerance.[19] Homozygous or compound heterozygous *PCBD1* mutations in humans are associated with transient neonatal hyperphenylalaninemia and high urinary levels of primapterin (HPABH4D, or primapterinuria [MIM 264070]).[20-22] To date there have been no reports of late complications, or possible phenotypic consequences of impaired stimulation of the HNF1 transcription factors.

In our study, the occurrence of hypomagnesemia and MODY diabetes was investigated in two patients carrying *PCBD1* mutations. We evaluated whether PCBD1 plays a role in renal Mg^{2+} reabsorption by directly affecting HNF1B-regulated *FXYD2* transcription, in order to gain new insight into the molecular basis of the PCBD1-HNF1B interaction.

Materials and Methods

Patients

The two patients reported in this study were ascertained by contacting authors of papers related to hyperphenylalaninemia, tetrahydrobiopterin-deficient, due to pterin-4-alpha-carbinolamine dehydratase deficiency (HPABH4D [MIM 264070]) as listed in the International Database of Tetrahydrobiopterin Deficiencies (BIODEF database, Opladen T, Blau N., http://www.biopku.org/biodef/).[23] Informed consent to participate in this study was obtained and the procedures followed were in accordance with the standards of the medical ethics committee of each participating institution. Patients were not treated with any medication that could affect serum levels of Mg^{2+} (e.g. diuretics, calcineurin inhibitors, corticosteroids).

Patient 1 (BIODEF 272) was reported in detail previously.[20] In summary, he was found to have borderline hyperphenylalaninemia on newborn screening, but this markedly increased to a peak of 2589 μmol/L at 3.5 weeks. The diagnosis of HPABH4D was suspected because of primapterinuria (7-biopterin), as well as a marked response to BH_4. There was no parental consanguinity; molecular analysis confirmed the diagnosis showing that he was homozygous for a c.312C>T (p.Gln97Ter) mutation in the *PCBD1* gene. He was treated with phenylalanine restriction and BH_4 until 4 months of age, at which time his phenylalanine levels normalised without further treatment, on a normal diet. Intermittent follow-up into adolescence revealed normal health, growth and cognitive development. He was re-contacted for this study at age of 19 years; at that time it was noticed that he had become an insulin-dependent diabetic about 6 months previously.

Patient 2 (BIODEF 329) Initial neonatal screening had shown hyperphenylalaninemia with high urinary levels of primapterin (7-biopterin).[20, 21] This resolved after daily treatment with BH_4. He was followed by paediatricians at the Klinik für Kinder- und Jugendmedizin (Karlsruhe, Germany). There was parental consanguinity, and molecular studies showed that he was homozygote for two separate mutations in the *PCBD1* gene: c.99G>T p.Glu26Ter, and 283G>A p.Arg87Gln.

Animal study

All the experimental procedures are in compliance with the animal ethics board of the Radboud University Nijmegen. The transgenic parvalbumin-eGFP mice were a kind gift from Dr. Monyer (University of Heidelberg, Germany).[24] In the Mg^{2+} diet experiment, the

mice were fed low (0.02% wt/wt) or high (0.48% wt/wt) Mg^{2+}-containing diets for 15 days (SSNIFF spezialdiäten GmbH, Soest, Germany). During the last 48h of the experiment the mice were housed in metabolic cages for urine collection (24h adaptation, 24h sampling). Blood samples were taken at the start of the experiment and just before the sacrifice. PV-eGFP positive tubules were isolated as described previously.[25] In short, mice aged from 4 to 6 weeks were anesthetized and perfused transcardially with ice-cold KREBS buffer (in mmol/L: 145 NaCl, 5 KCl, 1 NaH_2PO_4, 2.5 $CaCl_2$, 1.8 $MgSO_4$, 10 glucose, 10 HEPES/NaOH pH: 7.4). Kidneys were harvested and digested in KREBS buffer containing 1 mg/ml collagenase (Worthington, Lakewood, NJ, USA) and 2000 units/ml hyaluronidase (Sigma, Houten, the Netherlands) for three cycles of maximal 15 min each at 37° C. Subsequently, the kidney tubules sized between 40-100 μm were collected by filtration. Tubules collected from the three digestions were ice-cooled and sorted by the Complex Object Parameric Analyzer and Sorter (COPAS, Union Biometrica, Holliston, MA, USA). Sorted tubules were directly collected in 1% (v/v) β-mercaptoethanol containing RLT buffer that was supplied by the RNeasy RNA extraction kit (Qiagen, Venlo, the Netherlands). Per mouse, 4,000 eGFP-fluo-rescent tubules were collected. 4,000 tubules were pooled on a micro column for RNA extraction according to the manufacturer's protocol. Subsequently, reverse transcription of the RNA by M-MLV reverse transcriptase (Invitrogen) was performed 1 h at 37 °C. Gene expression levels were determined by quantitative real-time PCR on a BioRad Analyzer and normalized for *Gapdh* expression levels. Real-time PCR primers (**Supplemental Table 1**) were designed using the online computer program NCBI/Primer-BLAST software.

Phenylalanine measurements

Phenylalanine in plasma and urine samples was measured by LC-MS/MS by a standardized method, in which the MS was operated in positive mode. For these MS measurements, a Waters Premier triple quadrupole mass spectrometer (tandem MS) interfaced with an electrospray ionization (ESI) source and equipped with an Alliance UPLC (Waters, Etten-Leur, the Netherlands) was used. Briefly, to 10 μL plasma or urine sample, $^{13}C_6$-labeled phenylalanine was added as an internal control to correct for possible ion-suppression. Samples were deproteinized with methanol, diluted with water and injected on a RP C18 column (Waters Atlantis T3, 3 μm, 2,1 x 100 mm). Samples were eluted in 17.5% methanol/0.1M formic acid. Positively charged $[M-H]^+$ phenylalanine (m/z 166) and $^{13}C_6$-phenylalanine (m/z172) were selected as parent ions. After collision induced dissociation (CID), the loss of $-NH_3$ and $-COOH$ groups were chosen as quantifier ion (m/z 120 and 126, respectively) and the loss of $-NH_3$, $-COOH$ and $-OH_2$ groups (m/z 103 and 109, respectively) as qualifier ions. In Multiple Reaction Monitoring (MRM) mode, the transitions m/z 166 _ 120and m/z 166 _ 103 (for phenylalanine) and m/z 172 _ 126 and m/z 172 _ 109 (for $^{13}C_6$-phenylalanine) were measured. Phenylalanine concentrations were calculated using a calibration curve.

DNA constructs

Human HNF1B wild-type and mutants were cloned into the pCINeo HA IRES GFP vector as described previously.[7] HNF1B was Flag-tagged at the NH_2-terminal by Phusion PCR using NheI and AgeI restriction sites. To obtain a HNF1B lacking the dimerization domain spanning residues 2-30 (Δ2-30), HNF1B was amplified using a Phusion polymerase (Finnzymes, Vantaa, Finland) with specific primers covering from residue p.Glu31. The HNF1B-Δ2-30 was ligated into pCINeo IRES GFP using AscI/AgeI restriction sites. The open reading frame of human *PCBD1* was amplified using Phusion polymerase from PCBD1 pCMV-SPORT6 (Genbank BC006324, ImaGenes) and subcloned into the pCINeo HA IRES GFP vector using AgeI/EcoRI restriction sites. Wild-type PCBD1 was Flag-tagged at the NH_2-terminal by PCR using NheI/XhoI restriction sites. PCBD1 mutations were inserted in the construct using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. All constructs were verified by sequence analysis. Primer sequences used for cloning or mutagenesis PCR are reported in **Supplemental Table 2**. The *FXYD2* promoter construct was cloned into pGL3-Basic (Promega, Fitchburg, USA) as described previously.[7] In short, the human *FXYD2* promoter region that controls transcription of the γ-subunit isoform alpha (-3229/+91 bp from the transcription initiation site) was amplified using Phusion polymerase (Finnzymes, Vantaa, Finland) from genomic DNA, and cloned into pGL3-Basic using KpnI/BglII sites. The pRL-CMV vector encoding Renilla luciferase under control of a CMV promoter was commercially available (Promega, Fitchburg, USA).

Cell culture

Human Embryonic Kidney cells (HEK293) were grown in DMEM (Bio Whittaker-Europe, Verviers, Belgium) containing 10% (v/v) FCS (Thermo Fisher HyClone), 10 µl/ml nonessential amino acids and 2 mmol/L L-glutamine at 37°C in a humidity-controlled incubator with 5% (v/v) CO_2. The cells were transiently transfected with the respective constructs using polyethylenimine cationic polymer (PEI, Polysciences Inc.) at 1:6 DNA:PEI ratio for 48h unless otherwise stated.

Western blotting

HEK293 cells were transfected for 24h with HA-PCBD1 mutants and treated at the same time with 10 mmol/L MG-132 (Company). Protein lysates were denatured in Laemmli containing 100 mmol/L DTT for 30 min at 37 °C and subsequently subjected to SDS-PAGE. Then, immunoblots were incubated with a mouse anti-HA (Roche, high affinity 3F10, 1:5,000) primary antibody and peroxidase conjugated sheep anti-mouse secondary antibodies (Jackson Immunoresearch, 1:10,000).

Immunocytochemistry

HEK293 cells were seeded in 12-well plates on glass cover slips and co-transfected with 400 ng PCBD1 constructs and 400 ng HNF1B constructs or empty pCINeo IRES GFP vector (mock

DNA). After 48h, HEK293 cells were fixed with 4% (w/v) paraformaldehyde (PFA) for 30 min at 4°C, followed by the subsequent steps at room temperature: permeabilization for 15 min with 0.3% (v/v) Triton X-100 in PBS, incubation for 15 min with 50 mmol/L NH_4Cl, and finally incubation in blocking buffer (16% (v/v) goat serum, 0.3% (v/v) Triton X-100 and 0.3 M NaCl in PBS). After incubation overnight at 4°C with a rabbit anti-Flag M2 (Sigma F7425, 1:100) or a mouse anti-HA (Cell signaling technology, 6E2, 1:100), cells were washed three times with Tris-buffered NaCl Tween-20 (TNT; 150 mmol/L NaCl, 0.1 mol/L Tris/HCl, pH 7.5, 0.05% (v/v) Tween-20) and subsequently incubated with a secondary goat anti-rabbit antibody (Sigma A4914, 1:300) or a sheep anti-mouse (Jackson Immunoresearch, 1:300), coupled to AlexaFluor 594, for 45 min at room temperature. After incubation with DAPI for 30 min at room temperature, cells were washed three times with TNT and finally mount with Fluoromount-G (SouthernBiotech). Photographs were taken using a Zeiss Axio Imager 1 microscope (Oberkochen, Germany) equipped with a HXP120 Kubler Codix fluorescence lamp and a Zeiss Axiocam MRm digital camera. Images were analysed by use of the software ImageJ.[26]

Co-Immunoprecipitation

HEK293 cells were seeded on 55 mm petri dishes and co-transfected with 5 µg PCBD1 constructs and 5 µg of HNF1B constructs or a empty pCINeo IRES GFP vector (mock DNA). 48h after transfection, nuclear and cytosolic fractions were prepared using the NE-PER Nuclear Protein Extraction Kit (Pierce). The next incubation steps were all done under rotary agitation at 4 °C. 35 µL of protein A-agarose beads (Santa Cruz Biotechnology) were previously incubated overnight at 4 °C with 2.5 µg rabbit anti-Flag antibody (Sigma F7425) and washed with IPP500 (500 mmol/L NaCl, 10 mmol/L Tris adjusted to pH 8.0 with HCl, 0.1% (v/v) NP-40, 0.1% (v/v) Tween-20, 0.1% (w/v) BSA and protein inhibitors) four times. After sampling 60µl as input control, the remaining 180 µl of the lysate samples was added to the antibody-beads mixture overnight at 4 °C. Then, the beads were collected by centrifugation at 2000 rpm for 2 min at 4 °C and washed four times with lysis buffer. The proteins were separated from the beads by incubation for 30 min at 37°C in 1x Laemmli sample buffer supplemented with 100 mmol/L DTT and detected by immunoblotting using a mouse anti-HA (Roche, high affinity 3F10, 1:5,000) or a mouse anti-Flag M2 (Sigma F3165, 1:5,000) primary antibodies, and peroxidase conjugated sheep anti-mouse secondary antibodies (Jackson Immunoresearch, 1:10,000).

Luciferase reporter assay

HEK293 cells were seeded on 12-well plates and transfected with the following DNA amounts: 700 ng of the *FXYD2* promoter-luciferase construct, 50 ng PCBD1 constructs, 50 ng HNF1B constructs or empty pCINeo IRES GFP vector (mock DNA). To correct for transfection efficiency, 10 ng of pRL-CMV was used as a reference. Firefly and Renilla luciferase activities were measured by use of a Dual-Luciferase Reporter Assay (Promega, Fitchburg, USA) 48h after transfection.

Homology modelling

A homology model was built using the modelling script in the WHAT IF & YASARA Twinset with standard parameters.[27, 28] The structure of the PCBD1 dimer in complex with the HNF1A dimerization domain dimer was used as a template for modelling of HNF1B (PDB file: 1f93). Our model contains only the dimerization domain (31 amino acids) of HNF1B and this has 68% sequence identity with the dimerization domain of HNF1A.

Statistical analysis

All results are depicted as mean ± standard error of the mean (SEM). Statistical analyses were conducted by unpaired student's t-test when comparing two experimental conditions, and one-way ANOVA with Bonferroni test when comparing more conditions, P values less than 0.05 were considered significant.

Results

Homozygous PCBD1 mutations are associated with hypomagnesemia and renal Mg^{2+} wasting

We diagnosed hypomagnesemia and hypermagnesuria in two patients carrying mutations on both alleles in the *PCBD1* gene (**Table 1**). The patients were previously reported to suffer from transient neonatal hyperphenylalaninemia caused by a homozygous c.312C>T (p.Gln97Ter) mutation and two homozygous c.99G>T/283G>A (p.Glu26Ter/p.Arg87Gln) mutations, respectively.[20, 21] In patient 1, hypomagnesemia was corrected with oral Mg^{2+} supplements at a dose of 500 mg/day (0.64 mmol/L to 0.76 mmol/L, N 0.7-1.1 mmol/L), though at the expense of increased magnesuria (FEMg 4.6% to 7.8%, N<2%). Patient 1 suffered from Mg^{2+}-deficiency related symptoms like fatigue, muscular pain, weakness and cramps in arms, numbness, difficulty with memory, chest pains, and blurred vision. All symptoms improved after Mg^{2+} supplementation. An abdominal ultrasound in patient 1 showed slightly increased echogenicity of both liver and kidney of uncertain cause, but there was no evidence of renal cysts. Renal function resulted to be normal (GFR 128 mL/min per 1.73 m²). Serum and urinary values of other electrolytes and uric acid were within the normal range. Laboratory investigations of patient 2 revealed a relatively high 24h urinary Mg^{2+} excretion (5.25 mmol/24h, N 2-8; **Table 1**) in the presence of hypomagnesemia (0.65 mmol/L), with no secondary symptoms. Serum and 24h urinary Ca^{2+} excretion were within the normal range as well as creatinine clearance (126 mL/min, N 89-143).

Table 1 Pertinent laboratory investigations in patient 1 (BIODEF 272) and patient 2 (BIODEF 329).

Parameter	Patient 1 (BIODEF 272)		Patient 2 (BIODEF 329)	Reference range
	Baseline	On 500mg/day Mg^{2+}	Baseline	
Serum indices				
Na^+ (mmol/L)	141	-	138	135-145
K^+ (mmol/L)	4.0	-	4.5	3.5-5.0
Mg^{2+} (mmol/L)	0.64*	0.76	0.65*	0.7-1.1
Ca^{2+} (mmol/L)	2.41	2.55	2.25	2.20-2.65
Pi (mmol/L)	1.13	1.16	-	0.8-1.4
Creatinine (mmol/L)	0.069	0.066	0.062	0.045-0.110
HbA1c (%)	7.2*	-	4.95	4.3-6.1
Uric acid (μmol/L)	310	-	-	135-510
Urinary indices				
Mg^{2+}				
(mmol/L)	6.16	3.96	-	-
(mmol/24h)	-	-	5.25	2-8
FEMg (%)	4.6*	7.8*	-	<2
Ca^{2+}				
(mmol/L)	4.47	3.04	-	-
(mmol/24h)	-	-	5.61	<7.5
FECa (%)	0.9	1.8	-	>1
Pi (mmol/L)	40.29	13.61	-	-
FEPi (%)	17	17.6	-	5-20
Creatinine				
(mmol/L)	14.29	4.40	-	-
(mmol/24h)	-	-	14	9-18
GFR (mL/min per 1.73 m^2)	128	135	-	>60
CCr (mL/min)	-	-	126	89-143

HbA1c: glycosylated haemoglobin; FEMg: fractional excretion of Mg^{2+}; FECa: fractional excretion of Ca^{2+}; FEPi : fractional excretion of phosphate (Pi) ; GFR: glomerular filtration rate; CCr: creatinine clearance.

Maturity Onset Diabetes of the Young (MODY) in a patient with PCBD1 mutations

In addition to hypomagnesemia, patient 1 was diagnosed with diabetes. Type 1 autoimmune diabetes was excluded, since the patient lacked islet-cell antibodies and showed normal serum C-peptide levels (0.69 nmol/L, N 0.3-1.32 nmo/L). To test for MODY due to reduced function of HNF1A and/or HNF1B, patient 1 was placed on Gliclazide 80 mg/bid instead of insulin 20-30 units/day. MODY patients are generally highly sensitive to sulphonylureas.[29] Patient 1 responded well to the new therapy, not requiring insulin. Additional extrarenal manifestations in *HNF1B* disease include liver test abnormalities.[30, 31] Liver function tests in patient 1 revealed that the plasma levels of C-reactive protein (CRP) and high sensitivity C-reactive protein (hs-CRP) were significantly low at <2mg/L (N<9mg/L) and <0.1mg/L (N<8mg/L), respectively (**Table 2**). IgG was slightly low at 5.66 g/L (N 6.94-16.18 g/L), whereas the remaining liver tests were all within the normal range. Based on his glycosylated haemoglobin levels, we concluded that patient 2 did not develop diabetes (HbA1c 4.95%, N 4.3-6.1%; **Table 1**).

Table 2 Liver function tests in patient 1 (BIODEF 272).

Parameter	Patient 1 (BIODEF 272)	Reference range
Albumin (g/L)	41	38-50
Prealbumin (g/L)	0.381	0.1-0.4
CRP (mg/L)	<2*	<9
Hs-CRP (mg/L)	<0.1*	<8
α-1 antitrypsin (g/L)	1.4	0.9-2.6
C3 (g/L)	1.17	0.8-2.1
C4 (g/L)	0.18	0.15-0.5
IgG (g/L)	5.66*	6.94-16.18
IgM (g/L)	0.75	0.6-3.0
IgA (g/L)	1.99	0.7-4.0
ALT (U/L)	20*	21-72
AST (U/L)	26	15-46
ALP (U/L)	108	30-130

CRP: C reactive protein; hs-CRP: high-sensitivity C reactive protein; C3: complement component 3; C4: complement component 4; IgG: immunoglobulin G; IgM: immunoglobulin M; IgA: immunoglobulin A; ALT: alanine transaminase; AST: aspartate transaminase; ALP: alkaline phosphatase.

Pcbd1 expression in the DCT is modulated by dietary Mg²⁺ content

We examined Pcbd1 mRNA expression levels in a mouse tissue panel using real-time RT-PCR. Highest expression was measured in kidney and liver (**Figure 1A**). To evaluate whether the renal abundance of Pcbd1 locates to the site of active Mg^{2+} transport, DCT
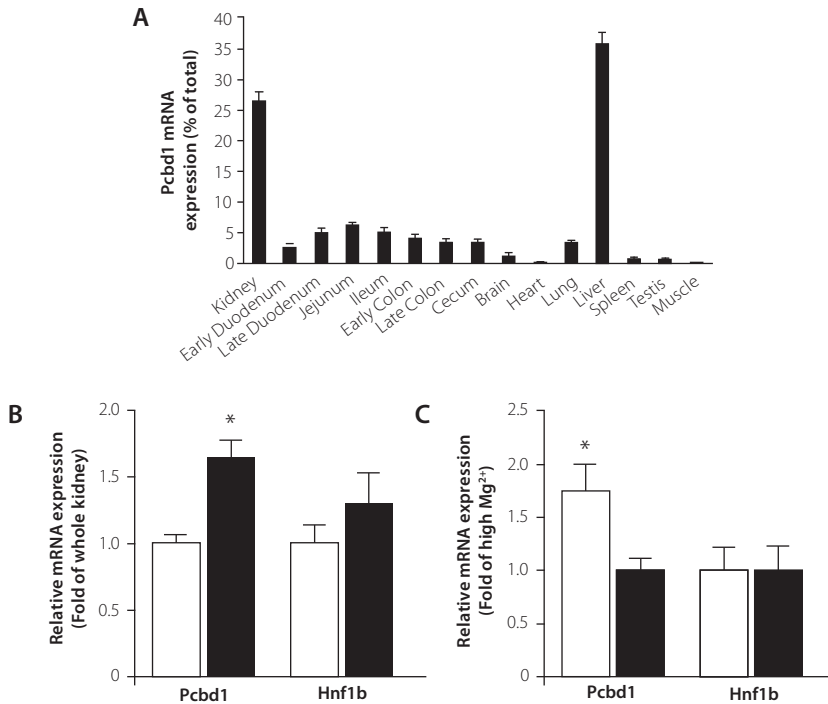

Figure 1 Pcbd1 is expressed in the DCT of the kidney.

(A) Tissue expression pattern of the Pcbd1 transcript. Pcbd1 mRNA expression level was measured in a panel of mouse tissues by quantitative RT-PCR and normalized for Gapdh expression. Data rep-resent the mean of 3 individual experiments ± SEM and are expressed as the percent-age of the total tissue expression. **(B)** Kidney expression pattern of Pcbd1 showed high-est expression in DCT. The mRNA expression levels of Pcbd1 and Hnf1b in COPAS-selected mouse DCT (black bars) and control (or non-selected; white bars) kidney tubules were measured by quantitative RT-PCR and normalized for *Gapdh* expression. Data represent the mean of 3 individual experiments ± SEM and are expressed as fold difference when compared with the expression in non-selected tubules. *, $p<0.05$ *versus* non-selected tubules. **(C)** DCT expression of Pcbd1 is regulated by dietary Mg^{2+} intake. The mRNA expression levels of Pcbd1 and Hnf1b in COPAS-selected mouse DCT kidney tubules form mice fed with low Mg^{2+}-containing diets (white bars) and high Mg^{2+}-containing diets (black bars) were measured by real-time RT-PCR and normalized for *Gapdh* expression. Data represent the mean of 4 individual experiments ± SEM and are expressed as fold difference when compared with the expression in high Mg^{2+}-containing diets. *, $p< 0.05$ *versus* high Mg^{2+}.

fragments were isolated from mice expressing enhanced green fluorescence protein (eGFP) after the parvalbumin promoter using a complex object parametric analyzer and sorter (COPAS). Pcbd1 expression was significantly higher in DCT in comparison with whole kidney (**Figure 1B**). Hnf1b transcript was not enriched in the DCT fraction. Furthermore, Pcbd1 expression in the DCT was significantly upregulated when mice were fed a low Mg^{2+}-containing diet compared to a high Mg^{2+}-containing diet (**Figure 1C**). Mice on a low Mg^{2+} diet displayed a significantly lower 24h Mg^{2+} excretion (**Figure 2B**) in response to the hypomagnesemia (1.1 mmol/L versus 2.2 mmol/L; Figure 2A). Of note: C57Bl/6 mice are known to have relatively high serum Mg^{2+} levels compared to humans (normal: 1.2-1.5 mmol/L).[32] Differences were not observed in the serum and urinary levels of phenylalanine between the experimental groups (**Figure 2C-D**).

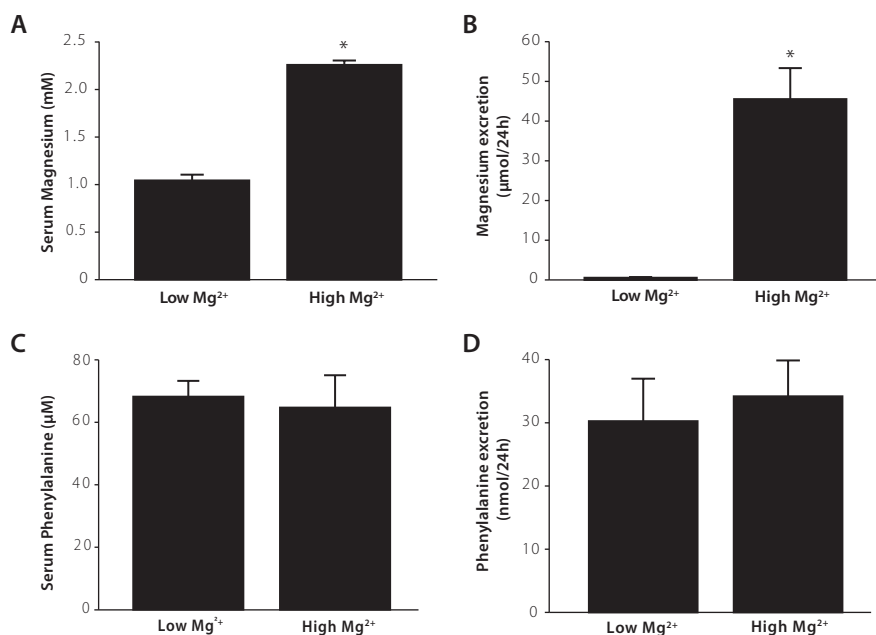**Figure 2** Effect of dietary Mg^{2+} on serum and urinary Mg^{2+} and phenylalanine levels.

C57Bl/6 mice were fed low (0.02% wt/wt) or high (0.48% wt/wt) Mg^{2+}-containing diets for 15 days. Before sacrifice, serum and 24h-urines samples were collected for Mg^{2+} **(A and B)** and phenylalanine **(C and D)** levels determination. Results are depicted as mean ± SEM. *, p< 0.05 versus low Mg^{2+} (n=10).

PCBD1 enhances FXYD2 promoter activation by HNF1B

In order to investigate whether the PCBD1 p.Gln97Ter and p.Glu26Ter/p.Arg87Gln mutations can lead to an impairment of the interaction with HNF1B, we generated a structural homology model of the PCBD1–HNF1B dimerization domain (HNF1B-D) complex using the structure of the PCBD1–HNF1A dimerization domain tetramer (**Figure 3A**). Analysis of the homology model indicates that the hydrogen bonds in the residue stretch from Asn44 to Glu58 in PCBD1 and from Leu5 to Val21 in HNF1B likely forms the protein-protein interaction domains (**Figure 3B**). The p.Glu26Ter mutation leads to a major protein truncation and complete loss of the interaction domain. Although p.Gln97Ter does not directly affect the HNF1-binding domain of PCBD1, the mutation results in a truncation of the central α-helix of the protein. Consequently, the interaction domain might be destabilized explaining the PCBD1 dysfunction.

Subsequently, to test the effects of the mutations on PCBD1 function, we studied all previously described patient mutations in their ability to co-activate *FXYD2* transcription in a luciferase assay (**Figure 3C**).[20-22] Renal Mg^{2+} transport has been suggested to depend on the transcriptional activation of the *FXYD2* gene promoter by HNF1B.[3, 7] HNF1B significantly enhanced the *FXYD2* promoter activity compared to mock-transfected cells

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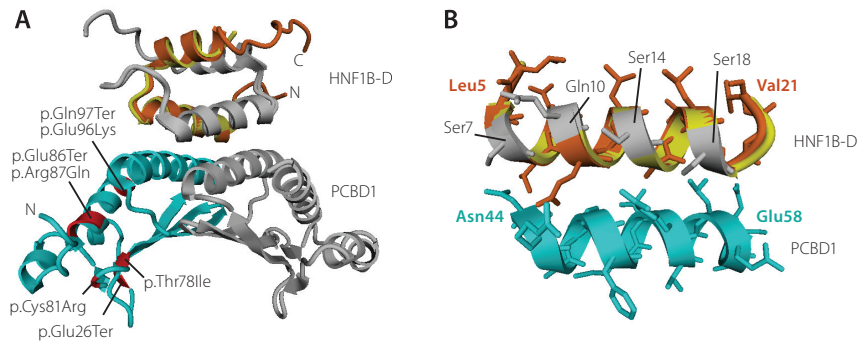
Figure 3 PCBD1 co-activates HNF1B-induced *FXYD2* promoter activity.

(A) Homology model of the PCBD1–HNF1B dimerization domain (HNF1B-D) tetramer, modelled using the structure of the PCBD1–HNF1A dimerization domain (HNF1A-D) complex (PDB file 1F93). The PCBD1 dimer (light blue and grey) binds the HNF1B dimer (orange and grey) via helix sequences. The HNF1A-D monomer is shown is yellow. Residues in the PCBD1 protein that were found mutated in patients affected by hyperphenylalaninemia are depicted in red. **(B)** Homology model of the interaction site within the PCBD1–HNF1B dimerization domain (HNF1-D) complex. The bound HNF1B monomer (orange) forms a helix bundle with PCBD1 monomer (light blue). The HNF1A-D monomer is shown in yellow. The residues that differ between HNF1B-D and HNF1A-D are visualized in grey.

Figure 3 Continued.

(C) Linear representation of the secondary structure elements of the human PCBD1 protein. Red arrowheads indicate the positions of the patient mutations described in literature. Green balls indicate the histidine residues involved in the dehydratase act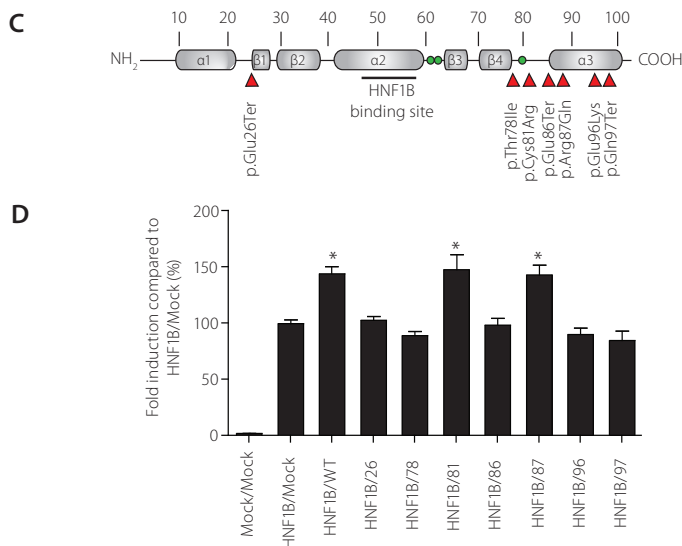ive site (His61, His62 and His79). **(D)** A luciferase assay was performed in HEK293 cells transiently co-transfected with a Firefly luciferase *FXYD2* promoter construct and HNF1B or mock DNA, in the presence of wild-type or mutant PCBD1. A Renilla luciferase construct was co-transfected to correct for transfection efficiency. Firefly/Renilla luciferase ratios were determined as a measure of promoter activity. Results are depicted as percentage compared to HNF1B/Mock transfected cells. *, $p < 0.05$ *versus* HNF1B/Mock (n=9). Mock: mock DNA; WT: wild-type; 26: p.Glu26Ter, 78: p.Thr78Ile, 81: p.Cys81Arg, 86: p.Glu86Ter, 87: p.Arg87Gln, 96: p.Glu96Lys, 97: p.Gln97Ter.

(**Figure 3D**). Interestingly, co-expression of wild-type PCBD1 further increased *FXYD2* promoter activation by HNF1B by ~1.5 fold (**Figure 3D**). However, among all PCBD1 mutants, only PCBD1 p.Arg87Gln and p.Cys81Arg maintained their co-activator activity (**Figure 3D**), showing that the p.Gln97Ter and the p.Glu26Ter mutation have lost their ability to stimulate HNF1B-induced transcription.

Mutations detected in HPABH4D patients cause protein degradation of PCBD1

To explain why the PCBD1 mutants are not capable of enhancing HNF1B-induced transcription, we examined their subcellular localization and their capacity to bind HNF1B. Immunostaining for PCBD1 in transiently transfected HEK293 cells revealed that wild-type PCBD1 translocates to the nucleus upon co-expression with HNF1B compared to mock DNA (**Figure 4A**).
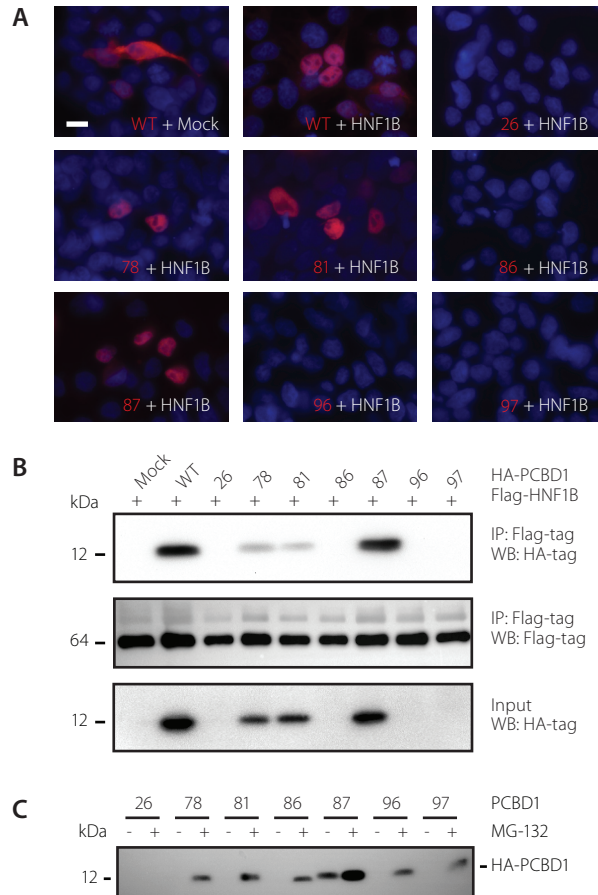
Figure 4 Several PCBD1 mutations lead to protein degradation in HEK293 cells.

(A) Immunocytochemistry analysis of the subcellular localization of HA-tagged PCBD1 wild-type or HA-tagged PCBD1 mutants when co-expressed in a 1:1 ratio with Flag-tagged HNF1B or mock DNA in HEK293 cells. Red signal represents immunodetected HA-epitopes. Nuclei stained with DAPI are shown in blue. The bar represents 10 μm in each panel. Representative immunocytochemical images are shown. **(B)** HA-tagged PCBD1 wild-type, PCBD1 mutants or mock DNA were transiently expressed in HEK293 cells with or without Flag-tagged HNF1B. Immunoprecipitations on nuclear extracts using an anti-Flag antibody were separated by SDS-PAGE, and western blots were probed with anti-HA (upper panel) or anti-Flag antibodies (middle panel). HA-PCBD1 input (25%) expression was also included in the analysis (lower panel). The immunoblots shown are representative for 3 independent experiments. **(C)** Western blot analysis of HA-tagged PCBD1 mutants expressed in HEK293 cells, treated with (+) or without (-) 10 nM MG-132 for 24h. A representative immunoblot is shown. Mock: mock DNA; WT: wild-type; 26: p.Glu26Ter, 78: p.Thr78Ile, 81: p.Cys81Arg, 86: p.Glu86Ter, 87: p.Arg87Gln, 96: p.Glu96Lys, 97: p.Gln97Ter.

Furthermore, PCBD1 p.Glu26Ter, p.Glu86Ter, p.Glu96Lys and p.Gln97Ter were not expressed, whereas p.Thr78Ile and p.Cys81Arg were detected significantly less than the wild-type protein. PCBD1 p.Arg87Gln was the only mutant showing a comparable expression level to wild-type PCBD1 (**Figure 4A**). In accordance with the PCBD1 expression levels observed in immunocytochemistry, co-immunoprecipitation studies confirmed that only PCBD1 p.Arg87Gln, p.Thr78Ile and p.Cys81Arg were expressed and able to bind to HNF1B (**Figure 4B**, upper and lower panel). Importantly HNF1B was equally expressed in all conditions (**Figure 4B**, middle panel). To examine whether mutated PCBD1 proteins are degraded by the proteasomal pathway for misfolded proteins, PCBD1-expressing HEK293 cells were treated for 24h with 10 nM of the proteasome inhibitor MG-132. Protein expression was restored for all of the PCBD1 mutants, with the only exception of p.Glu26Ter (**Figure 4C**).

HNF1B mutations affect the subcellular localization of PCBD1

We evaluated five HNF1B mutations (p.Lys156Glu, p.Gln253Pro, p.Arg276Gly, p. His324Ser325fsdelCA, p.Tyr352fsinsA) for their ability to bind and functionally respond to PCBD1 (**Figure 5A**).[3,4] All HNF1B mutants, except HNF1B Δ2-30, bound PCBD1 in co-immunoprecipitation studies in transiently transfected HEK293 cells (**Figure 5B**, upper panel). PCBD1 and HNF1B were expressed in all conditions tested (**Figure 5B**, middle and lower panel). *FXYD2* promoter-luciferase assays showed that only HNF1B p.His324Ser325fsdelCA and p.Tyr352fsinsA, that retained partial transcriptional activity, respond to the co-activation by PCBD1 to the same extent as HNF1B wild-type (~1.5 fold; **Figure 5C**). Importantly, immunocytochemical analysis revealed that co-expression of PCBD1 with the HNF1B mutants p.Gln253Pro and p.His324Ser325fsdelCA causes a predominant cytosolic localization of PCBD1 compared to the nuclear translocation observed upon co-expression with HNF1B wild-type (**Figure 5D-E**).

Discussion

Mutations in *PCBD1* have been shown to cause a transient and benign form of neonatal hyperphenylalaninemia.[20-22] Here, we present the first follow-up study of HPABH4D patients reporting the onset of late complications linked to the defected activity of PCBD1 as transcriptional co-activator of HNF1B. Our results suggest that PCBD1 acts as an important transcriptional regulator of FXYD2 contributing to renal Mg^{2+} reabsorption in DCT. Our observations are based on the following results: *i*) hypomagnesemia with renal Mg^{2+s} wasting was reported in two patients carrying homozygous mutations in the *PCBD1* gene; *ii*) one out of two patients was diagnosed with MODY; *iii*) *in vitro* data demonstrated that PCBD1 binds
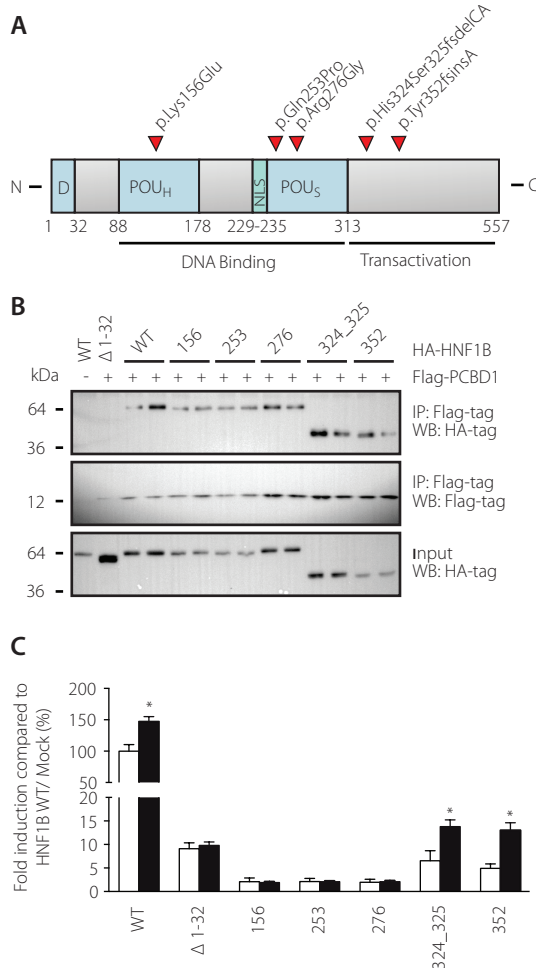
Figure 5 Effect of HNF1B mutations on PCBD1 binding, transcription co-activation and subcellular localization.

(A) Linear representation of the human HNF1B protein. Red arrowheads indicate patient mutations that were tested in this study. D: dimerization domain; POU$_H$: atypical POU homeodomain; POU$_S$: POU specific domain; NLS: nuclear localization signal. **(B)** Immunoprecipitations on nuclear extracts using an anti-Flag antibody were separated by SDS-PAGE, and western blots were probed with anti-HA (upper panel) or anti-Flag antibodies (middle panel). HA-HNF1B input (25%) expression was also included in the analysis (lower panel). The immunoblots shown are representative for 3 independent experiments. **(C)** A luciferase assay was performed in HEK293 cells transiently co-transfected with a Firefly luciferase *FXYD2* promoter construct and each of the HNF1B variants, with (black bars) or without (white bars) PCBD1. Results are depicted as percentage compared to HNF1B/Mock transfected cells. *, p< 0.05 compared to the HNF1B/Mock condition (n=9).
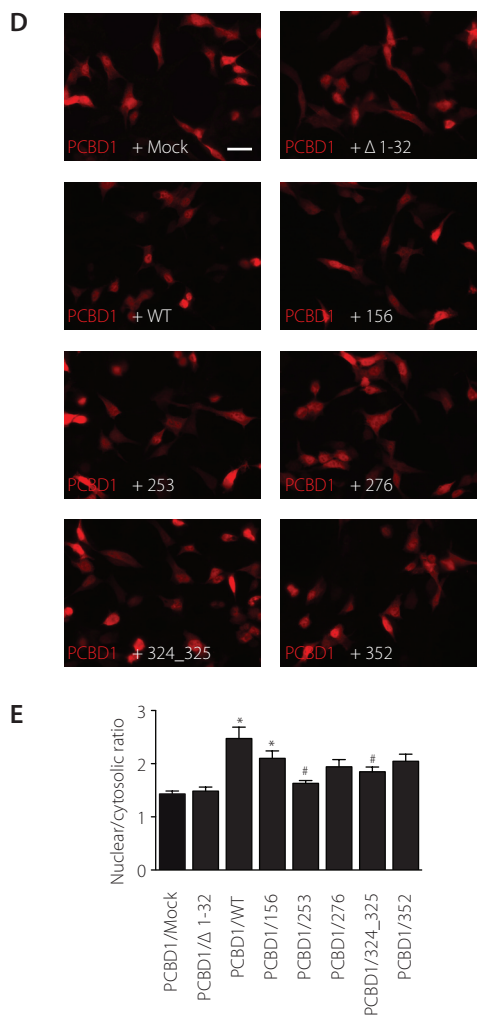
Figure 5 Continued.

(D) Immunocytochemistry analysis of the subcellular localization of Flag-tagged PCBD1 when co-expressed in a 1:1 ratio with HA-tagged HNF1B constructs or mock DNA in HEK293 cells. Red signal represents immunodetected Flag-epitopes. The bar represents 20 μm in each panel. The immunocytochemical images shown are representative for 3 independent experiments. **(E)** Quantification of the nuclear versus cytosolic localization of PCBD1 when co-expressed in a 1:1 ratio with mock DNA (n=24) or HNF1B Δ 1-32 (n=33), WT (n=41), 156 (n=38), 253 (n=35), 276 (n=33), 324_325 (n=37); 352 (n=36). *, p< 0.05 compared to mock; #, p<0.05 compared to WT. Mock: mock DNA; WT: wild-type; Δ 1-32: HNF1B lacking the dimerization domain; 156: p.Lys156Glu; 253: p.Gln253Pro; 276: p.Arg276Gly; 324_325: p.His324Ser325fsdelCA; 352: p.Tyr352fsinsA.

HNF1B to co-stimulate the *FXYD2*-promoter, whose activity contributes to Mg^{2+} reabsorption in DCT; *iv*) in the kidney, PCBD1 is highly expressed in DCT and sensitive to dietary Mg^{2+} content; *iv*) PCBD1 mutations reported in HPABH4D patients caused proteolytic instability leading to degradation via the proteasomal pathway.

To our knowledge, we are the first to report that *PCBD1* mutations associate with hypomagnesemia, renal Mg^{2+} wasting and MODY. Laboratory investigations of both patients revealed a defect in renal Mg^{2+} reabsorption, while serum Ca^{2+} levels and urinary Ca^{2+} excretion were not affected. In kidney, the key sites for Mg^{2+} reabsorption are the thick ascending limb of Henle's loop (TAL) and DCT. While defects in the molecular pathway for Mg^{2+} handling in TAL lead to a concomitant waste of Ca^{2+}, shortcomings in DCT cause hypermagnesuria associated with hypo- or normocalciuria. Thus, it is likely that in our patients the DCT is primarily affected. Fluorescence-based sorting of DCT-eGFP tubules using a COPAS apparatus coupled to real-time PCR analysis confirmed an enrichment of Pcbd1 expression in DCT tubules compared to other nephron segments. This is in line with a previous immunohistochemical study showing Pcbd1 expression in the cortex and outer medulla of the kidney.[33] Interestingly, our results evidence that Pcbd1 expression is increased in mice fed with a low Mg^{2+} diet, whereas *Hnf1b* levels remained stable. This suggests that Pcbd1 expression levels are an important regulating factor of Hnf1b-mediated transcription in DCT. We excluded that Pcbd1 expression is modulated by a change in phenylalanine metabolism, since serum and urinary phenylalanine levels were stable in our mice. Our results demonstrated that PCBD1 enhances the *FXYD2* promoter activation by HNF1B *in vitro*. The relevance of *FXYD2* activity in Mg^{2+} handling in DCT has been suggested previously, since both patients with *FXYD2* mutations and patients carrying *HNF1B* mutations may present with hypomagnesemia.[3, 6] The *FXYD2* gene encodes the γ-subunit of the Na^+-K^+-ATPase. To date, the exact molecular mechanism by which the γ-subunit regulates Mg^{2+} handling in DCT remains elusive.[5] Further evidence for an impaired HNF1-mediated transcription in HPABH4D patients was provided by the diagnosis of MODY in patient 1. MODY is a monogenic form of autosomal dominant type II diabetes characterized by age of onset often below 25 years and negative pancreatic autoantibodies. Heterozygous mutations in HNF1B or its homolog HNF1A associate with MODY type 5 and type 3, respectively.[34] While MODY5 is mainly linked to pancreas hypoplasia secondary to agenesis,[35] MODY3 shows an abnormal insulin secretion due to impaired growth and function of pancreatic β cells. Knowing that PCBD1 acts as transcriptional co-activator of both HNF1B and HNF1A, a dysregulation of HNF1B and/or HNF1A is potentially responsible for the MODY diagnosed in patient 1. Interestingly, our finding of low plasma hs-CRP levels in patient 1 is in line with two recent studies showing that subjects with MODY3 maintain substantially lower levels of hs-CRP than individuals with other forms of diabetes, including MODY5, or nondiabetic control subjects.[36, 37] This evidence favors the diagnosis of MODY3-like diabetes in patient 1. Patient 2 was not diagnosed with diabetes, but he will be monitored for later onset.

In this study, we showed that the HPABH4D patient mutations reported in literature lead to protein degradation of PCBD1 via the proteasome pathway. Proteasomal degradation of misfolded proteins occurs often within minutes of protein synthesis, explaining why many of the PCBD1 mutants are not expressed in our experiments.[38] Our data together with previous studies on proteolytic instability for the same mutants in both mammalian and bacterial expression systems support the hypothesis that this degradation process may also occur in HPABH4D patients.[20, 21, 39] Considering the inheritance of the disease, HNF1-mediated transcription seems to be sensitive to changes in PCBD1 quantity only when both *PCBD1* alleles are affected, suggesting that PCBD1 probably belongs to an ancillary regulatory mechanism to which other HNF1B partners may participate.[40] *In vitro* data in HEK293 cells revealed that PCBD1 p.Arg87Gln was the only mutant showing both a functional activity and expression level comparable to the wild-type protein. Nevertheless, in patient 2 p.Arg87Gln is homozygously present on both alleles with a p.Glu26Ter mutation.[21] Thus, this condition most probably mimics a homozygous p.Glu26Ter mutation leading to a significant decrease in the cellular content of PCBD1. PCBD1 p.Glu26Ter degradation could not be rescued by proteasome inhibition, suggesting that its transcript is degraded by mRNA surveillance mechanisms.[41, 42] PCBD1 p.Thr78Ile and p.Cys81Arg were significantly less expressed than the wild-type protein. Both mutants showed binding to HNF1B, but only PCBD1 p.Cys81Arg co-activated the *FXYD2* promoter. In the near future, it would be of interest to screen patients with PCBD1 p.Thr78Ile and p.Cys81Arg mutations for complications related to the HNF1B disease.

PCBD1 monomers are small molecules that can passively diffuse from the cytosol into the nucleus where they form heterotetramers with the nuclear HNF1 transcription factors.[43] It was suggested that, by assembling via the same interface, PCBD1 homotetramer and PCBD1–HNF1 complexes are mutually exclusive.[18] Furthermore, the high stability of the PCBD1 homotetramer in the cytosol may be essential to compete with the formation of the nuclear PCBD1–HNF1 complex or to serve as a cytosolic reserve for transcriptionally inactive PCBD1.[18, 44] In our immunocytochemical analysis, the HNF1B mutants p.Gln253Pro and p.His324Ser325fsdelCA significantly stimulated a cytosolic localization of PCBD1 compared to the nuclear PCBD1 localization observed in the presence of wild-type HNF1B. This suggests that HNF1B mutations may disturb the stability of the PCBD1–HNF1 complexes in the nucleus and therefore favour the formation of PCBD1 homotetramers in the cytosol of the cell.[3, 4] Although PCBD1 is still capable of interacting with HNF1B mutants, the reduced nuclear localization of PCBD1 will indirectly result in a decreased co-activation of HNF1B. Thus, an increased cytosolic localization of PCBD1 could contribute to hypomagnesemia in HNF1B patients. Since the presentation and development of HNF1B disease is diverse, variations in HNF1B interacting proteins may be responsible for the phenotypic heterogeneity. Screening of HNF1B patients for polymorphisms in *PCBD1* should, therefore, be considered.

In conclusion, we identified PCBD1 as a new molecular player in HNF1B nephropathy. Our results suggest that PCBD1 regulates the HNF1B-mediated *FXYD2* transcription, influencing active renal Mg^{2+} reabsorption in DCT. So far, HPABH4D due to *PCBD1* mutations has been considered a transient, benign condition, primarily related to impaired BH_4 regeneration. To date, 23 patients with *PCBD1* mutations linked to HPABH4D are listed in the International Database of Tetrahydrobiopterin Deficiencies (BIODEF database, Opladen T, Blau N., http://www.biopku.org/biodef/).[23] Here, we suggest that patients affected by HPABH4D should be monitored for late complications related to the interactions with HNF1 transcription factors, including hypomagnesemia and MODY.

Acknowledgements

4

Supplemental data

Supplemental Table 1 Primer sequences used for real-time PCR analysis.

Gene product	Forward (5'-3')	Reverse (5'-3')
Pcbd1	TGGACATGGCCGGCAAGGC	CCCACAGCCCTCAGGTTTG
Hnf1b	CAAGATGTCAGGAGTGCGCTAC	CTGGTCACCATGGCACTGTTAC
Gapdh	TAACATCAAATGGGGTGAGG	GGTTCACACCCATCACAAAC

Pcbd1: pterin-4 alpha-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha; *Hnf1b*: hepatocyte nuclear factor b; *Gapdh*: glyceraldehyde 3-phosphate dehydrogenase.

Supplemental Table 2 Primer sequences used for cloning or mutagenesis PCR.

Gene product		Primer sequence (5'-3')
PCBD1		
HA- wild-type	F	CGACCGGTGGCTGGCAAGCACACAGG
	R	CGCTCGAGCTATGTCATGGACACTGCTAC
Flag-wild-type	F	GCGCTAGCGCCACCATGGACTACAAGGATGACAAGGCTGGCAAAGCACACAGGCTG
	R	CGCTCGAGCTATGTCATGGACACTGCTAC
HA-Glu26Ter	F	GCTGTGGGGTGGAATTAGCTGGAAGGCCGTGAT
	R	ATCACGGCCTTCCAGCTAATTCCACCCCACAGC
HA-Thr78Ile	F	CACATCACGCTGAGCATCCATGAGTGTGCCGGC
	R	GCCGGCACACTCATGGATGCTCAGCGTGATGTG
HA-Cys81Arg	F	AGCACCCATGAGCGTGCCGGCCTTTCA
	R	TGAAAGGCCGGCACGCTCATGGGTGCT
HA-Glu86Ter	F	TGTGCCGGCCTTTCATAACGGGACATAAACCTG
	R	CAGGTTTATGTCCCGTTATGAAAGGCCGGCACA
HA-Arg87Gln	F	GCCGGCCTTTCAGAACAGGACATAAACCTGGCC
	R	GGCCAGGTTTATGTCCTGTTCTGAAAGGCCGGC
HA-Glu96Lys	F	CTGGCCAGCTTCATCAAACAAGTAGCAGTGTCC
	R	GGACACTGCTACTTGTTTGATGAAGCTGGCCAG
HA-Gln97Ter	F	GCCAGCTTCATCGAATAAGTAGCAGTGTCCATG
	R	CATGGACACTGCTACTTATTCGATGAAGCTGGC

Supplemental Table 2 Continued.

Gene product		Primer sequence (5'-3')
HNF1B		
Flag-wild-type	F	CGGCTAGCCCACCATGGACTACAAGGATGACGATGACAAGTGGC GCGCCGTGTCCAAG
	R	GGCCCCCATTTGAACCGGTCG
HA-wild-type	F	GGCGCGCCATGGTGTCCAAGCTCACGTCGC
	R	TCTAGATCACCAGGCTTGTAGAGGACAC
HA-Δ2-30	F	CGGGCGCGCCGAGGAGTTGCTGCCATC
	R	GGCCCCCATTTGAACCGGTCG
HA-Lys156Glu	F	CCCAGCATCTCAACGAGGGCACCCCTATG
	R	CATAGGGGTGCCCTCGTTGAGATGCTGGG
HA-Gln253Pro	F	CAGGCCTACGATCGGCCAAAGAACCCCAGCAAG
	R	CTTGCTGGGGTTCTTTGGCCGATCGTAGGCCTG
HA-Arg276Gly	F	GCAGAATGTTTGCAGGGAGGGGTGTCCCCCTC
	R	GAGGGGGACACCCCTCcCTGCAAACATTCTGC
HA-His324Ser325fsdelCA	F	CTCCAACCAGACTCAGCCTGAACCCTCTGC
	R	GCAGAGGGTTCAGGCTGAGTCTGGTTGGAG
HA-Tyr352fsinsA	F	GTCAGGAGTGCGCTAACAGCCAGCAGGGAAAC
	R	GTTTCCCTGCTGGCTGTTAGCGCACTCCTGAC

PCBD1: pterin-4 alpha-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha; HNF1B: hepatocyte nuclear factor B.

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Vitamin D downregulates TRPC6 expression in podocyte injury and proteinuric glomerular disease

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Abstract

The cation channel transient receptor potential channel C6 (TRPC6) is a slit diaphragm protein expressed by podocytes. *TRPC6* gain-of-function mutations cause autosomal dominant focal segmental glomerulosclerosis. In acquired proteinuric renal disease, glomerular TRPC6 expression is increased. We previously demonstrated that acquired increased TRPC6 expression is ameliorated by anti-proteinuric angiotensin receptor blockers and angiotensin converting enzyme inhibitors. Vitamin D also has an anti-proteinuric effect. We hypothesized that vitamin D reduces proteinuria by affecting TRPC6 expression in podocytes.

Adriamycin-induced nephropathy increased TRPC6 mRNA and glomerular TRPC6 protein expression, and induced proteinuria in rats. Treatment with 1α,25-dihydroxy-vitamin D_3 (1,25-D_3) normalized TRPC6 expression and reduced proteinuria. *In vitro*, podocyte injury was induced by adriamycin exposure in cultured mouse podocytes, which increased TRPC6 expression. Treatment of injured podocytes with 1,25-D_3 resulted in a dose-dependent reduction of adriamycin-induced TRPC6 expression. Of note, chromatin immunoprecipitation analysis demonstrated that the vitamin D receptor directly binds to the *TRPC6* promoter, and 1,25-D_3 reduced *TRPC6* promoter activity in a luciferase reporter assay. In 1,25-D_3-deficient 25-hydroxy-1α-hydroxylase knockout mice, TRPC6 mRNA and glomerular TRPC6 protein expression were increased, accompanied by podocyte foot process effacement and proteinuria. Importantly, 1,25-D_3 supplementation normalized TRPC6 expression, podocyte morphology and proteinuria in these mice.

We demonstrated that vitamin D downregulates the enhanced TRPC6 expression in *in vivo* and *in vitro* podocyte injury, possibly through a direct effect on *TRPC6* promoter activity. Thus, our results suggest that this TRPC6 downregulation could contribute to the anti-proteinuric effect of vitamin D.

Introduction

Podocytes play a crucial role in the glomerular filtration barrier. Podocyte foot processes are connected by specialized proteins, such as nephrin and neph1, to form the glomerular slit diaphragm complex. This structure is closely connected to the actin skeleton through proteins like podocin and CD2AP, and plays an important role in cell signaling in podocytes.[1-5] Injury to the podocyte and its slit diaphragm can lead to proteinuria and eventually chronic renal function decline.[2] Various studies suggest that podocyte injury plays a crucial initiating role in several hereditary and acquired proteinuric diseases, including focal segmental glomerulosclerosis (FSGS) and diabetic nephropathy.[6-9]

Transient receptor potential cation channel, subfamily C, member 6 (TRPC6) is a calcium-conducting ion channel, which is expressed in podocytes and suggested to function as a slit diaphragm-associated protein.[10] TRPC6 is thought to play a role in signaling processes at the slit diaphragm complex which, in turn, might influence the cytoskeleton of the podocyte.[10, 11] *TRPC6* gain-of-function mutations are associated with a hereditary form of FSGS.[10, 12] Moreover, in several acquired proteinuric diseases, an increased glomerular TRPC6 expression was demonstrated. Therefore, it appears that TRPC6 plays a role in the pathogenesis of podocyte injury in both hereditary and acquired proteinuric diseases.[13] Previously, we described a correlation between TRPC6 expression and severity of FGS score in an animal model for acquired FSGS.[14] Furthermore, we demonstrated that angiotensin II activates TRPC6 and increases TRPC6 expression via a nuclear factor of activated T-cells (NFAT)-mediated positive feedback signaling pathway, which contributes to podocyte injury. Moreover, we demonstrated that angiotensin converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARBs), pivotal therapies to reduce proteinuria, decrease TRPC6 expression in injured podocytes as well as in animal models for proteinuric disease. Thus, enhanced TRPC6 activity and/or expression appears to mediate podocyte and glomerular injury, whereas decreasing TRPC6 expression is associated with reduced injury and amelioration of proteinuria.

In addition to ACEi and ARBs, new anti-proteinuric therapies have recently emerged, including treatment with vitamin D analogs.[15] Cholecalciferol (vitamin-D_3) is taken up in the gastrointestinal tract or synthesized by the effect of sunlight in the skin, after which it can be converted by the liver into 25-hydroxyvitamin D_3 and, subsequently, to 1,25-dihydroxyvitamin D_3 (1,25-D_3) in the proximal tubule of the kidney. 1,25-D_3 is classically defined as the active form of vitamin D, which plays a central role in Ca^{2+} and PO_4^{3-} metabolism. With the progression of renal insufficiency, deficiency of 1,25-D_3 ensues.[16] However, 1,25-D_3 deficiency may not only be a consequence of, but recent studies indicated that 1,25-D_3 deficiency itself could also be the cause of renal injury. Clinical and pre-clinical studies demonstrated that treatment with vitamin D analogs reduces proteinuria and podocyte loss.[17-22] It was demonstrated that podocytes express the vitamin D receptor (VDR) and undergo ultrastructural changes when exposed to 1,25-D_3.[23, 24] More recently,

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it was shown that 1,25-D_3 regulates the expression of several key podocyte proteins, like nephrin and podocin.[25, 26] Other TRP channel family members, such as TRPV5 and TRPV6, are also regulated by 1,25-D_3.[27, 28]

Taken together, this suggests a role for 1,25-D_3 in the regulation of TRPC6 expression in podocyte injury and proteinuric disease. Therefore, we investigated whether vitamin D regulates TRPC6 expression in cultured podocytes as well as in animal models for FSGS or 1,25-D_3 deficiency.

Materials and Methods

Adriamycin nephropathy (AN) rats
The AN model for human FSGS was induced in 8 week old Wistar rats (Charles River, Wilmington, USA) by a single tail vein injection with 5 mg/kg body weight adriamycin (Sigma-Aldrich, St. Louis, USA). Hereafter, rats were treated with daily intraperitoneal injections of 2.5 μg/kg bodyweight 1,25-D_3 or vehicle for 6 weeks. At the end of the experiment, rats were housed in metabolic cages to collect 24 hour urine samples. Subsequently, animals were sacrificed, and kidneys and blood samples were collected. All animals were kept at the Central Animal Facility of the Radboud University Nijmegen in a standard room at a temperature of 21°C and controlled humidity. Animals were exposed to a 12 hours light/dark cycle with *ad libitum* access to food and water. All procedures involving animals were approved by the Animal Ethics Committee of the Radboud University Nijmegen, The Netherlands, in accordance with the guidelines of the Dutch Council for Animal Care and the European Communities Council Directive (86/609/EEC).

Podocyte cell culture
Conditionally immortalized mouse podocytes (MPC-5) were cultured at 33°C/5% CO_2 and differentiated at 37°C in Roswell Park Memorial Institute (RPMI) Dutch modified medium (Invitrogen, Carlsbad, USA) supplemented with 10% v/v fetal calf serum (FCS), 1% w/v glutamine, 10 units/ml interferon-gamma and 1% penicillin/streptomycin as described previously.[29] Depending on the exact experimental set-up, differentiated podocytes were treated with 0.25 μg/ml adriamycin and 100 nM 1,25-D_3 or vehicle for 24 hours (Sigma-Aldrich, St. Louis, USA). In these experiments, N=4-5 separate podocyte cultures were used per experimental condition per experiment, and all experiments were repeated at least twice for confirmatory purposes.

Construction of TRPC6 promoter luciferase reporter construct and luciferase activity assay
The 5'-promoter region of the mouse *TRPC6* gene (–1500/+32; +1 designates the transcription start site, NM_013838.2) was obtained by amplification of genomic DNA using primers

5'-GACGCTCGAGTGTTGCTTCTGCAGCCCGAGTG-3' and 5'-GATCAAGCTTAGC CGCGAAAG-GAACCTTGACC-3'. The PCR product was cloned into the pGL3-Basic luciferase reporter vector and the cloned promoter sequence was verified by sequence analysis. The pRL-CMV vector encoding Renilla luciferase under control of a CMV promoter was used as control for transfection efficiency (Promega, Fitchburg, USA).

Opossum kidney (OK) cells were cultured in DMEM/F12 (1:1) medium supplemented with 10% FCS, 15 mM Hepes, 2.5 mM L-glutamine and 1% penicillin/streptomycin at 37°C in a humidity-controlled incubator with 5% (v/v) CO_2.[30] Briefly, cells were seeded in a 12-well plate and transfected the following day in serum free medium. The transfection mixture was prepared in 60 µl Optimem (Invitrogen, Carlsbad, USA) and consisted of 1 µg of either *TRPC6* promoter construct or empty pGL3-basic vector, 50 ng of pRL-CMV and 1.25 µl Lipofectamin2000 (Invitrogen, Carlsbad, USA). Four hours after transfection, cells were washed with PBS and incubated with culture medium containing 1% FCS, in the presence of 100 nM 1,25-D_3 or vehicle. Cells were harvested 48 hours post-transfection and luciferase activity was determined using a dual luciferase reporter assay system (Promega, Fitchburg, USA). In these experiments, N=4-5 separate cultures were used per experimental condition per experiment, and all experiments were repeated at least twice for confirmatory purposes.

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed using OK cells transfected with the *TRPC6* promoter luciferase reporter construct or the empty pGL3-basic vector without promoter. Cells were treated with 100 nM 1,25-D_3 as described above. Cells were harvested 4 hours after transfection and a Magna ChIP A assay was performed according to the manufacturer's protocol (Merck Millipore, Billerica, USA). Briefly, proteins were cross-linked to the DNA with formaldehyde, subsequently cells were lysed and samples were sonicated two times for 30 seconds on ice at 22µm amplitude using a Soniprep 150 (MSE, London, UK). Samples were incubated with 5.0 µg of rabbit polyclonal anti-vitamin D receptor antibody (ab3508, Abcam, Cambridge, USA) or with rabbit IgG isotype antibodies as control. Immunopre-cipitates were enriched with protein A magnetic beads. Subsequently chromatin complexes were eluted, the cross-links reversed, and the DNA was isolated. The presence of *TRPC6* promoter DNA was evaluated in real-time PCR with specific primers corresponding to the *TRPC6* promoter region (5'-CTCAACGCATGTCCCCATAC-3' and 5'-GTAACACCAAG-GGAGGGC-3'). Subsequently, samples were loaded on a 2% agarose gel and visualized using Proxima C16 (Isogen, De Meern, the Netherlands).

1,25-D_3-deficient 25-hydroxy-1α-hydroxylase KO mice

The 1,25-D_3-deficient 25-hydroxy-1α-hydroxylase KO mice were previously generated by targeted ablation of exon 8 encoding the heme binding domain of the enzyme. Mice were genotyped using PCR and Southern blot analysis, as described previously.[31]

Five-week-old WT and KO mice were given daily intraperitoneal injections with 500 pg 1,25-D$_3$ (Sigma-Aldrich, St. Louis, USA) or vehicle for 6 weeks. At the end of the experiment mice were housed in metabolic cages to collect 24 hour urine samples. Subsequently, animals were sacrificed, kidneys and blood samples were collected.

Analytical procedures

Urinary albumin and creatinine levels were determined by radial-immunodiffusion and enzymatic colorimetry, respectively. Serum Ca^{2+} levels were measured by spectro-photometry.[32]

Real-time PCR analysis

RNA was isolated from cultured podocytes or kidney cortex and reverse transcribed (Transcriptor Kit, Roche Diagnostics, Mannheim, Germany). Real-time quantitative PCR was performed using SYBR Green Supermix (Roche) on a MyiQ Real-Time PCR detection system (Bio-Rad Laboratories, CA, USA) as described previously.[14] TRPC6 expression was quantified by the delta-delta cycle threshold (Ct) method using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. In these experiments, N=4-5 separate cultures were used per experimental condition per experiment, and all experiments were repeated at least twice for confirmatory purposes.

Immunohistochemistry

Glomerular expression of TRPC6 and desmin was determined by semi-quantitative scoring of immunofluorescence staining in 2 μm cryosections as described previously.[14] In rat kidneys, TRPC6 was probed using a rabbit polyclonal antibody against the C-terminal tail of rat TRPC6 (Abcam, Cambridge, USA). TRPC6 expression in mice kidneys was detected by a rabbit polyclonal antibody against the N-terminal tail of mouse TRPC6 (Alomone, Jerusalem, Israel). Desmin expression was detected using a goat polyclonal antibody against the C-terminus of mouse and rat desmin (Santa Cruz, Santa Cruz, USA). Alexa-conjugated secondary antibodies were used subsequently. Glomerular TRPC6 and desmin expression was scored semi-quantitatively on a scale from 0 to 5 based on the extent of TRPC6 immunofluorescence staining and 0 to 10 for desmin staining in the glomerulus as described previously.[14] Scoring was performed independently by two investigators, who scored 35 to 50 glomeruli per animal on blinded sections.

Transmission Electron Microscopy

For electron microscopy, we used immersion fixation. Small fragments of cortex were fixed in 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4°C and washed in the same buffer. The tissue fragments were postfixed in palade-buffered 2% OsO$_4$ for 1h, dehydrated, and embedded in Epon812, Luft's procedure (Merck, Darmstadt, Germany). Ultrathin sections were contrasted with 4% uranyl acetate for 45

min and subsequently with lead citrate for 5 min at room temperature. Podocyte effacement was analysed by blinded scoring of the glomeruli in a Jeol 1200 EX2 electron microscope (JEOL, Tokyo, Japan).

Statistical analysis

All results are depicted as mean ± SEM. All statistical analyses were conducted by two-tailed student's t-test when comparing 2 treatment groups or experimental conditions, and ANOVA when comparing 3 or more treatment groups or conditions, using SPSS (IBM, New York, USA). P values less than 0.05 were considered significant.

Results

Effect of 1,25-D$_3$ on TRPC6 expression in an *in vivo* FSGS model

To study the *in vivo* effects of vitamin D on TRPC6 expression and proteinuria in an animal model for FSGS, control and adriamycin-exposed rats (adriamycin nephropathy; AN) were treated with 1,25-D$_3$ or vehicle. Vehicle-treated AN rats exhibited an increased albumin/creatinine ratio compared to vehicle-treated control rats, which was significantly ameliorated by 1,25-D$_3$ treatment (**Figure 1A**). 1,25-D$_3$ treatment did not alter urinary albumin/creatinine ratio in control rats. AN rats showed increased TRPC6 mRNA (**Figure 1B**) and glomerular TRPC6 protein expression (**Figure 1C**). By co-staining for TRPC6 and nephrin, we could demonstrate that the enhanced TRPC6 expression occurs primarily in podocytes (data not shown). Importantly, 1,25-D$_3$ treatment significantly reduced adriamycin-induced TRPC6 mRNA as well as protein expression. Furthermore, TRPC6 expression was not significantly altered by 1,25-D$_3$ in control animals. In addition, glomerular desmin expression, as a measure of podocyte damage, was increased in AN rats, but significantly reduced upon treatment with 1,25-D$_3$ (**Figure 1D**).

Effect of 1,25-D$_3$ on TRPC6 expression in podocyte injury *in vitro*

In the adriamycin-induced podocyte injury model, TRPC6 expression was significantly increased when compared to vehicle-treated control cells (**Figure 2A**). When injured podocytes were treated for 24 hours with 100 nM 1,25-D$_3$, TRPC6 expression was similarly reduced. Furthermore, a dose-dependent reduction of adriamycin-induced TRPC6 expression was observed when adriamycin-injured podocytes were treated with increasing concentrations of 1,25-D$_3$ (**Figure 2B**). In contrast, no effect of 1,25-D$_3$ on TRPC6 expression was seen in uninjured control podocytes.

Effect of 1,25-D$_3$ on TRPC6 promoter activity

In order to evaluate whether 1,25-D$_3$ directly regulates *TRPC6* transcription, possibly through vitamin D-responsive elements (VDRE) in the TRPC6 promoter, the 1500 bp

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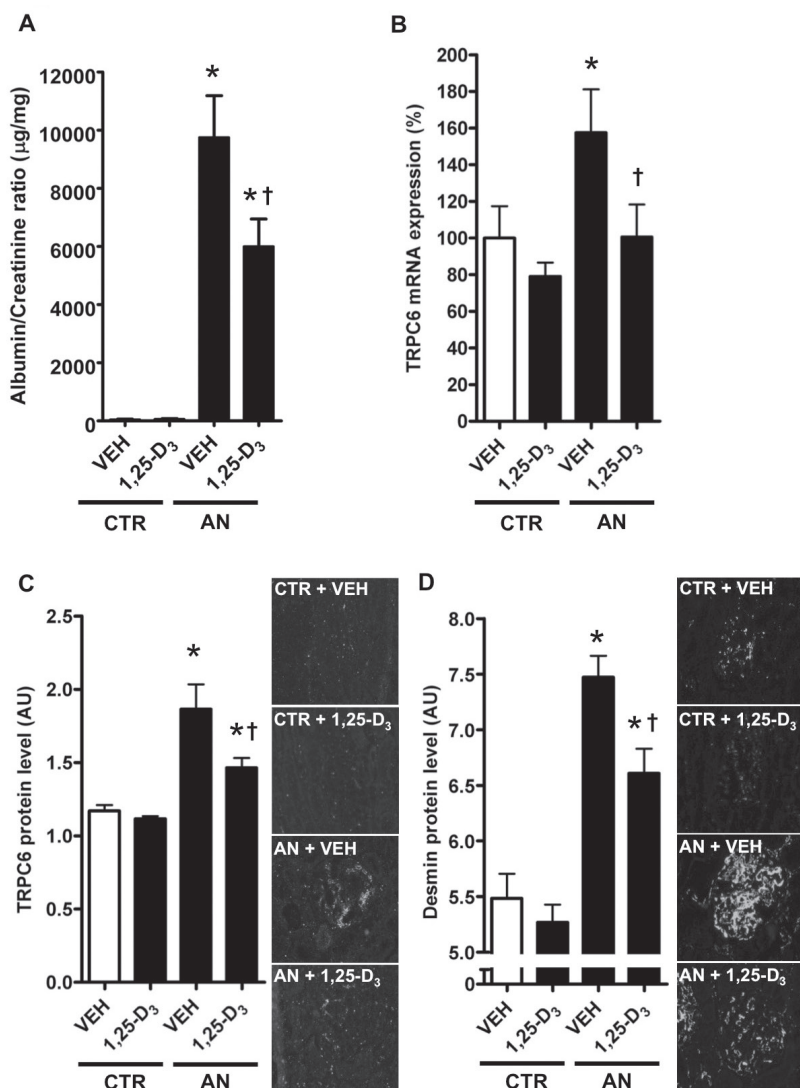
Figure 1 Effect of 1,25-D$_3$ on TRPC6 expression and proteinuria in the rat adriamycin-induced nephropathy (AN) model for FSGS.

AN was induced in Wistar rats by a single injection of adriamycin (1 mg/ml). Subsequently, AN or control (CTR) rats were treated with 1,25-D$_3$ (2.5 µg/kg) or vehicle (VEH) for 6 weeks. Proteinuria is depicted as albumin/creatinine ratio (**A**), TRPC6 mRNA expression (**B**) was determined by real-time qPCR and glomerular TRPC6 protein expression (**C**) and desmin protein expression (**D**) were determined by semi-quantitative immunohistochemistry. Representative immunohistochemical images are shown (**C** and **D**) *, $p < 0.05$ vs VEH-treated controls, †, $p < 0.05$ vs VEH-treated AN.
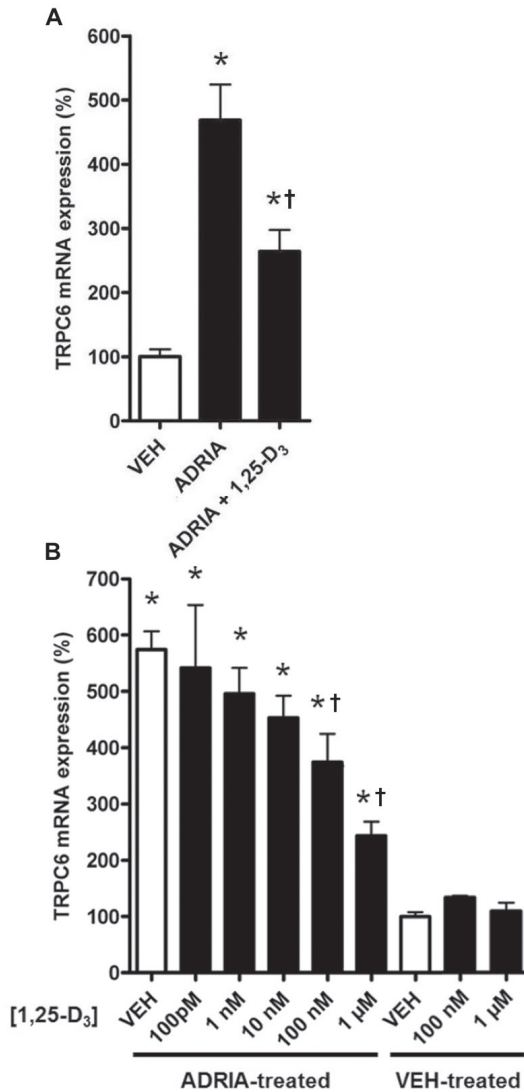
Figure 2 Dose-dependent effect of 1,25-D_3 on TRPC6 expression in podocyte injury.

Cultured podocytes were exposed to adriamycin (ADRIA) or vehicle (VEH) in absence or presence of 1,25-D_3 (100nM) for 24 hours (**A**). Cultured podocytes were treated with adriamycin (ADRIA-treated) or vehicle (VEH) and subsequently treated with different concentrations of 1,25-D_3 (100 pM – 1 μM) or vehicle (VEH) for 24 hours (**B**. TRPC6 mRNA levels were determined by real-time qPCR and quantified by the delta-delta cycle threshold (Ct) method using GAPDH as the house-keeping gene. Results are shown as percentage compared to vehicle-treated control podocytes. *, $p < 0.05$ vs VEH/VEH-treated, †, $p < 0.05$ vs ADRIA.

upstream of the mouse *TRPC6* transcription start site were cloned upstream of the luciferase reporter gene. Opossum kidney (OK) cells were subsequently transfected with either the mouse *TRPC6* promoter luciferase reporter construct or an empty vector that does not contain transcriptionally active elements, and treated for 48 hours with 100 nM 1,25-D_3 or vehicle. Treatment with 1,25-D_3 significantly reduced the activity of the *TRPC6* promoter by about 25%, compared to the vehicle-treated cells (**Figure 3**). Luciferase activity was not altered by 1,25-D_3 in cells expressing the empty vector.

ChIP analyses of VDR binding to the TRPC6 promoter region

To determine whether the above described effect of 1,25-D_3 on *TRPC6* promoter activity is mediated by direct binding of the VDR to the *TRPC6* promoter, we performed a ChIP analysis. OK cells were transfected with the mouse *TRPC6* promoter luciferase construct (TRPC6) or empty vector (empty) and treated with 100 nM 1,25-D_3 for 4 hours. After the ChIP assay, we performed a real-time PCR analysis, which showed a 16-fold enrichment of the *TRPC6* promoter when precipitated with the anti-VDR antibody (**Figure 4**: anti-VDR lane) compared to the rabbit IgG isotype control (**Figure 4**: IgG lane). Two percent of the
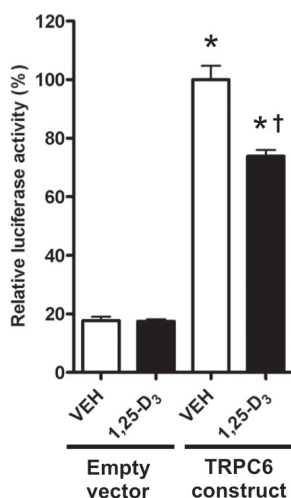
Figure 3 Effect of 1,25-D_3 on *TRPC6* promoter activity.

A luciferase assay was performed in OK cells transiently transfected with a Firefly luciferase *TRPC6* promoter construct or empty vector, after 48 hours of treatment with 100 nM 1,25-D_3 or vehicle (VEH). A Renilla luciferase construct was co-transfected to correct for transfection efficiency. Firefly/Renilla luciferase ratios were determined as a measure of promoter activity. Results are depicted as percentage compared to vehicle-treated cells transfected with the *TRPC6* promoter construct. *, $p < 0.05$ vs VEH-treated empty vector, †, $p < 0.05$ vs VEH-treated TRPC6 construct.

chromatin used for immunoprecipitation was included as a control (**Figure 4**: input lane). Using the empty vector no significant difference was seen between both antibodies (data not shown).

Figure 4 Vitamin D receptor (VDR) binds to the *TRPC6* promoter region.

To determine whether the VDR directly binds the *TRPC6* promoter region we performed a chromatin immunoprecipitation (ChIP) analysis. OK cells were transfected with the mouse *TRPC6* promoter luciferase construct or the empty vector without promoter, and treated with 100 nM 1,25-D_3. After chromatin immunoprecipitation using an anti-VDR antibody or a rabbit IgG isotype control antiserum, DNA was isolated and a (real-time) PCR with specific primers designed for the *TRPC6* promoter was performed. Real-time PCR showed a 16-fold enrichment of signal using the anti-VDR antibody (anti-VDR lane) compared to the isotype control (IgG lane) in the presence of the *TRPC6* promoter construct, whereas this enrichment was absent using the empty vector (not shown). As a control 2% of the chromatin used for the immunoprecipitation is shown (input lane).

TRPC6 expression and glomerular injury in 1,25-D_3-deficient mice

To study the *in vivo* effect of 1,25-D_3 deficiency, we used 25-hydroxy-1α-hydroxylase knockout (KO) mice, which are unable to synthesize 1,25-D_3.[33] These KO mice showed a significantly increased albumin/creatinine ratio compared to their wild-type (WT) littermates (**Figure 5A**). Subsequently, 1,25-D_3 supplementation in 25-hydroxy-1α-hydroxylase KO mice reduced albumin/creatinine ratio to WT levels, without restoring the hypocalcemia in these mice. At sacrifice, serum Ca^{2+} concentrations were 2.09±0.01mM, 1.37±0.03mM and 1.39±0.04mM for WT, KO and KO supplemented with 1,25-D_3, respectively. Importantly, 25-hydroxy-1α-hydroxylase KO mice demonstrated an increased TRPC6 mRNA (**Figure 5B**) and glomerular TRPC6 protein expression (**Figure 5C**). By co-staining for TRPC6 and podocin, we could demonstrate that the enhanced TRPC6 expression occurs in podocytes (data not shown). Furthermore, 1,25-D_3 treatment normalized TRPC6 mRNA and protein expression in these mice. Expression of desmin was significantly increased in 25-hydroxy-1α-hydroxylase KO mice, which was restored to normal levels by 1,25-D_3 treatment (**Figure 5D**). Electron microscopy analysis clearly demonstrated more, and in some segments total, podocyte foot process effacement in proteinuric 1,25-D_3-deficient mice. Hardly any effacement could be detected in their WT non-proteinuric littermates, as shown in the representative images in **Figure 5E**. The extent of podocyte effacement was quantified as 1.9±0.1% in WT versus 15.3±2.9% in the 25-hydroxy-1α-hydroxylase KO animals. When treated with 1,25-D_3, the podocyte foot

process effacement in 25-hydroxy-1α-hydroxylase KO animals completely recovered to 2.2±0.7%, and glomerular ultrastructure was not distinguishable from WT mice.

Discussion

The present study demonstrated that 1,25-D_3 downregulates TRPC6 expression both in injured podocytes as well as in animal models for FSGS and 1,25-D_3 deficiency. The increased glomerular TRPC6 expression and proteinuria in the AN rat model for human FSGS, were both significantly ameliorated by 1,25-D_3 treatment. *In vitro*, the enhanced TRPC6 expression in injured podocytes was dose-dependently reduced by 1,25-D_3 application. We demonstrated that the VDR binds directly to the *TRPC6* promoter region and, accordingly, 1,25-D_3 inhibits *TRPC6* promoter activity. Interestingly, 25-hydroxy-1α-hydroxylase KO mice, which are 1,25-D_3 deficient, showed significantly enhanced glomerular TRPC6 expression levels along with a remarkable proteinuria associated with podocyte foot process effacement. In line with the aforementioned results, 1,25-D_3 supplementation reversed both the increased TRPC6 expression as well as the proteinuria, and normalized podocyte morphology. Taken together, we demonstrated that vitamin D downregulates the enhanced TRPC6 expression in *in vitro* and *in vivo* podocyte injury, possibly through a direct effect on *TRPC6* promoter activity.

The present study is the first to show that *TRPC6* promoter activity and expression is regulated by means of a nuclear hormone receptor, the VDR. TRPC6 was generally regarded to be a primarily receptor-operated channel, regulated by cell surface receptors like the angiotensin II type 1 receptor (AT1R).[14 34] The TRP channel family members TRPV5 and TRPV6 are also transcriptionally regulated by vitamin D. However, these channels are not regarded as primarily receptor-mediated signaling proteins, but rather involved in transcellular Ca^{2+} transport across the gastrointestinal and renal epithelia, processes known to be governed by vitamin D.[35-37]

While the *TRPC6* promoter activity can be inhibited by 1,25-D_3 in uninjured OK cells, in cultured podocytes and *in vivo* in the rat kidney 1,25-D_3 appears to reduce TRPC6 expression only in injured podocytes, but not in uninjured podocytes. One explanation could be the length (~1500bp) of the cloned *TRPC6* promoter region for the luciferase reporter construct. In this cloned region there could be less transcription sites compared to the native promoter in its chromatin context. Therefore the native *TRPC6* gene in the cultured or *in vivo* podocytes might be regulated more strictly by other (transcription) factors, which are unable to affect our 1500 bp promoter construct or are not even present, like higher order chromatin structures. A second explanation could be that the kinetics of the turnover and relative expression of the luciferase protein is different from the TRPC6 mRNA and protein. A third explanation could be, that the basal *TRPC6* promoter activity is regulated by another transcriptional complex in a specific chromatin context in uninjured
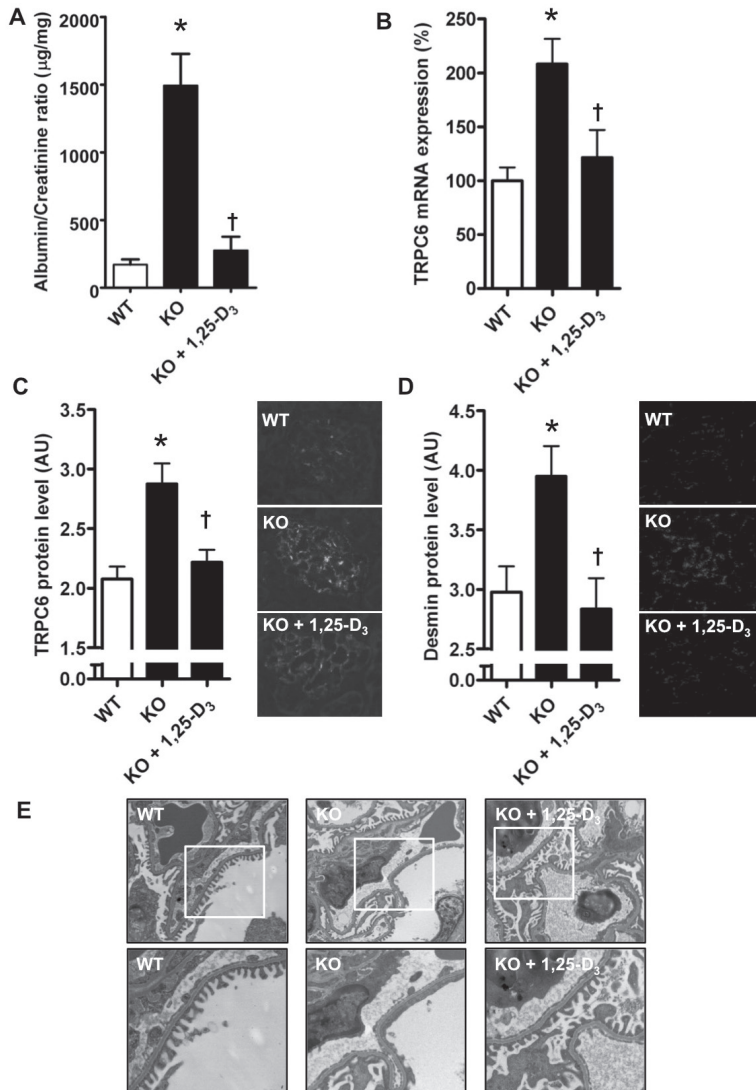
Figure 5 TRPC6 expression, proteinuria and podocyte foot process effacement in 1,25-D$_3$-deficient 25-hydroxy-1α-hydroxylase KO mice.

25-hydroxy-1α-hydroxylase KO mice were supplemented daily with 1,25-D$_3$ (500 ng) or vehicle for 6 weeks. Proteinuria is depicted as albumin/creatinine ratio (**A**), TRPC6 mRNA expression (**B**) was determined by real-time qPCR and glomerular TRPC6 protein expression (**C**) and desmin protein expression (**D**) were determined by semi-quantitative immunohistochemistry. *, p < 0.05 vs VEH-treated controls, †, p < 0.05 vs VEH-treated AN. Representative images showing podocyte morphology as determined by electron microscopy (**E**).

cultured or *in vivo* podocytes compared to the *TRPC6* promoter activity in injured podocytes and/or OK cells.

In general, transcriptional regulation of vitamin D-responsive genes occurs through interaction of the nuclear VDR complex with vitamin D responsive elements (VDRE) in the promoter region of these target genes.[38] In this study, we demonstrated that the VDR indeed binds to the *TRPC6* promoter region. For positively regulated genes, a VDRE consensus sequence is available. Genes that are negatively regulated by vitamin D are sparse and, next to TRPC6, include the genes encoding parathyroid hormone and renin.[39-41] When the negative vitamin D-responsive promoter sequences (nVDRE) in these genes were identified, they did not comply with the consensus sequence.[42, 43] Similarly, the *TRPC6* promoter region did not contain sequences complying with the VDRE consensus sequence (data not shown). Thus, TRPC6 appears to be part of a select group of genes that are negatively regulated by vitamin D.

The relationship between proteinuria and increased TRPC6 activity and/or expression was demonstrated in several acquired human proteinuric diseases and animal models.[10, 12, 13, 44-46] In addition, downregulation of TRPC6 expression by e.g. ARBs, ACEi or calcineurin inhibitors correlated with reduced proteinuria in proteinuric animal models.[14, 47, 48] Recently, Eckel *et al.* illustrated the potential beneficial anti-proteinuric effect of downregulating TRPC6 expression by showing reduced AngII-mediated albuminuria in TRPC6 KO compared to WT mice.[49] In the present study, we demonstrated that 1,25-D_3 treatment significantly reduces glomerular TRPC6 expression and proteinuria, as well as expression of the podocyte injury marker desmin in the rat AN FSGS model. To further evaluate the importance of 1,25-D_3, we used the 25-hydroxy-1α-hydroxylase KO model, which displays undetectable levels of 1,25-D_3.[33, 50] Glomeruli showed increased TRPC6 expression, which was paralleled by a similarly increased expression of the podocyte damage marker desmin. Electron microscopy showed podocyte foot process effacement in the KO mice. To confirm that the enhanced TRPC6 expression was 1,25-D_3-mediated and to test whether damage could be prevented, mice were supplemented with 1,25-D_3, which normalized glomerular TRPC6 expression and proteinuria. Theoretically, the observed effects could also result from e.g. the striking hypocalcemia interfering with TRPC6 function in the podocytes of 25-hydroxy-1α-hydroxylase KO mice, but the relatively low dose of 1,25-D_3 supplementation did not significantly increase serum Ca^{2+} levels in these mice. The current study is the first to describe the glomerular and proteinuric phenotype in this animal model of 1,25-D_3-deficiency. However, our results by no means prove a causal relationship between increased TRPC6 expression and the glomerular phenotype in this particular model. Taken together though, our data do show that 1,25-D_3 downregulates the enhanced TRPC6 expression in FSGS, while 1,25-D_3 deficiency results in increased TRPC6 expression, which was proven 1,25-D_3-sensitive.

While our *TRPC6* promoter luciferase reporter and ChIP analyses suggested that *TRPC6* promoter activity is a specific target of vitamin D, this does not rule out that *in vivo*,

other mechanisms could also contribute to the downregulation of TRPC6 expression by vitamin D. Importantly, the gene encoding renin, important in AngII biosynthesis, is negatively regulated by vitamin D. As we previously showed that AngII enhances TRPC6 expression by stimulating a calcineurin/NFAT-mediated feed-forward pathway in adriamycin-induced podocyte injury, this could certainly be an additional mechanism reducing TRPC6 expression [14]. Interestingly, recent studies in mice with cardiomyocyte-specific deletion of the VDR demonstrated that vitamin D inhibits the calcineurin/NFAT signaling pathway in the cardiomyocyte, which also includes TRPC6.[51] Thus, the demonstrated vitamin D-mediated inhibition of *TRPC6* promoter activity could serve to counteract the effect of the transcription factor NFAT on the *TRPC6* promoter. Furthermore, TRPC6 will certainly not be the only mediator of the effect of vitamin D on glomerular injury and proteinuria. Several studies described the protective role of $1,25-D_3$ in different proteinuric disorders such as FSGS and diabetic nephropathy, in which the authors hypothesized that $1,25-D_3$ acts on various pathways.[18-20] For example, $1,25-D_3$ upregulates expression of various structural podocyte proteins such as podocin and nephrin.[52, 53] Nephrin was shown to inhibit TRPC6-phospholipase C complex formation, surface expression and activation.[54] Therefore, in addition to inhibiting TRPC6 expression directly, $1,25-D_3$ could also affect TRPC6 indirectly via nephrin.

Altogether, our results add important new data to our understanding of the regulation of TRPC6 expression in podocytes. The transcriptional regulation of TRPC6 by $1,25-D_3$ demonstrated in cultured podocytes and illustrated in a rat FSGS model as well as in $1,25-D_3$-deficient mice, suggests that TRPC6 downregulation could indeed contribute to the anti-proteinuric effect of vitamin D.

Acknowledgements

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Rapamycin-induced renal magnesium wasting associates with decreased TRPM6 expression and EGF stimulation

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Abstract

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that has recently emerged as an important signaling molecule in several renal diseases. The inhibitor of mTOR, rapamycin, is a potent immunosuppressant used in the anti-rejection therapy after organ transplantation and known to induce renal magnesium (Mg^{2+}) wasting. However, the underlying molecular mechanism remains unknown. The present study investigated the effects of rapamycin on the regulation of the epithelial Mg^{2+} channel, transient receptor potential melastatin 6 (TRPM6), the key molecular player in the fine-tuning of renal Mg^{2+} excretion in the distal convoluted tubule (DCT). Administration of rapamycin to mice by daily intraperitoneal injections for one week significantly reduced the renal mRNA expression of TRPM6. The mRNA expression levels of hepatocyte nuclear factor homeobox B (HNF1B) and epidermal growth factor (EGF), which play a role in Mg^{2+} reabsorption in DCT, displayed a similar downregulation. The expression levels of the marker genes for the thick ascending limb of Henle (TAL), claudin-16 (*CLDN16*) and claudin-19 (*CLDN19*), were upregulated in the rapamycin-treated group compared to the control group. None of the other tested genes known to be involved in renal Mg^{2+} reabsorption either in TAL or DCT were affected by the rapamycin treatment. Patch-clamp analysis revealed that rapamycin does not directly affect TRPM6-mediated currents in human embryonic kidney cells transiently transfected with TRPM6, but significantly inhibited the EGF-stimulated TRPM6. Stimulation of TRMP6 activity by insulin, another magnesiotropic hormone, was not influenced by rapamycin. In conclusion, inhibition of the mTOR pathway by the immunosuppressant rapamycin decreased renal TRPM6 expression *in vivo* and inhibited the stimulatory effect of EGF, but not insulin, on TRPM6 activity *in vitro*, providing a molecular explanation for the rapamycin-induced hypermag-nesuria.

Introduction

Rapamycin has been used for many years as chemotherapeutic agent and component of antirejection therapy for recipients of organ transplants who mainly developed toxicity to other immunosuppressive medications, like the calcineurin inhibitors (CNI, cyclosporine A [CsA] and tacrolimus [FK506]). It is a potent inhibitor of the mammalian target of rapamycin (mTOR).[1, 2] The availability of a specific inhibitor of mTOR, such as rapamycin, helped to identify a large signaling network that integrates information on nutrient availability and growth factors to control protein synthesis and cell size.[3, 4] mTOR is a serine/threonine kinase that exists in two separate complexes, mTORC1 and mTORC2.[4] These complexes differ in two scaffolding proteins, raptor and rictor, respectively, which connect mTOR to distinct intracellular pathways. mTORC1 is involved in nutrient sensing and growth factors signaling, whereas mTORC2 primarily regulates cytoskeleton dynamics.[3-5] Initially identified as mTORC1-specific inhibitor, rapamycin has been recently shown to also affect signaling through mTORC2.[6, 7] Upon entering the cell, rapamycin binds the cytosolic FK506-binding protein of 12 kDa (FKBP12) to finally act as allosteric inhibitor of the mTOR kinase activity.[4] Activation of mTORC1 begins with stimulation of the lipid kinase phosphatidylinositol 3-kinase (PI3K) that leads to the phosphorylation of Akt at the amino acid residue Thr308.[8] Phosphorylated Akt in turn activates mTORC1 through a cascade of downstream intermediates that include the tuberous sclerosis complex (TSC) and Rheb, a Ras family GTPase that directly activates mTOR.[9] Stimulation of mTORC1 results in the phosphorylation of 4EBP and p70S6K that promote cell growth and proliferation.[10] Relative to mTORC1, little is known regarding the upstream regulation and downstream functions of mTORC2. Insulin and growth factors directly stimulate the kinase activity of mTORC2,[11] but the mechanism is currently unknown. The best-characterized downstream events to mTORC2 are the phosphorylation of Akt at Ser473 and the regulation of actin cytoskeleton through PKC.[12] Interestingly, mTORC1 and p70S6K can exert negative feedback on the upstream signaling molecules, like the insulin receptor substrate (IRS) proteins, which are required to activate the PI3K-Akt pathway downstream of the insulin receptor,[13] and the core mTORC2 component rictor, which leads to an attenuation of Akt activation.[14]

Hyperactivation or overexpression of the mTOR molecule was found in various solid tumor malignancies,[3] while, more recently, mTOR has emerged as an important modulator of several forms of renal diseases.[15, 16] Inhibition of the mTOR pathway by rapamycin in a variety of animal models revealed beneficial effects on the progression of diabetic nephropathy,[17] non-diabetic forms of chronic kidney disease[18] and polycystic kidney disease.[19] Furthermore, inhibitors of mTOR improve survival in patients with metastatic renal cell carcinoma.[20, 21] On the other hand, inhibition of mTOR with rapamycin-based regimen delays recovery of renal function after acute kidney injury,[22, 23] increases risks of allograft failure and mortality compared to CNI,[24-26] and associates with inappropriately high fractional excretion of Mg²⁺.[27-30] In the kidney, the bulk of Mg²⁺ in the pro-urine is

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reabsorbed in the proximal tubule (PT) and thick ascending limb of Henle (TAL) of the nephron by a passive transport route.[31] The fine-tuning of the final Mg^{2+} excretion occurs in the early distal convoluted tubule (DCT) via an active transcellular process. In DCT, the epithelial cation channel TRPM6 facilitates transport of Mg^{2+} from the pro-urine into the cell.[31] So far, a single study investigated the renal TRPM6 expression in rats administrated with rapamycin.[28] The authors showed that TRPM6 expression in DCT is upregulated as a compensatory mechanism to the upstream defect in Mg^{2+} reabsorption in the TAL.[28] On the other hand, an *in vitro* study suggested that rapamycin treatment of renal tubular epithelial cells decreased endogenous TRPM6 expression by affecting the stability of TRPM6 mRNA.[32] Many extracellular and intracellular mediators influence TRPM6 protein abundance and activity at the plasma membrane.[33] Recently, epidermal growth factor (EGF) and insulin, two well-known upstream activators of the mTOR pathway, have been implicated as magnesiotropic hormones.[34, 35] The stimulation of the EGF receptor (EGFR) and insulin receptor (IR) lead to an intracellular cascade involving Rac1 that promotes trafficking of TRPM6 to the plasma membrane.[34-38] Nevertheless, the effects of rapamycin on the EGF- and insulin-mediated TRPM6 stimulation are unknown. Thus, the role of mTOR in the cascade of molecular events that lead to impaired renal Mg^{2+} handling during rapamycin treatment is still vague. The aim of the present study was, therefore, to determine the effect of rapamycin on the regulation of the epithelial channel TRPM6, also in response to the magnesiotropic hormones EGF and insulin.

Materials and Methods

Animal model

The animal experiment reported in this study was previously described by the Chien-Te Lee *et al*.[39] Briefly, adult male C57BL6 mice (weight: 15–20 g) were maintained in a temperature-controlled and light-cycled environment, and had *ab libitum* access to water and food (1.0% wt/wt Ca^{2+} and 0.5% wt/wt Mg^{2+}). According to the experimental design, animals were divided into two groups: control group and rapamycin-treated group (n=8 per group). All the treatment animals received daily administration via intraperitoneal injections for 1 week (1 mg/kg/day, Sigma, St. Louis, Mo., USA). After this period, mice were sacrificed and kidneys were harvested. Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Chang-Gung Memorial Hospital, Taiwan, and all animal procedures were performed according to the IACUC policy.

Cell culture and transfection

Human embryonic kidney (HEK) 293 cells seeded in 12-well plates were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Bio Whittaker Europe, Vervier, Belgium) supplemented with 10% (v/v) fetal calf serum (PAA, Linz, Austria), 2% (v/v) L-glutamine, 10

μl/mL essential aminoacids and 0.01 mg/mL ciproxin at 37 °C in a humidity controlled incubator with 5% (v/v) CO_2 atmosphere. For patch clamp experiments, cells were transiently transfected with 1 μg of pCINeo-IRES-GFP construct encoding wild-type TRPM6 or mock DNA using Lipofectamine 2000 (Invitrogen Life technologies, Breda, The Netherlands).

Electrophysiology

After 48 h transfected cells were plated at low density on 18 mm glass coverslips coated with fibronectin (Roche Diagnostics, Almere, Netherlands). The whole-cell configuration of the patch-clamp technique was used. Experiments were done in an EPC-9 patch-clamp amplifier controlled by the Pulse software (HEKA Electronik, Germany). Borosilicate patch pipettes had resistances between 2-3 MΩ after being filled with intracellular solution. Series resistances (≤5 MΩ) were monitored after each sweep with the automatic capacitance compensation option of Pulse software. Experiments were performed at room temperature (22 °C). 24 h prior to experiments, medium was replaced to FCS-free DMEM medium. To study TRPM6-evoked outward Na$^+$ currents, a stimulation protocol consisting of repetitive voltage-ramps from -100 mV to +100 mV over 450 ms duration from a V_h of 0 mV was applied. TRPM6 currents were allowed to develop for 200 s to reach steady-state. Extracting the current amplitudes at +80 and -80 mV from individual ramp current records provided an assessment of the temporal development of membrane currents. Current densities were obtained by normalizing the current amplitude to the cell membrane capacitance. Current-voltage (I/V) relations were established from the ramp protocols. The extracellular bath solution consisted of (in mmol/L): 150 NaCl, 1 CaCl$_2$, 10 HEPES (pH 7.35 adjusted with NaOH). The intracellular solution consisted of (in mmol/L): 150 NaCl, 10 Na$_2$EDTA, 10 HEPES (pH 7.2 adjusted with NaOH). The analysis and display of patch-clamp data were performed using Igor Pro software version 6.0 (WaveMetrics, Lake Oswego, OR, USA). To test the effect of EGF and insulin on TRPM6, HEK293 cells were treated for 60 min with 10 nmol/L EGF (Sigma Aldrich, St. Louis, USA) or 10 nmol/L insulin (Sigma Aldrich, St. Louis, USA) in the presence and absence of 100 nmol/L rapamycin (LC Laboratories, Woburn, MA, USA). Either rapamycin or DMSO was added to cells 30 min after starting EGF or insulin treatment. 10 nmol/L EGF, 10 nmol/L insulin and 100 nmol/L rapamycin were added to the extracellular solution while running the patch clamp experiments.

Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR) analysis

A quarter of a mouse kidney was homogenized in 800 μl TRIzol, and mRNA was extracted using a mix of isoaminoalcohol/phenol/chloroform (1:25:25) and then precipitated by isopropanol. Subsequently, reverse transcription (RT) of the RNA by M-MLV reverse transcriptase was performed 1 h at 37°C according to manufacturer's specifications (Invitrogen). RT-PCR reactions were performed using samples pooled from four to five

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animals. The cDNA was mixed with Power SYBR green PCR Mastermix (Applied Biosystems, Foster City, CA) and the exon-intron-exon overlapping primers reported in **Table 1**.

Table 1 Oligonucleotide sequences used for RT-PCR analysis.

Gene product	Forward (5'-3')	Reverse (5'-3')
TRPM6	AAAGCCATGCGAGTTATCAGC	CTTCACAATGAAAACCTGCCC
EGF	GAGTTGCCCTGACTCTACCG	CCACCATTGAGGCAGTATCC
NCC	CTTCGGCCACTGGCATTCTG	GATGGCAAGGTAGGAGATGG
HNF1B	CAAGATGTCAGGAGTGCGCTAC	CTGGTCACCATGGCACTGTTAC
FXYD2a	GATCTGTCAGCGAACAGTG	GCGGACGGTTTCATAGTCGTAC
FXYD2b	CTACCATGGACAGGTGGTA	GCGGACGGTTTCATAGTCGTAC
CNNM2 v.1	GTCTCGCACCTTTGTTGTCA	GTCGCTCCGACTGAGAGAAT
CNNM2 v.2	CTCACAGCCTCTCCAGGG	AGGAAGAGCTGAGCTGGTTG
CLDN16	GTTGCAGGGACCACATTAC	GAGGAGCGTTCGACGTAAAC
CLDN19	GGTTCCTTTCTCTGCTGCAC	CGGGCAACTTAACAACAGG
NKCC2	GGCTTGATCTTTGCTTTTGC	CCATCATTGAATCGCTCTCC
GAPDH	TAACATCAAATGGGGTGAGG	GGTTCACACCCATCACAAAC

TRPM6: transient receptor potential melastatin 6; EGF: pro-epidermal growth factor; NCC: Na$^+$-Cl$^-$-cotransporter; HNF1B: hepatocyte nuclear factor B; FXYD2a: γ-subunit of the Na$^+$-K$^+$-ATPase, isoform a; FXYD2b: γ-subunit of the Na$^+$-K$^+$-ATPase, isoform b; CNMM2 v.1: cyclin M2, variant 1; CNNM2 v.2: cyclin M2, variant 2; CLDN16: claudin-16; CLDN19: claudin-19; NKCC2: Na$^+$-K$^+$-Cl$^-$-cotransporter; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Gene expression levels were determined by quantitative real-time PCR on a BioRad Analyzer and normalized for *GAPDH* expression levels. Real-time PCR primers were designed using the online computer program NCBI/Primer-BLAST software.

Statistical analysis

All results are depicted as mean ± standard error of the mean (SEM). Statistical analyses were conducted by unpaired student's t-test when comparing two experimental conditions, and one-way ANOVA with Bonferroni test when comparing more conditions. P values less than 0.05 were considered significant.

Results

Rapamycin treatment in mice modulates the renal expression of magnesiotropic genes

Lee *et al.* previously reported that rapamycin treatment induces severe renal Mg^{2+} wasting in mice (fractional excretion of Mg^{2+} [FEMg] 8.8% in the control group *versus* FEMg 29% in the rapamycin-treated group) without affecting serum Mg^{2+} concentrations.[39] The authors also showed that rapamycin treatment has no effects on Ca^{2+} homeostasis in mice.[39] Changes in the expression levels of many proteins that are involved in the paracellular Mg^{2+} reabsorption in TAL and in the transcellular Mg^{2+} reabsorption in DCT could contribute to the observed phenotype (**Figure 1**).

To elucidate the molecular mechanisms of the rapamycin-induced hypermagnesuria, the renal mRNA levels of several magnesiotropic genes were determined by real-time quantitative PCR analysis. Among the genes involved in the Mg^{2+} handling in DCT, rapamycin treatment significantly decreased TRPM6, EGF and hepatocyte nuclear factor homeobox B (HNF1B) expression in the mouse kidney (**Figure 2**). The mRNA levels of the Na$^+$-Cl$^−$-cotransporter (NCC), the two splice variants of cyclin-M2 (CNNM2 v.1 and CNNM2
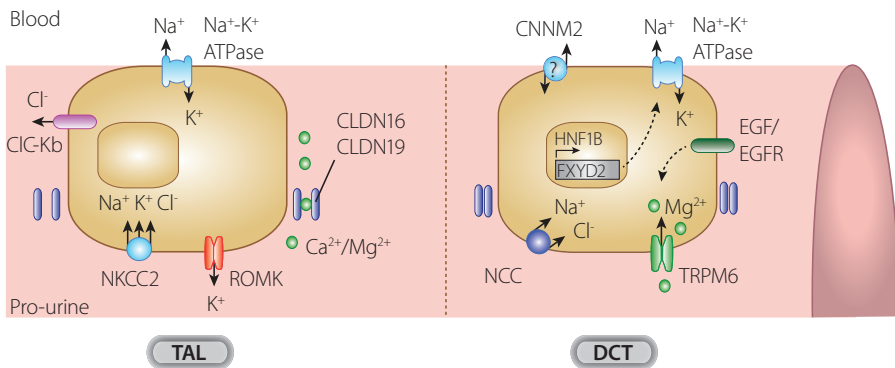
Figure 1 Cartoon depicting the molecular players involved in renal Mg^{2+} reabsorption in TAL and DCT.

After the filtration in the glomeruli, the bulk of Mg^{2+} in the pro-urine is reabsorbed in the proximal tubule and TAL by a passive transport route. Fine-tuning of Mg^{2+} reabsorption occurs by regulation of an active transcellular route in DCT. TAL: thick ascending limb of Henle; DCT: distal convoluted tubule; NKCC: Na$^+$-K$^+$-2Cl$^-$ cotransporter; ROMK: renal outer medullary K$^+$ channel; ClC-Kb: Cl$^-$ channel Kb; CLDN16: claudin-16; CLDN19: claudin-19; NCC: Na$^+$-Cl$^-$-cotransporter; TRPM6: transient receptor potential cation channel subfamily M member 6; HNF1B: hepatocyte nuclear factor 1 homeobox B; FXYD2: γ-subunit of the Na$^+$-K$^+$-ATPase; EGF: epidermal growth factor; EGFR: epithelial growth factor receptor; CNNM2: cyclin-M2.

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v.2) and the two isoforms of the γ-subunit of the Na$^+$-K$^+$-ATPase (FXYD2a and FXYD2b) were not affected (**Figure 2**).

Figure 2 Effect of rapamycin treatment on mRNA expression levels of genes involved in the active transcellular Mg^{2+} transport in DCT.

The effect of rapamycin (1 mg/kg/day via daily intrapertioneal injections for one week) on renal mRNA expression levels of TRPM6, HNF1B, EGF, NCC, CNNM2 v.1, CNNM2 v.2, FXYD2a and FXYD2b in mice were determined by real-time quantitative PCR analysis. Data are presented as mean +/- SEM. *, $p<0.05$ versus control, n=3.

Subsequently, the mRNA expression levels of the Na$^+$-K$^+$-2Cl$^-$ cotransporter (NKCC2) and the tight junction proteins claudin-16 (CLDN16) and claudin-19 (CLDN19), all located in the thick ascending limb of Henle (TAL), were determined. The NKCC2 expression levels were not significantly changed, whereas CLDN16 and CLDN19 were both upregulated in the rapamycin-treated group (**Figure 3**).

Rapamycin inhibits TRPM6 activation by EGF, but not by insulin

We further investigated the possibility of an effect of rapamycin on TRPM6 activation by use of the patch-clamp technique. Incubation with rapamycin of HEK293 cells transiently transfected with TRPM6 did not affect TRPM6 activity compared to mock-transfected cells after 30 min treatment (**Figure 4**). Nevertheless, rapamycin significantly inhibited the stimulatory effect of the magnesiotropic hormone EGF, but not of insulin, on the TRPM6-mediated currents (**Figure 4**).

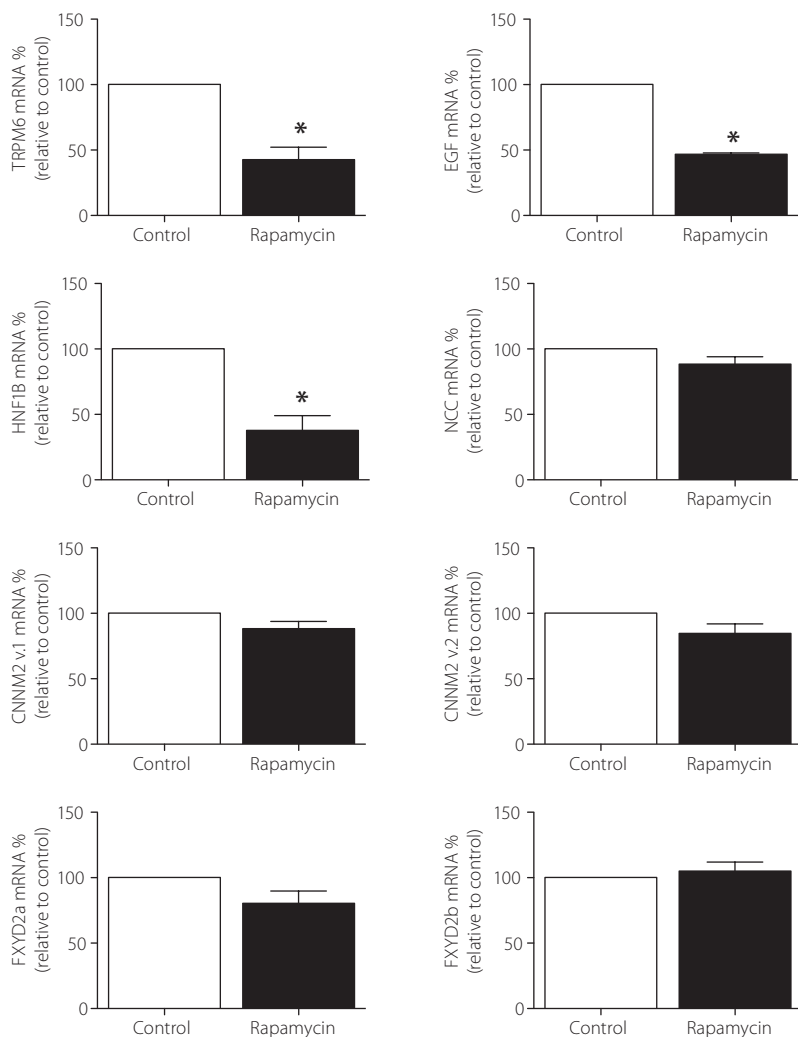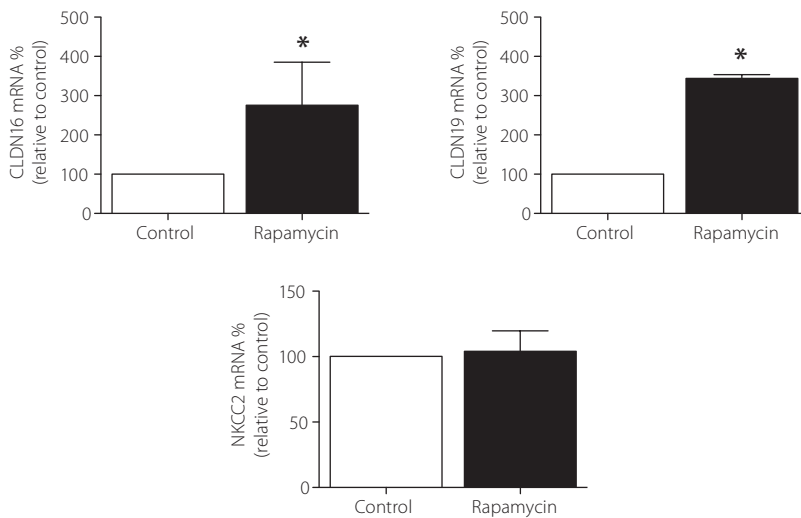**Figure 3** Effect of rapamycin treatment on mRNA expression levels of genes relevant to the passive paracellular Mg^{2+} transport.

The effect of rapamycin (1 mg/kg/day via intrapertioneal injection) on renal mRNA expression levels of CLDN16, CLDN19 and NKCC2 in mice were determined by real-time quantitative PCR analysis. Data are presented as mean +/- SEM. *, $p < 0.05$ versus control, n=3.
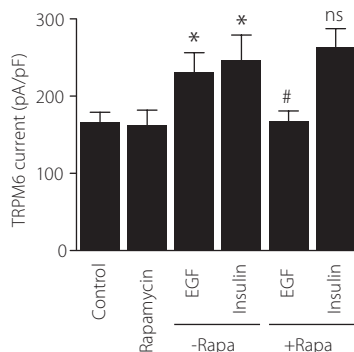
Figure 4 Rapamycin inhibits TRPM6 activation by EGF, but not by insulin.

Average current density (pA/pF) of TRPM6 channels was measured in HEK293 cells after overnight starvation in 0% v/v FCS followed by treatment with vehicle (control, n=20), 100 nmol/L rapamycin (n=13) for 30 min, 10 nmol/L EGF (n=22) for 1 h or 10 nmol/L insulin (n=12) for 1 h. Co-incubation experiments were conducted by adding 100 nmol/L rapamycin to EGF- (n=13) or insulin-(n=10) treated cells during the last 30 min. Data are presented as means +/- SEM. *, p<0.05 versus control; #, p<0.05 versus EGF treatment in the absence of rapamycin; ns: non significant compared to insulin treatment in the absence of rapamycin.

Discussion

In this study, we demonstrated that the rapamycin-induced renal Mg^{2+} wasting in mice associates with decreased mRNA levels of the epithelial Mg^{2+} channel TRPM6 in DCT, and of the gene encoding the EGF precursor protein pro-EGF, involved in the hormonal regulation of TRPM6.[34] The renal mRNA level of HNF1B that is relevant to Mg^{2+} reabsorption in DCT was also decreased in mice treated with rapamycin. Overall these observations indicate that rapamycin interferes with the cross-talk between TRPM6 and EGF inhibiting the mTOR-mediated pathway that possibly involves HNF1B. These molecular events will ultimately lead to the renal Mg^{2+} wasting observed *in vivo*.

In rats, administration of human EGF significantly upregulated TRPM6 mRNA levels in parallel with an amelioration of the renal ability to reabsorb Mg^{2+}.[40] These findings suggest that administration of EGF has a positive effect on the Mg^{2+} homeostasis *in vivo*.[40] Furthermore, long-term treatment with EGF increased TRPM6 expression via the ERK-AP1 (c-Fos/c-Jun) pathway *in vitro*.[41, 42] Thus, rapamycin could indirectly inhibit TRPM6 expression by decreasing EGF levels. Alternatively, rapamycin may directly interfere with the activation of AP1 transcription factors or other mTOR-mediated transcriptional events responsible for *TRPM6* gene transcription. Interestingly, Ikari *et al.* suggested that a PI3K/Akt/mTOR pathway sensitive to rapamycin is involved in the regulation of the stability of

TRPM6 mRNA, rather than a direct effect on gene expression. However, our evidence that rapamycin, as calcineurin-inibitors (CNI),[40, 43] associates with a significant decrease in TRPM6 mRNA levels *in vivo* opens the possibility of common transcriptional networks between the calcineurin/NFATc and mTOR pathways important to TRPM6 expression. It was reported that mTOR phosphorylates the transcription factor NFATc4, one of the substrates of the phosphatase calcineurin.[44] Moreover, a complex network of HNF transcription factors is under the control of both the calcineurin/NFATc[45, 46] and the mTOR pathway.[47] Among the HNF factors, HNF1B plays an important role in renal Mg^{2+} reabsorption, but no HNF1B binding sites were predicted in the *TRPM6* gene promoter.[48] Noteworthy, in our experiment, HNF1B mRNA expression was significantly downregulated in mice treated with rapamycin. Future investigations should address the role of HNF1B in the calcineurin/NFATc and mTOR pathways, and its relevance to *TRPM6* gene transcription. Interestingly, the expression of the magnesiotropic gene *FXYD2* that is under the transcriptional control of HNF1B was not affected by rapamycin. This finding indicates that other HNF1B target genes may be involved in the rapamycin-induced renal Mg^{2+} loss. A previous study in rats treated with rapamycin[28] suggests a decrease in the Na$^+$-K$^+$-2Cl$^-$ cotransporter (NKCC2) expression in TAL as the main cause for the observed hypermagnesuria with Na$^+$ and K$^+$ wasting, indicating a disturbance in the Mg^{2+} paracellular reabsorption. In the latter study, renal TRPM6 mRNA levels were increased. Our results not only show that TRPM6 is decreased, but also that NKCC2 expression remains stable upon rapamycin treatment in mice. Differences in the genetic background of the animals and in the methodologies used for rapamycin administration may be responsible for these discrepancies. Surprisingly, in our study two major players in the paracellular Mg^{2+} reabsorption, the tight-junction proteins claudin-16 (CLDN16) and claudin-19 (CLDN19), were significantly upregulated. The increased expression of the heteromeric complex CLDN16/19 should augment the paracellular cation permeability instead of associating with Mg^{2+} wasting in urine.[49, 50] Recently, it was shown that claudin-14 (CLDN14) directly interacts with CLDN16 to act as a negative regulator of paracellular cation permeability.[51] Therefore, the upregulation of CLDN16 and CLDN19 could be a compensatory response to changes in CLDN14 expression.

We decided to further investigate the effect of rapamycin on TRPM6 stimulation by EGF. *In vitro*, EGF induces rapid incorporation of functional TRPM6 channels into the plasma membrane via activation of the PI3K-Akt-Rac1 axis.[37] Rac1 is a member of the Rho family GTPases implicated in actin remodeling and membrane trafficking.[37] In particular, Rac1 is an important PI3K-Akt effector responsible for TRPM6 insertion into the membrane upon exposure to EGF and insulin.[35, 38] In the present study, rapamycin inhibited the EGF-dependent stimulation of TRPM6 activity at the cell surface of HEK293 cells. It is reasonable to believe that this effect is probably mediated by a block of the mTOR-rictor (mTORC2) complex rather than by inhibition of the mTOR-raptor (mTORC1) complex.[6, 52, 53] Indeed mTORC2 is a known regulator of cytoskeletal organization through the Rho family

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GTPases.[54] Furthermore, knockdown of rictor, but not of raptor, has been previously described to affect epithelial transport processes.[55] However, in many cell lines, the mTORC2 assembly is inhibited only after prolonged rapamycin treatment.[6] Thus, it cannot be excluded that inhibition of mTOR within the mTORC1 complex may participate in the inhibitory effect of rapamycin on the EGF-stimulated TRPM6 activity.

Although insulin and EGF initiate similar signaling cascades that ultimately increase the cell membrane abundance of TRPM6 (**Figure 5**),[35, 38] rapamycin failed to inhibit the stimulatory effect of insulin on TRPM6. This observation may have different causes. Firstly, a dose-related effect of rapamycin could be important. Possibly, the signaling transduction initiated by IR and EGFR may require different rapamycin concentrations to be inhibited in our cell model due to the differential expression levels of the receptors. Secondly, it is
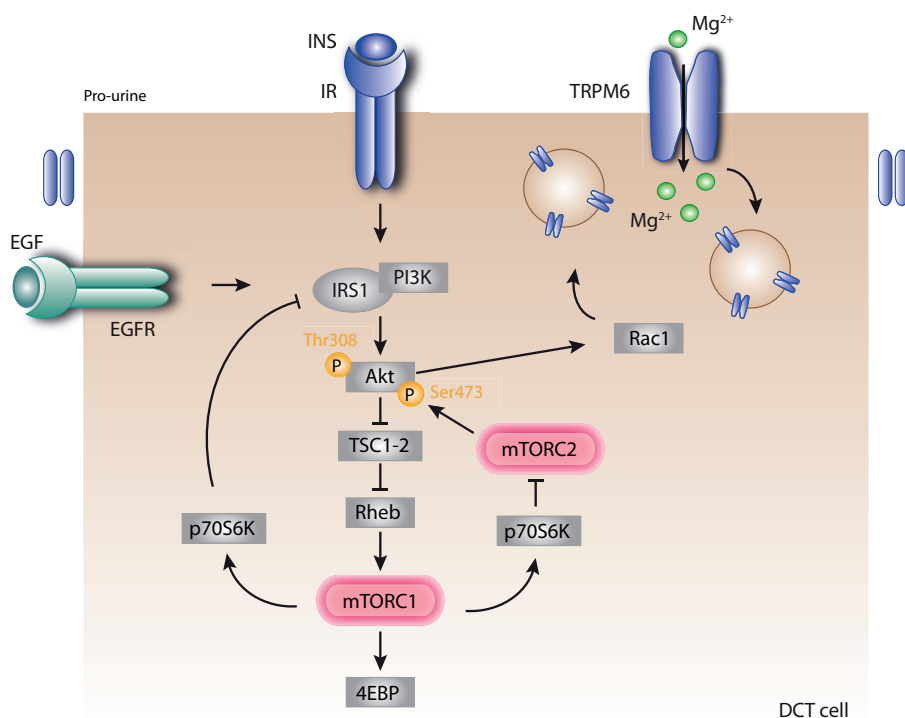

Figure 5 Schematic representation of the role of the mTOR pathway in the vesicular insertion of TRPM6.

EGFR activation by EGF binding and IR stimulation by insulin leads to the activation of a PI3K/Akt/mTOR/Rac1 pathway. Rac1 in turn increases the distribution of the endomembrane TRPM6 channel to the plasma membrane and augments the influx of Mg^{2+}.

possible that insulin and EGF-mediated pathways are not equivalent and/or that they are subjected to different feedback mechanisms.[56] Future investigations should aim to understand how EGF and insulin treatment differentially modulate the mTOR pathway and its final targets responsible for TRPM6 insertion in the plasma membrane.

The evidence that rapamycin inhibits the EGF-dependent stimulation of TRPM6 has significant relevance in cancer therapy. One of the main causes of acquired hypomagnesemia due to renal Mg²⁺ wasting is the use of the anticancer treatments cetuximab and erlotinib, two EGFR inhibitors.[34, 57, 58] Both cetuximab and erlotinib reduce EGF-stimulated TRPM6 activity and consequently impair Mg²⁺ reabsorption in the kidney.[34, 58] Of note, it is increasingly recognized that disruption of the mTOR signaling by rapamycin has an important role in cancer likely due to inhibition of cell growth and proliferation.[21, 59, 60] Temsirolimus, an analogue of rapamycin, has been recently approved by the Food and Drug Administration for the treatment of metastatic renal cell carcinoma.[20, 21] Interestingly, by the use of xenograft models derived from a variety of tissue types, it was shown that rapamycin produces a synergistic effect with erlotinib on the cell growth inhibition.[61, 62] Nevertheless, in patients, this synergistic effect could also occur with respect to the renal Mg²⁺ wasting. Thus, caution should be given when treating cancer patients receiving combinational treatment with Mg²⁺ lowering compounds, like rapamycin and EGFR inhibitors.

The clinical relevance of studying a relation between rapamycin treatment and Mg²⁺ balance extends to diabetes research. Several studies suggest that treatment with rapamycin leads to hyperglycemia and insulin resistance.[63] In two recent randomized controlled trials, rapamycin was more diabetogenic than CNI.[25, 64] Moreover, the replacement of CNI by rapamycin was associated with a worsening of insulin resistance.[65] Besides a direct diabetogenic effect of the immunosuppressive treatment,[66-68] the drug-induced hypomagnesemia can be an independent predictor of new-onset diabetes after transplantation (NODAT) in renal transplant recipients.[69] There is growing evidence suggesting that Mg²⁺ deficiency is a significant risk factor for the development of insulin resistance and subsequently diabetes mellitus type 2.[35, 70-72] In a retrospective study, van Laecke *et al.* reported that hypomagnesemia was in part causative for the CNI-induced diabetes in their cohort of patients, whereas rapamycin appeared to have an intrinsic diabetogenic effect that was not linked to Mg²⁺ levels.[69] Our observation that rapamycin does not affect the stimulatory effect of insulin on TRPM6 trafficking is in line with the hypothesis of a Mg²⁺-independent origin of the rapamycin-induced NODAT.

In conclusion, elucidation of the molecular mechanisms responsible for the rapamycin-induced renal Mg²⁺ wasting extended our knowledge of the regulation of the epithelial channel TRPM6 to the mTOR pathway, and will refine the pharmacologic therapy for the pathophysiological conditions that require rapamycin treatment.

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Acknowledgments

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General discussion

7

Introduction

The maintenance of Ca^{2+} and Mg^{2+} homeostasis is essential for many physiological functions including intracellular signaling processes, enzymatic reactions, neuronal excitability, muscle- contraction and bone formation. The human body preserves plasma Ca^{2+} and Mg^{2+} concentrations within a narrow range due to an efficient homeostatic system encompassing the parathyroid gland, intestine, bone and kidney. The last determines the final amount of Ca^{2+} and Mg^{2+} excreted in the urine by controlling the reabsorption of these cations from the pro-urine back into the blood. Overall, Ca^{2+} and Mg^{2+} fluxes across the plasma membrane of epithelial renal cells are facilitated by *i)* sensors sensitive to extracellular fluctuations in Ca^{2+} and Mg^{2+} concentrations; *ii)* endo- and paracrine hormones; *iii)* cation-selective ion channels and transporters. In general, the total capacity of a cell to mediate transport of a specific ion through the plasma membrane is determined by both the activity and the amount of ion channels and transporters at the cell surface. Many intracellular processes regulate the abundance of these molecules at the plasma membrane, including gene transcription, post-transcription, translation, post-translational modifications and trafficking.

This thesis is mainly directed at elucidating novel transcriptional networks that control gene expression of ion channels, transporters and hormones relevant to Ca^{2+} and Mg^{2+} handling in the kidney. In particular: *i)* new roles for the transcription factor HNF1B in *PTH* and *FXYD2* gene transcription are unraveled, which extend our knowledge on renal Ca^{2+} and Mg^{2+} transport; *ii)* the HNF1B-interacting protein, PCBD1 is proposed as a new molecular player in renal Mg^{2+} reabsorption in the distal convoluted tubule (DCT); *iii)* it is demonstrated that $1,25(OH)_2D_3$ downregulates *TRPC6* gene expression in podocytes injury, which is important for understanding the regulation of Ca^{2+} fluxes in glomerular (patho)physiology; *iv)* finally evidence for a role of rapamycin in the regulation of TRPM6 in the DCT is provided, which contributes to the molecular explanation of the rapamycin-induced hypermagnesuria.

Postnatal roles of HNF1B

Hepatocyte nuclear factor 1 homeobox B (HNF1B) is a developmental transcription factor first isolated in human hepatocytes.[1] HNF1B plays a crucial role in the organogenesis of several tissues, such as gut, pancreas, liver, lung, and kidney.[2] The syndrome associates with deletions and/or single point mutations in *HNF1B* is a dominantly inherited disease and is generally referred to as renal cysts and diabetes syndrome (RCAD [MIM 137920]). *HNF1B* abnormalities have drawn significant attention in pediatric nephrology as an important cause of prenatally hyperechogenic kidneys with or without cysts, whereas renal hypodysplasia with few or multiple cysts is the most frequent presentation in early

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childhood.[3, 4] The HNF1B nephropathy also comprises slow renal decline and tubular transport abnormalities, and is often accompanied by a highly heterogeneous non-renal phenotype including maturity-onset diabetes of the young (MODY [MIM 606391]) type 5, gout, pancreas hypoplasia as well as liver and genital tract abnormalities.[5, 6] More recently, hypomagnesemia with hypocalciuria and hypermagnesuria due to defective renal Mg^{2+}

Table 1 Overview of some HNF1B and HNF1A target genes identified experimentally.

Tissue	HNF1B				
	Gene	Protein	+/-	Ref.	
Kidney	URAT1	Urate anion exchanger 1	+	8	
	OAT1	Organic anion transporter 1	+	9	
	OAT3	Organic anion transporter 3	+	10	
	OAT4	Organic anion transporter 4	+	11	
	TMEM27	Collectrin	+	12	
	SOCS3	Suppressor of cytokine signaling 3	-	13	
	PkhD1	Polycystic kidney and hepatic disease 1 protein	+	14	
	Umod	Uromodulin	+	14	
	Pkd2	Polycystin-2	+	14	
	Kif12	Kinesin-like protein Kif12	+	15	
	CDH16	Ksp-cadherin	+	16	
	FXYD2	γa-subunit Na^+-K^+-ATPase	+	7 This thesis	
Liver	Vitamin D-binding protein	Vitamin D-binding protein	-	17	
	AFM	α-albumin	+	18	
Pancreas	GLUT2	Glucose transporter member 2	+	19	
Parathyroid	PTH	Parathyroid hormone	-	This thesis	

Tissue-speficic expression of genes whose transcription is regulated by HNF1B and/or HNF1A. +/-: positive or negative transcriptional regulation of the target genes by the HNF1 factors; Ref.: number of the corresponding reference in the text.

reabsorption in DCT was observed in up to 50% of children with *HNF1B* abnormalities.[7] **Table 1** summarizes some relevant target genes of HNF1B and of its homolog HNF1A in postnatal tissues.[7-32] HNF1B and HNF1A form heterotetramers and bind to the same DNA consensus sequence, but may regulate gene transcription with different potency.[9, 18] Of note, HNF1B can act both as inhibitor and as stimulator of gene transcription, most

		HNF1A			
		Gene	Protein	+/-	Ref.
		URAT1	Urate anion exchanger 1	+	8
		OAT1	Organic anion transporter 1	+	9
		OAT3	Organic anion transporter 3	+	10
		OAT4	Organic anion transporter 4	+	11
		TMEM27	Collectrin	+	12
		NPT1	Na+-dependent Pi transport protein 1	+	20
		NPT4	Na+-dependent Pi transport protein 4	+	20
		SGLT2	Na+-glucose cotransporter 2	+	21 22
		GLUT2	Glucose transporter member 2	+	23
		CLCN5	H+-Cl--exchange transporter 5	+	24
		Vitamin D-binding protein	Vitamin D-binding protein	-	17
		AFM	α-albumin	+	18
		PAH	Phenylalanine-4-hydroxylase	+	25
		Serpina1	α1-antitrypsin	+	26
		FGA/FGB	α/β-fibrinogen	+	27
		OATP1B1-3	Solute carrier organic anion transporter member 1B1-3	+	28
		GLUT2	Glucose transporter member 2	+	29
		INS-1	Insulin	+	30
		L-PK	L-type pyruvate kinase	+	29
		TMEM27	Collectrin	+	31
		PDX-1	Pancreas/duodenum homeobox protein 1	+	32

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probably due to the binding to alternative interacting proteins and/or due to different epigenetic modifications. Importantly, it was demonstrated that HNF1B plays a role in the epigenetic bookmarking of gene expression throughout mitotic chromatin condensation.[33]

To date, several questions remain unanswered concerning the role of HNF1B in renal electrolyte handling, and many of its upstream regulators and downstream targets are still unknown. The studies presented in **chapters 2**, **3** and **4** of this thesis extend our knowledge about the (patho)physiological relevance of the HNF1B-mediated transcription in the homeostatic control of Ca^{2+} and Mg^{2+} via the identification of the HNF1B target genes, *FXYD2a* and *PTH* (**Chapter 2** and **3**, respectively), and the functional characteriza-tion of mutations in the HNF1B interacting protein PCBD1 (**Chapter 4**).

HNF1B stimulates *FXYD2a* gene expression in the DCT

HNF1B nephropathy is one of the few monogenic forms of renal hypomagnesemia identified at the molecular level so far.[34] In the postnatal kidney, HNF1A expression is restricted to the proximal tubule (PT), whereas HNF1B is expressed in the epithelial cells throughout the entire nephron. However, the hypomagnesemia reported in almost 50% of the patients with *HNF1B* mutations specifically originates in the DCT, where a defect in active Mg^{2+} reabsorption leads to renal Mg^{2+} loss accompanied by secondary hypocalciuria. The mechanism leading to this phenotype is ascribed to the direct control of the *FXYD2* gene expression by HNF1B in the DCT. Bioinformatical prediction tools in combination with luciferase reporter assays and chromatin immunoprecipitation (ChIP) experiments confirmed the presence of HNF1B binding sites in the *FXYD2* gene promoter.[7] *FXYD2* encodes the γ-subunit of the Na^+-K^+-ATPase.[35] At the basolateral membrane of the DCT, the Na^+-K^+-ATPase generates opposing Na^+ and K^+ gradients that allow high transcellular transport rate of Na^+ and favor the formation of a negative membrane potential. In DCT, active Mg^{2+} reabsorption from the pro-urine into the blood occurs via the apical epithelial Mg^{2+} channel, transient receptor potential melastatin 6 (TRPM6). In this segment of the nephron, the chemical driving force for Mg^{2+} is limited because the extra- and intracellular Mg^{2+} concentrations are in the same millimolar range. Thus, Mg^{2+} reabsorption through TRPM6 is primarily driven by a favorable membrane potential established by the voltage-gated K^+ channel, Kv1.1, and energized by the action of the Na^+-K^+-ATPase. Other proteins play key roles in regulating Mg^{2+} balance within the DCT, either by directly affecting TRPM6 or by altering the driving force for Mg^{2+} influx via the channel, like the thiazide-sensitive Na^+-Cl^--cotransporter (NCC), the Cl^- channel CLC-Kb and the K^+ channel Kir4.1.[36] Interestingly, the c.121G>A (p.Gly41Arg) mutation in *FXYD2* is the underlying defect in isolated dominant hypomagnesemia with hypocalciuria (IDHH; OMIM 154020).[37, 38]

The *FXYD2* gene can be alternatively transcribed by activation of the promoter prior to exon γa or exon γb (**Figure 1**). Therefore, the γ-subunit of the Na$^+$-K$^+$-ATPase exists in two isoforms, γa- and γb-subunit, that differ just at their extracellular N-termini. Because the

Distal convoluted tubule cell
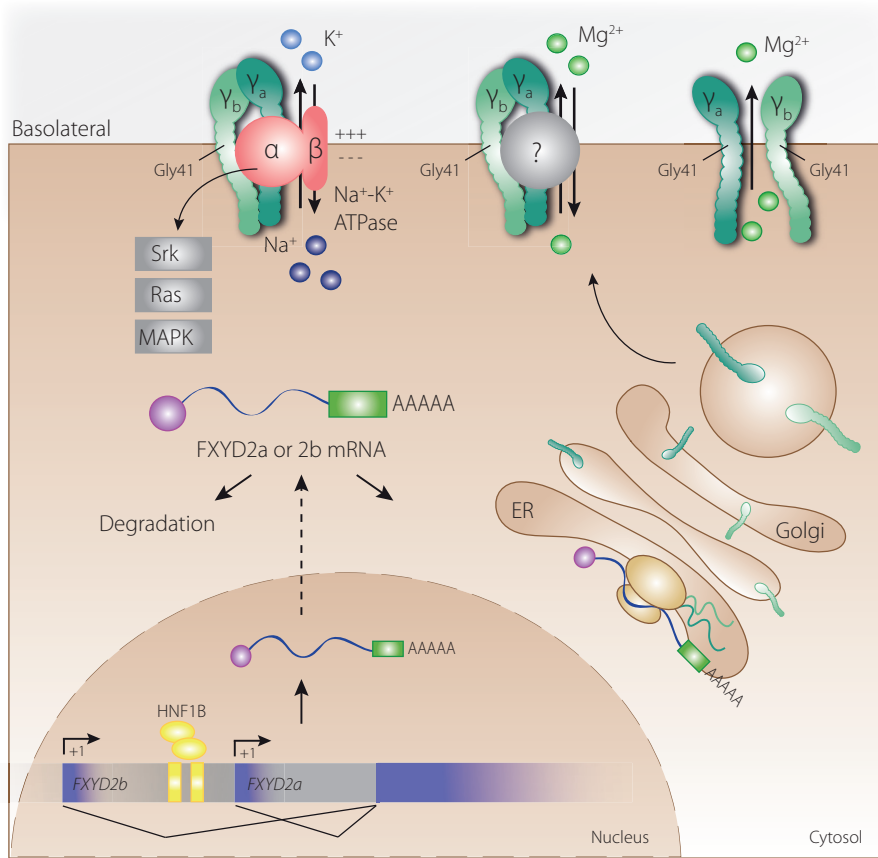
Figure 1 Overview of the regulatory mechanisms of the γ-subunit isoforms in a DCT cell model.

HNF1B exclusively transactivates *FXYD2a* expression. Subsequently, FXYD2a and FXYD2b transcripts can encounter degradation or translation into proteins. Changes in the γa/γb-subunits abundance at the basolateral membrane due to either a γ-subunit p.Gly41Arg mutation or HNF1B mutations could lead to hypomagnesemia via several mechanisms: i) misregulation of the Na$^+$-K$^+$-ATPase activity, as pump or signaling molecule; ii) dysregulation of a still unknown Mg^{2+} extrusion mechanisms; ii) impaired Mg^{2+} extrusion by γa/γb oligomers. ER: endoplasmic reticulum; Src: proto-oncogene tyrosine-protein kinase; MAPK: mitogen-activated protein kinase; +1: transcription initiation site.

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HNF1B binding sites are located upstream the transcription initiation site of exon γa but downstream exon γb, in **chapter 2** of this thesis it was tested whether both promoters are regulated by HNF1B. By use of two different reporter gene assays, it was demonstrated that HNF1B specifically acts as an activator of *FXYD2a* promoter activity, whereas *FXYD2b* expression was not affected. Moreover, the HNF1B mutations p.His69fsdelAC, p. His324Ser325fsdelCA, p.Tyr352fsinsA and p.Lys156Glu prevented transcriptional activation of *FXYD2a* via a dominant negative effect on wild-type HNF1B. Renal Na^+-K^+-ATPase is associated with the γ-subunit at the basolateral membrane of all nephron segments, but to a less extent in cortical TAL (cTAL) and cortical collecting ducts (cCD), where the γ-subunit is expressed at lower levels.[39, 40] In the kidney cortex, γa-subunit was described to be the predominant isoform in PT, whereas γb-subunit was the main isoform in cTAL, DCT and CNT.[39, 40] In **chapter 2**, it was demonstrated by immunohistochemistry on mouse kidney sections that γ*a*-subunit is also expressed in the DCT where it co-localizes with the γb-subunit. In conclusion, it is suggested that abnormalities in *HNF1B* impair the relative abundance of γa- and γb-subunit, thus affecting the transcellular Mg^{2+} reabsorption in DCT.

Physiological implications of FXYD2 expression

FXYD proteins are a family of seven small single span membrane proteins that regulate the Na^+-K^+-ATPase by modifying its kinetic properties in a tissue-specific manner.[35] The γ-subunit is the most abundant protein of the FXYD family expressed in the kidney. Both γa and γb variants modify the Na^+-K^+-ATPase activity via reduction of the affinity for Na^+ and decrease of the V_{max}.[40-42] Furthermore, *in vitro* assays using recombinant proteins and functional studies using the γ-deficient renal Na^+-K^+-ATPase isolated from *FXYD2* knockout (*FXYD2$^{-/-}$*) mice demonstrated that the γ-subunit strongly protects the pump against thermal denaturation.[43, 44] This evidence indicates that the γ-subunit plays a structural role that is important for the stability of the pump at the basolateral membrane of renal cells.[43, 44] It is paradoxical that on one side the γ-subunit reduces the Na^+-K^+-pumping capacity, whereas on the other side it stabilizes the Na^+-K^+-ATPase at the cell surface, which over time increases the pump density and capacity. Most probably a combination of these two actions determines the final biological effect on the Na^+-K^+-ATPase. In order to understand the physiological role of the γ-subunit, it is important to consider that none of the established kidney-derived cell lines express the γ-subunit under regular culture conditions. However, it is possible to induce *FXYD2* gene expression in kidney cells by several stress-inducers, for example hypertonicity, heat shock, exogenous oxidation and chemical stress.[45, 46 47] Importantly, induction of *FXYD2* upon cellular insults appears to be a protective mechanism essential for cell survival.[48]

Of note, the γ-subunit p.Gly41Arg mutation prevents trafficking of wild-type γ-subunit, but not of the α/β subunits of the Na^+-K^+-ATPase, to the cell surface where the lack of

wild-type γ-subunit abrogates its functional effects on the pump.[37, 49] To date, it is still unknown how changes in the γ-subunit expression at the basolateral membrane of DCT, due to γ-subunit p.Gly41Arg mutation or HNF1B mutations, cause hypomagnesemia. Several hypotheses can be postulated (**Figure 1**): *i*) over time, the lack of association with γ-subunit and loss of stabilization of α/β subunits compromise the ability of the cell to maintain the driving forces, i.e. trans-epithelial voltage and Na^+ gradients necessary for active Mg^{2+} transport via TRPM6;[43, 46] *ii*) changes in the relative abundance of γa- and γb-subunit can modulate the Na^+-K^+-ATPase activity and thereby affect the transcellular Mg^{2+} reabsorption. Although, both γa and γb isoforms decrease the Na^+ affinity of the pump, it was shown that post-translation modifications can alter the affinity of the pump for Na^+, K^+ and ATP in a isoform-specific manner;[42, 50] *iii*) a diminished γ-subunit oligomer-ization may affect the ability of this protein to directly mediate extrusion of Mg^{2+} towards the blood.[51, 52] However, this hypothesis seems unlikely due to the unfavorable electro-chemical Mg^{2+} gradient across the basolateral membrane *in vivo*; *iv*) a decrease in γ-subunit expression may affect a still unknown Mg^{2+} extrusion mechanism. Recently, the CNNM2 protein was proposed to participate in the basolateral Mg^{2+} extrusion in DCT.[53] It would be of interest to test the effect of the γ-subunit on the Mg^{2+}-sensitive Na^+ currents mediated by CNNM2 *in vitro*; *v*) finally, γa- and γb-subunit could differentially modulate the ability of the Na^+-K^+-ATPase to act as a receptor and to initiate the ouabain signaling pathway. The latter, in turn, could affect the activation status of important molecular players involved in Mg^{2+} reabsorption in the DCT.[54, 55] Surprisingly, *FXYD2^{-/-}* mice exhibit normal blood and urine Mg^{2+} values,[44] indicating that the presence of wild-type γ-subunit *per se* is not required for the maintenance of the Mg^{2+} balance. This evidence may suggest the existence of a compensatory mechanism by other renal FXYD proteins in mice compared to humans. Furthermore, inducing hypomagnesemia in *FXYD2^{-/-}* mice, for example via a low Mg-containing diet, may be necessary to generate a stress in the tubular cells of the kidney and therefore observe a renal phenotype in these animals. Overall, the high complexity in the regulation of the renal Na^+-K^+-ATPase via the γ-subunit isoforms suggests how small changes in the activity of these proteins can significantly unbalance the electrolyte handling by the kidney. Further investigation is needed to unravel if and how γ-subunit isoforms differentially affect active Mg^{2+} reabsorption in the DCT. So far, Mg^{2+} research has been obstructed by the absence of appropriate techniques to monitor changes in Mg^{2+} concentrations in cell samples. Recently, our laboratory successfully implemented the use of the stable isotope ^{25}Mg to measure Mg^{2+} transport by cell monolayers. The measurement of ^{25}Mg content in the apical and basolateral medium of polarized kidney cells co-transfected with TRPM6 and each of the γ-subunit isoforms represents a valuable approach to study the role of the γ-subunit in Mg^{2+} transcellular transport.

HNF1B downregulates *PTH* gene expression in parathyroid glands

Chapter 3 describes *PTH* as previously unrecognized HNF1B target gene. Parathyroid hormone (PTH) is released into the circulation by the parathyroid glands in response to changes in the extracellular Ca^{2+} and PO_4^{3-} concentrations. PTH subsequently restores Ca^{2+} and PO_4^{3-} homeostasis by regulating the synthesis of active vitamin D (1,25-dihydroxyvitamin D_3, or $1,25(OH)_2D_3$) in PT, altering Ca^{2+} and PO_4^{3-} (re)absorption in the kidney and intestine, and modulating bone metabolism.[56-58] Serum PTH levels depend on both the rapid secretion of PTH stored in secretory granules within the parathyroid gland and the synthesis of new PTH molecules secondary to *PTH* gene transcription (**Figure 2**). Of note, secondary hyperparathyroidism (HPT) classically occurs during the course of chronic kidney disease (CKD). Under physiological conditions, a low plasma Ca^{2+} reduces the activity of the Ca^{2+}-sensing receptor (CaSR) on the surface of parathyroid glands, which leads to the rapid release of PTH from the secretory granules and promotes PTH mRNA stability.[59, 60] Conversely high Ca^{2+} inhibits PTH secretion and favors PTH mRNA degradation.[59, 60] Contrary to Ca^{2+}, high PO_4^{3-} leads to increased PTH levels.[60, 61] Furthermore, *PTH* transcription is repressed by binding of a complex of $1,25(OH)_2D_3$, the $1,25$-D_3 receptor (VDR) and retinoic acid receptor (RXR), to vitamin D responsive elements (VDRE) in the promoter region of the *PTH* gene.[62, 63] In CKD, the increase in plasma PO_4^{3-}, the prolonged decrease in plasma Ca^{2+} as well as a reduced 1α-hydroxylase-mediated $1,25(OH)_2D_3$ conversion lead to a secondary increase in serum PTH.[64] The study reported in **chapter 3** provides evidence of early HPT disproportionate to the renal function in a cohort of eleven patients with *HNF1B* deletions or mutations, due to the direct involvement of HNF1B in *PTH* gene transcription. In our cohort of *HNF1B* patients, two cases showed HPT compatible with eGFR or creatinine clearance values, whereas the remaining patients displayed a discrepant correlation between PTH levels and kidney function. The possibility of diabetic nephropathy is excluded since our patients were not diagnosed MODY. Serum Ca^{2+} and PO_4^{3-} levels were both within the normal range, as well as the renal tubular reabsorption of PO_4^{3-}. Hypomagnesemia was diagnosed in the majority of individuals. Importantly, hypomagnesemia is clinically associated with a paradoxical block of PTH secretion and a resulting hypoparathyroidism rather than HPT.[65, 66] This supports the hypothesis of a putative role of *HNF1B* abnormalities in the development of HPT independent from extracellular Mg^{2+} levels. Interestingly, most of the HNF1B patients in the cohort displayed hypocalciuria. It is suggested that the early HPT may contribute to this phenotype. Due to the retrospective character of the study, data concerning $1,25(OH)_2D_3$ and 25-OH-D_3 levels were not available for analysis. Immunohistochemical analysis demonstrated that HNF1B is expressed in the nuclei of PTH-positive cells of human parathyroid gland and in the rat parathyroid PT-r cell line. PT-r cells were the first available immortalized cell line that retained characteristics of parathyroid cells, i.e.

secretion of PTH into the cell culture medium and sensitivity to Ca^{2+} regulation.[67] Subsequent characterizations of these cells reported that PT-r cells retain PTH expression sensitive to Ca^{2+} and $1,25(OH)_2D_3$ treatment, but did not endogenously express CaSR and VDR.[68, 69] Furthermore, they mainly express and secrete PTH-related peptide (PTHrP) compared to PTH.[70] Based on these studies, HNF1B over- and under-expression levels in PT-r cells would provide limited advances in the understanding of the role of HNF1B in parathyroid (patho)physiology. Alternatively, the human embryonic kidney cells (HEK293) were used to perform further investigations. *In vitro* studies showed that wild-type HNF1B
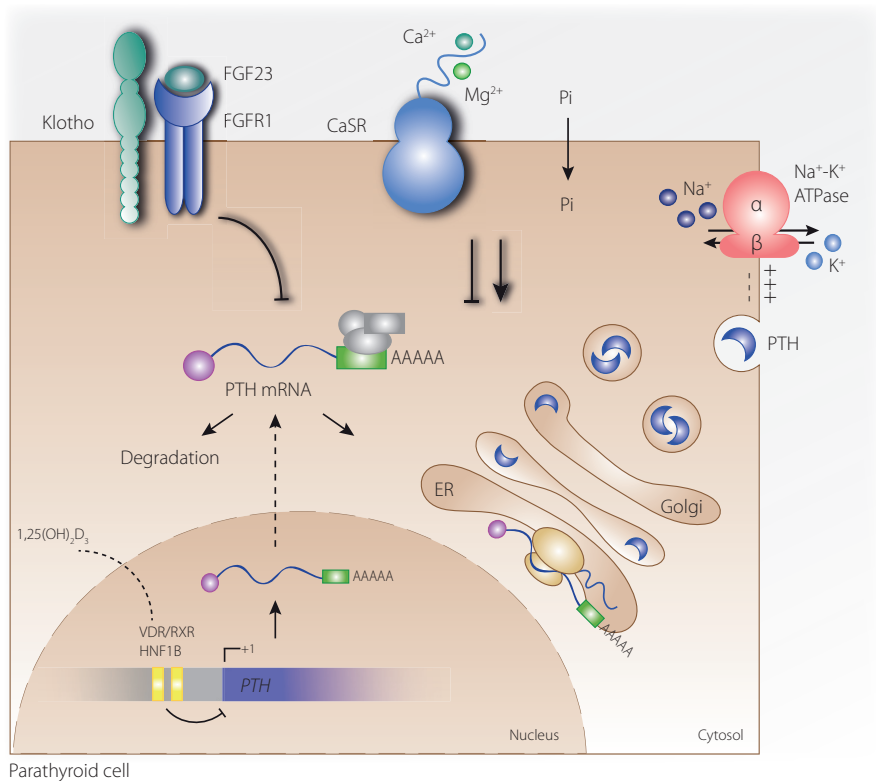
Parathyroid cell

Figure 2 Overview of the regulatory mechanisms of PTH expression and secretion in the parathyroid gland.

HNF1B and $1,25(OH)_2D_3$ negatively regulate *PTH* gene expression. Activation of the complex Klotho/FGFR1 by FGF23 and changes in the extracellular Ca^{2+} and Pi modulate instead *PTH* mRNA stability and secretion. VDR: vitamin D receptor; RXR: retinoic acid X receptor; FGF23: fibroblast growth factor 23; FGFR1: fibroblast growth factor receptor 1; CaSR: Ca^{2+} sensing receptor; Pi: inorganic phosphate; ER: endoplasmic reticulum; +1: transcription initiation site.

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inhibits *PTH* promoter activity with a maximal reduction of 30% upon binding to responsive elements that resides -200/-70 bp from the transcription initiation site, while HNF1B mutants lack this inhibitory effect. Taken together, these data suggest that HNF1B is a transcriptional repressor of *PTH* gene expression in the parathyroid gland, which could explain the early development of HPT in patients with *HNF1B* mutations or deletions. An intriguing additional explanation for the phenotype observed in our cohort of patients could involve a putative regulation of CaSR by HNF1B. Interestingly, patients affected by familial hypocalciuric hypercalcaemia (FHH [MIM 145980]) due to inactivating mutations in *CaSR* present with normal or increased PTH levels and hypocalciuria. Therefore, a hypothesis could be that *HNF1B* mutations downregulate *CaSR* expression leading to a FHH-like phenotype including HPT and hypocalciuria. This clinical presentation would be the result of the effect of HNF1B on *PTH* and *CaSR* expression in the parathyroid together with an effect of HNF1B on *CaSR* expression in kidney. *In silico* and *in vitro* data should investigate the presence of functional HNF1B binding sites in the promoter region of *CaSR*. Other downstream HNF1B target genes that could be relevant in setting circulating PTH levels are the *FXYD* genes. Since the Na^+-K^+-ATPase pump is essential to generate the driving force for PTH secretion,[71] the impaired transcriptional regulation of *FXYD* family members due to *HNF1B* abnormalities may contribute to the onset of HPT. Preliminary data in **chapter 3** showed that neither *FXYD2a* nor *FXYD2b* are expressed in human cadaveric parathyroid samples. This does not exclude that *FXYD2* expression may be induced under certain pathological conditions (e.g. primary or secondary HPT due to uremia or parathyroid cancer), or that other FXYD protein may be involved. Overall, many regulatory pathways that control PTH secretion, both transcriptionally and post-transcriptionally, have been defined (**Figure 2**) and several are currently therapeutic targets for the treatment of secondary HPT in the course of CKD, including active vitamin D analogs and calcimimetics.[57, 72-75] Recently, it was shown that *PTH* gene transcription is also inhibited by Fibroblast Growth Factor 23 (FGF23), a novel phosphaturic hormone that acts though the FGFR1/Klotho receptor complex present in parathyroid cells.[76, 77] In this respect, little is known about the signaling cascades in which HNF1B is the final effector in adult tissues. Signaling downstream the FGF23-FGFR1/Klotho axis as well as the CaSR and interaction with the VDR/RXR complex may affect HNF1B-mediated transcription. Further studies are needed to evaluate the role of HNF1B in the responsiveness of parathyroid cells to extracellular Ca^{2+}, $1,25(OH)_2D_3$ and FGF23.

PCBD1: an important player in the HNF1-mediated transcription

The bifunctional protein pterin-4 alpha-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (PCBD1) is able to co-activate the HNF1-mediated

transcription in the nucleus of the cell.[78] In **figure 3** the main organs co-expressing PCBD1, HNF1A and HNF1B are reported together with representative HNF1 target genes relevant to tissue physiology. Additionally, in the cytosol of kidney and liver cells, PCBD1 (EC 4.2.1.96) is required for tetrahydrobiopterin (BH_4) regeneration during the catalytic event of phenylalanine hydroxylation by the enzyme phenylalanine-4-hydroxylase (PAH [EC 1.14.16.1]). **Chapter 4** describes PCBD1 as an important regulator of the transcriptional machinery that associates with HNF1B in kidney and that contributes to Mg^{2+} reabsorption
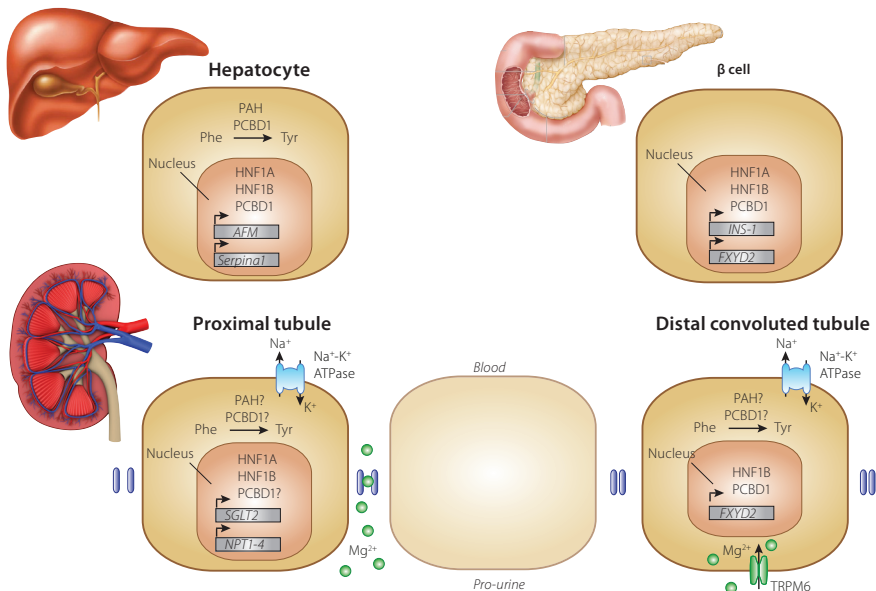
Figure 3 Schematic representation of the PCBD1, HNF1A and HNF1B transcriptional network.

In the adult pancreas, HNF1A regulates the expression of *INS-1*, possibly via HNF1B and PCBD1 interaction. A putative role of HNF1 factors in the transcriptional regulation of *FXYD2* in pancreas can be speculated. The transcription of many liver-specific proteins is under the control of HNF1 factors. Furthermore, in the liver, cytosolic PCBD1 is involved in the phenylalanine metabolism together with the enzyme phenylalanine-4-hydroxylase (PAH). After the liver, the kidney is the second tissue with the highest abundance of the *PAH* transcript. In the kidney, *HNF1A* is mainly expressed in the proximal tubule (PT), whereas *HNF1B* is expressed in all segments of the nephron. In the PT, HNF1A regulates the expression of many genes, among others *SGLT2* and *NPT1-4*. In the distal convoluted tubule (DCT), HNF1B is involved in the transcellular reabsorption of Mg^{2+} from the pro-urine into the blood through TRPM6 via regulation of *FXYD2*. ?: question marks indicate the still not defined localization of PCBD1 and PAH in PT and DCT. *AFM*: α-albumin; *FXYD2*: γ-subunit of the Na+-K+-ATPase; *INS-1:* insulin; *NPT*: Na+-dependent Pi transport protein; *Serpina 1*: α1-antitrypsin; *SGLT2*: Na+-glucose co-transporter 2.

in the DCT. Hypomagnesemia with renal Mg^{2+} wasting was reported in two patients who were diagnosed neonatal hyperphenylalaninemia and primapterinuria (HPABH4D [MIM 264070]) due to homozygous p.Gln97Ter and p.Glu26Ter/p.Arg87Gln mutations in the *PCBD1* gene. A similar renal manifestation is reported in 50% of the patients with *HNF1B* mutations and, therefore, suggest that the ability of PCBD1 to co-activate HNF1-mediated transcription may be affected in patients with HPABH4D. HPABH4D is a benign and transient condition that resolves after daily BH_4 supplementation and/or diet control, and the patients demonstrated normal growth and development.[79] Importantly, the study presented in **chapter 4** is the first follow-up investigation of patients affected by HPABH4D reporting the onset of late complications linked to the defected activity of PCBD1 as transcriptional co-activator. In patient 1, hypomagnesemia was corrected with oral Mg^{2+} supplements at a dose of 500 mg/day, though at the expense of hypermagnesuria. Furthermore, symptoms of the hypomagnesemia like fatigue, muscular pain, weakness and cramps in arms improved after Mg^{2+} supplementation. Renal function (GFR 128 mL/min per 1.73 m^2) resulted to be normal whereas ultrasonography showed slightly increased echogenicity of both liver and kidney with no evidence of renal cysts. The absence of any abnormality in blood and urine Ca^{2+} levels in the reported patients pinpoints to a primary defect in Mg^{2+} reabsorption in DCT, rather than impairment of Ca^{2+} and Mg^{2+} reabsorption in the TAL. Gene expression analysis in sorted DCT-eGFP tubules proved that PCBD1 is present in the DCT, where HNF1B regulates active Mg^{2+} reabsorption by controlling *FXYD2* expression (**chapter 2**). Future immunohistochemical analysis should aim to identify the exact (sub)cellular localization of PCBD1 in the different nephron segments. Based on the nuclear or cytosolic localization of PCBD1, it will be possible to speculate on segment-specific functions of the protein. To study the effect of PCBD1 patient mutations on the interaction with HNF1B (**Figure 4**), HEK293 cells were transiently co-transfected with PCBD1 mutants and wild-type HNF1B, and co-immunoprecipitation experiments were performed. Briefly, PCBD1 p.Glu26Ter, p.Glu86Ter, p.Glu96Lys and p.Gln97Ter were not expressed, whereas p.Thr78Ile and p.Cys81Arg were expressed significantly less than the wild-type protein due to proteasomal degradation. PCBD1 p.Arg87Gln was the only mutant showing a comparable expression level to the wild-type protein. Nevertheless, in patient 2 p.Arg87Gln is homozygously present on both alleles with a p.Glu26Ter mutation that most probably lead to the early degradation of the PCBD1 transcript by mRNA surveillance mechanisms.[80, 81] All the PCBD1 mutants that show protein expression (p.Thr78Ile, p.Cys81Arg and p.Arg87Gln) retained the ability to bind HNF1B suggesting that the mutations did not cause structural rearrangements at the interaction interface with HNF1B. Based on a structural homology model of the PCBD1–HNF1B dimerization domain (HNF1B-D) complex, the residue stretches from Asn44 to Glu58 in PCBD1 and from Leu5 to Val21 in HNF1B are essential for protein-protein interactions (**Figure 4**). *FXYD2*-promoter co-activation was compatible to the expression levels of the PCBD1 proteins. Wild-type PCBD1 increased *FXYD2* promoter activation by

HNF1B of approximately 1.5 fold. However, among all PCBD1 mutants, only PCBD1 p. Arg87Gln and p.Cys81Arg maintained their co-activator activity. Surprisingly, despite similar protein expression to p.Cys81Arg, PCBD1 p.Thr78Ile was not functional. Prediction tools excluded Thr78 to be a putative phosphorylation site that could therefore play a role in the regulation of PCBD1 activity (**Figure 4**). Alternatively, mutations at Thr78 or Cys81 may have different structural consequences on the PCBD1-HNF1B interaction site. The screening of patients with PCBD1 p.Thr78Ile and p.Cys81Arg mutations for complications related to the HNF1B disease could confirm the *in vitro* findings. Subsequently, the hypothesis that HNF1B mutations could affect PCBD1 binding and FXYD2-promoter co-activation was tested. All the HNF1B mutants (p.Lys156Glu, p.Gln253Pro, p.Arg276Gly, p.His324Ser325fsdelCA, p.Tyr352fsinsA) had an intact dimerization domain and, therefore, showed binding to wild-type PCBD1, whereas only the HNF1B p.His324Ser325fsdelCA and p.Tyr352fsinsA mutants, that retained a residual transcriptional activity, showed response to PCBD1 co-activation. Interestingly, immunocytochemical experiments showed that HNF1B p.His324Ser325fsdelCA and p.Gln253Pro mutations disturb PCBD1 localization in the nucleus compared to wild-type HNF1B, leading to an accumulation of PCBD1 in the cytosol. Of note, HNF1B p.Gln253Pro was previously associated with hypomagnesemia, whereas no definitive correlation has been reported for the other mutant.[82] This finding
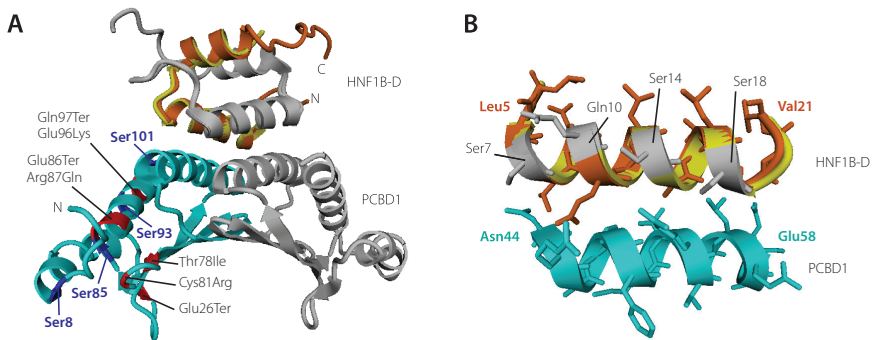
Figure 4 Structural model of the PCBD1–HNF1B interaction.

(A) Homology model of the PCBD1–HNF1B dimerization domain (HNF1B-D) tetramer, modeled using the structure of the PCBD1–HNF1A dimerization domain (HNF1A-D) complex (PDB file 1F93). The PCBD1 dimer (light blue and grey) binds the HNF1B dimer (orange and grey) via helix sequences. The HNF1A-D monomer is shown is yellow. Residues in the PCBD1 protein that were found mutated in patients affected by hyperphenylalaninemia are depicted in red. In blue, the amino acid residues predicted as putative phosphorylation sites by NetPhos 2.0. **(B)** Homology model of the interaction site within the PCBD1–HNF1B dimerization domain (HNF1-D) complex. The bound HNF1B monomer (orange) forms a helix bundle with PCBD1 monomer (light blue). The HNF1A-D monomer is shown in yellow. The residues that differ between HNF1B-D and HNF1A-D are visualized in grey.

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suggests that the instability of the PCBD1-HNF1B complex in the nucleus will favor a cytosolic localization of PCBD1 resulting in a decreased co-activation of HNF1B at the promoter of its target genes. Thus, an increase of PCBD1 in the cytosol of the cell could contribute to the onset of hypomagnesemia in some HNF1B patients. To date, five additional proteins, apart from PCBD1, were identified by two-hybrid system as HNF1B-interacting partners in the human fetal kidney, i.e. DAK, E4F1, HADH, TRIM26 and ZFP36L1.[83] It is not known whether the binding of HNF1B to all these proteins is relevant in the adult kidney. Assuming that genes encoding for proteins binding HNF1B are potential magnesiotropic genes, subjects with idiopathic hypomagnesemia could be screened for mutations in those genes. Furthermore, screening of HNF1B patients for polymorphisms in interacting proteins should be considered. Since the presentation and development of HNF1B disease is diverse, variations in HNF1B binding partners may be responsible for the phenotypic heterogeneity.

Extra-renal findings in patients with PCBD1 mutations

In the study reported in **chapter 4**, patient 1 was also diagnosed with MODY. MODY is a monogenic form of autosomal dominant type II diabetes. Mutations in *HNF1B* or its homolog *HNF1A* associate with MODY type 5 and type 3, respectively.[84] While MODY5 is mainly linked to pancreas hypoplasia secondary to agenesis, MODY3 shows an abnormal insulin secretion due to impaired growth and function of β–cells. Knowing that PCBD1 acts as transcriptional co-activator of both HNF1B and HNF1A, it cannot be excluded that a concomitant impaired regulation of HNF1B and HNF1A activity may be responsible for the diabetes observed in the patient. The fact that MODY characterizes for dominant inheritance, while mutations in *PCBD1* associate with a recessive phenotype, suggests that HNF1 factors are important in controlling the expression of genes essential for glucose metabolism. On the other hand, HNF1-mediated transcription is impaired only when both *PCBD1* alleles are affected, meaning that PCBD1 probably belongs to an ancillary regulatory mechanism to which other HNF1B partners may participate.[83, 85] Accordingly, HNF1 function in *PCBD1* knockout (*PCBD1$^{-/-}$*) mice is only slightly impaired, as animals are mildly glucose-intolerant in contrast to the *HNF1A* knockout (*HNF1A$^{-/-}$*) mice, which are diabetic.[86] Of note, the gene expression of *PCBD1*, glucose transporter member 2 (*GLUT2*), insulin (*INS-1*) and L-type pyruvate kinase (*L-PK*) was significantly reduced after birth in the pancreatic β-cells of *HNF1A$^{-/-}$* mice, suggesting that these changes are caused by HNF1A deficiency and are likely to contribute to the molecular defects in *HNF1A$^{-/-}$* islets.[87] Interestingly, FXYD2 was recently proposed as a novel pancreatic β-cell biomarker.[88] Furthermore, *FXYD2$^{-/-}$* mice are more glucose tolerant compared to the wild-type animals which probably resides in the substantial hyperplasia in pancreatic β-cells observed in the knockout mice.[89] In the future, the study of the regulatory axis including PCBD1, HNF1B/HNF1A and FXYD2 in the pancreas will provide new insights into insulin sensitivity and glucose metabolism.

Additional extra-renal findings in PCBD1 patients include unexplained elevated liver enzymes.[82] However, in patient 1, ALT, AST, and ALP were in the lower-to-normal range. Interestingly, low plasma hs-CRP levels were detected, which is in line with two recent studies showing that subjects with MODY3 maintain substantially, lower levels of hs-CRP than individuals with other forms of diabetes, including MODY5, or nondiabetic control subjects.[90, 91] This evidence favors the diagnosis of MODY3-like diabetes in patient 1.

In conclusion, patients affected by HPABH4D should be monitored for late complications related to the lack of interaction between PCBD1 and HNF1 factors, including hypomagnesemia and MODY.

1,25(OH)$_2$D$_3$ regulates TRPC6 expression in podocyte injury

1,25(OH)$_2$D$_3$ plays an important role in the hormonal regulation of Ca^{2+} homeostasis. Two key channels involved in Ca^{2+} (re)absorption, TRPV5 and TRPV6, as well as PTH and the Ca^{2+} binding proteins calbindin-D$_{28K}$ and calbindin-D$_{9K}$, are transcriptionally regulated by 1,25(OH)$_2$D$_3$.[92-94] Beyond its homeostatic role, clinical and pre-clinical studies demonstrated that treatment with vitamin D analogs reduces proteinuria and podocyte loss in glomerular disease.[95-98] The anti-proteinuric activity of 1,25(OH)$_2$D$_3$ involves the transcriptional regulation of some key podocyte proteins, like nephrin and podocin, that preserve glomerular filtration by maintaining a functional glomerular slit diaphragm complex.[99, 100] Interestingly, the receptor-mediated TRPC6 channel was recently shown to be part of this complex.[101] *TRPC6* gain-of-function mutations are associated with a hereditary form of focal segmental glomerulosclerosis (FSGS).[101, 102] Furthermore, several other inherited and acquired proteinuric diseases associate with an increased glomerular TRPC6 expression and activity.[101-106] The data presented in **chapter 5** demonstrated that 1,25(OH)$_2$D$_3$ is able to reduce the enhanced *TRPC6* expression in *in vitro* and *in vivo* models of podocyte injury, suggesting that the TRPC6 downregulation potentially contribute to the anti-proteinuric effect of 1,25(OH)$_2$D$_3$. By use of an adriamycin nephropathy (AN) model for human FSGS in rats, it was shown that increased glomerular *TRPC6* expression and proteinuria were both significantly ameliorated by 1,25(OH)$_2$D$_3$ treatment, in association with a decreased expression of the podocyte injury marker desmin. *In vitro*, the enhanced *TRPC6* expression in injured podocytes was dose-dependently reduced by 1,25(OH)$_2$D$_3$ application, whereas no effect of 1,25(OH)$_2$D$_3$ on *TRPC6* expression was seen in uninjured podocytes. ChIP experiments using chromatin from opossum kidney (OK) cells expressing a TRPC6 promoter construct demonstrated that the downregulation of *TRPC6* promoter activity upon 1,25(OH)$_2$D$_3$ treatment occurs via direct binding of the VDR/RXR complex with the TRPC6 promoter. Interestingly, 25-hydroxy-1α-hydroxylase$^{-/-}$ mice showed significantly enhanced glomerular *TRPC6* expression levels, podocyte foot process effacement and remarkable proteinuria. 1,25(OH)$_2$D$_3$ supplementation reversed both the increased *TRPC6*

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expression as well as the proteinuria, and normalized podocyte morphology. The possibility that low concentrations in serum Ca^{2+} in the 25-hydroxy-1α-hydroxylase$^{-/-}$ mice may interfere with TRPC6 function and, therefore, contribute to the observed phenotype, can be excluded, because the relatively low dose of $1,25(OH)_2D_3$ supplementation did not significantly normalize the hypocalcemia in these mice. Anyway, it is important to consider that additional mechanisms could indirectly contribute to the downregulation of TRPC6 expression by $1,25(OH)_2D_3$ in podocyte injury. Firstly, in adriamycin-induced podocytes damage, angiotensin II (AngII) enhances TRPC6 expression by activation of a calcineurin/ nuclear factor of activated T-cells (NFAT)-mediated pathway.[107] Since $1,25(OH)_2D_3$ negatively regulates the gene encoding for renin, essential in AngII biosynthesis, renin downregulation could indirectly contribute to the reduction in *TRPC6* expression by $1,25(OH)_2D_3$.[107] Furthermore, there are indications that $1,25(OH)_2D_3$ directly inhibits the calcineurin/NFAT signaling pathway.[108] Finally, $1,25(OH)_2D_3$ increases the expression of various structural podocytes proteins, such as podocin and nephrin.[99, 100] Nephrin in turn was shown to inhibit TRPC6-phospholipase C complex formation, surface expression and activation.[109] Thus, follow-up experiments should reveal whether the expression of other structural podocyte proteins and the activation of, for example, the calcineurin/NFAT pathway are affected in 25-hydroxy-1α-hydroxylase$^{-/-}$ mice. These experiments should aim to ultimately define the regulatory role of $1,25(OH)_2D_3$ on the functional network existing between TRPC6 and the other glomerular slit diaphragm-associated proteins in the course of podocytes injury.

Targeting TRPC6 regulatory mechanisms in the treatment of proteinuric kidney diseases

Increased TRPC6 activity or expression causes podocyte dysfunction in patients affected by inherited and acquired proteinuric diseases, possibly due to the detrimental increase in Ca^{2+} influx across the cell membrane.[110] This Ca^{2+} influx is thought to activate deleterious intracellular signaling cascades leading to disruption of the actomyosin contractile apparatus essential to normal podocyte function[111] and, in the most severe cases, to apoptosis. Indeed, transgenic podocyte-specific overexpression of a TRPC6 active mutant induced podocyte foot process effacement and albuminuria in mice.[104] Therefore, downregulating or blocking TRPC6 may be therapeutically beneficial in proteinuric kidney diseases. Accordingly, the study reported in **chapter 5** of this thesis suggested that inhibition of TRPC6 gene expression by $1,25(OH)_2D_3$ could be one of the molecular mechanisms at the basis of the anti-proteinuric effect of vitamin D analogs observed in many clinical and pre-clinical investigations (**Figure 5**).[95-98]

TRPC6 is also known to be a receptor-operated channel, regulated by cell surface receptors like the angiotensin II type 1 receptor (AT1R). The renin-angiotensin system (RAS) plays a critical role in modulating proteinuria and progression of kidney injury.[112, 113] It was demonstrated that binding of AngII to AT1R activates TRPC6 channels and increases *TRPC6*

gene expression via a calcineurin/NFAT-mediated signaling pathway, which contributes to podocyte injury (**Figure 5**).[107] Most importantly, angiotensin converting enzyme inhibitors (ACEi), angiotensin receptor blockers (ARBs) and calcineurin inhibitors, pivotal therapies in
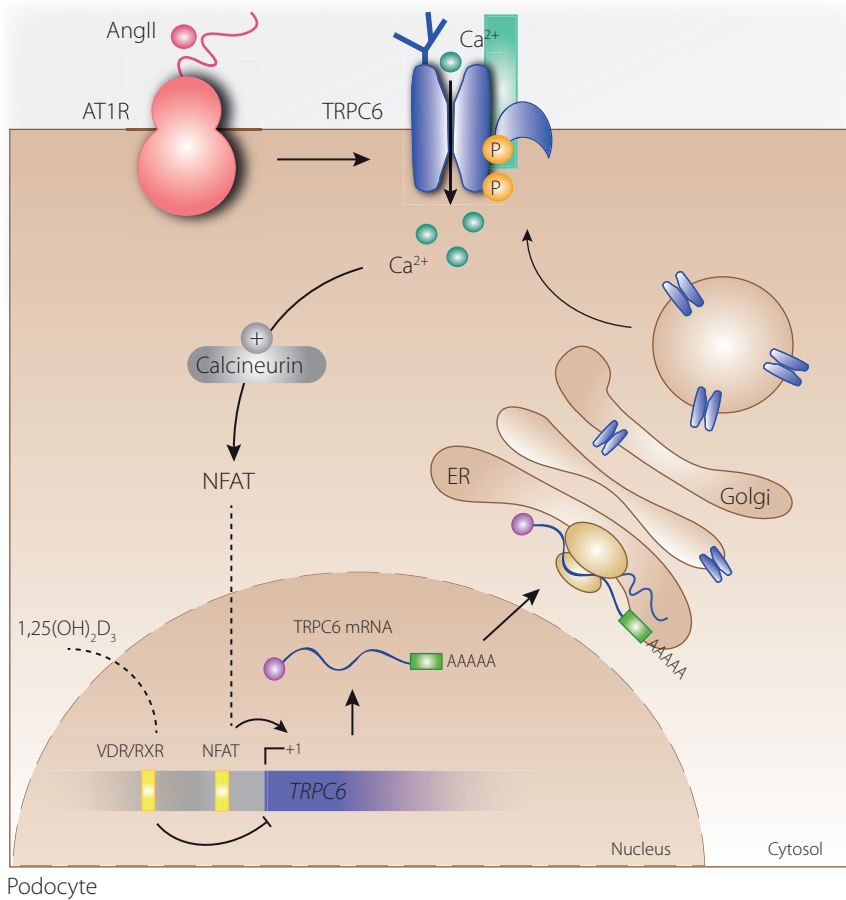
Figure 5 Overview of the regulatory mechanisms of TRPC6 expression and activity in a podocyte cell model.

Binding of AngII to AT1R induces opening of the TRPC6 channels at the plasma membrane leading to increased intracellular Ca^{2+} concentrations, activation of calcineurin and thus translocation of NFAT to the nucleus where it ultimately enhances *TRPC6* transcription. $1,25(OH)_2D_3$ instead negatively regulates *TRPC6* gene expression. Post-translation modifications, like phosphorylation and glycosylation, can further regulate TRPC6 expression and activity at the plasma membrane within the slit diaphragm complex. AT1R: angiotensin type I receptor; AngII: angiotensin II; NFAT: nuclear factor of activated T cells; VDR: vitamin D receptor; RXR: retinoic acid X receptor; ER: endoplasmic reticulum; +1: transcription initiation site.

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reducing proteinuria, decreased TRPC6 expression in *in vitro* podocyte studies as well as in animal models for human FSGS.[107, 114-116] Moreover, experimental data in cultured podocytes suggests that blocking Ca^{2+} influx by applying $LaCl_3$ or blocking TRPC6 using the TRPC channel blocker 2-aminoethyldiphenylborane (2-APB), abrogates the deleterious activation of calcineurin/NFAT signaling, further supporting that specific TRPC6 blocking compounds could show therapeutic promise.[107] Recently, Eckel *et al.* illustrated the potential beneficial anti-proteinuric effect of downregulating TRPC6 expression by showing reduced AngII-mediated albuminuria in *TRPC6* knockout (*TRPC6*-/-) compared to wild-type mice.[116] Most importantly, they demonstrated that the lack of TRPC6 is generally not harmful in mice, and thus that reducing TRPC6 expression is a plausible therapeutic approach. This also raises the question whether blockade of TRPC6 channel activity would be favorable, both in acquired proteinuric disease and in patients with TRPC6 mutations. While the precise mechanism underlying the development of kidney disease caused by mutations in *TRPC6* remains to be defined, fifteen *TRPC6* mutations have been described in literature to associate with FSGS, of which ten have demonstrated increased Ca^{2+} flux due to either enhanced channel activity or increased plasma membrane expression.[101, 102, 105, 106, 117-120] Three of the *TRPC6* mutations identified were not functionally tested, whereas two mutations did not produce apparent changes in current amplitude. These data suggests that other abnormalities, rather than increased Ca^{2+} influx, could result in the disease in the affected patients. Most probably these mutations lead to altered interactions with other slit-diaphragm proteins or downstream signaling proteins possibly due to impaired post-translational modifications (e.g. phosphorylation)[109, 121] or structural remodeling. Noteworthy, all *TRPC6* mutations identified so far map to the terminal domains of the protein that have been proven to be essential regions for protein-protein interactions in analogous with other TRP channels.[122, 123]

Overall, the multiple mechanisms involved in TRPC6 regulation, its role in activating deleterious signaling cascades in podocytes and its identification as a target for known anti-proteinuric agents identifies TRPC6 as a new putative therapeutic target in the treatment of proteinuric kidney diseases. However, the most important question will be whether specific TRPC6 targeting provides clinical advantage over the currently available treatment modalities. In the future, a synergistic multidrug therapy blocking various molecular players in this signaling cascade could represent an eligible approach for the treatment of proteinuric diseases.

Molecular mechanisms of the rapamycin-induced renal Mg^{2+} wasting

Perturbations in mineral homeostasis can represent severe side effects of immuno-suppressive therapies. Rapamycin has been used for many years as chemotherapeutic

agent and component of anti-rejection therapy for recipients of organ transplants who mainly developed toxicity to other immunosuppressive medications, like the calcineurin inhibitors (CNI) cyclosporine A (CsA) and tacrolimus (FK506). Inhibition of the mammalian target of rapamycin (mTOR) with rapamycin-based regimen associates with inappropriately high urinary fractional excretion of Mg^{2+}. So far, contrasting *in vivo* and *in vitro* data did not fully elucidate whether impaired passive paracellular Mg^{2+} reabsorption in the TAL or defected active Mg^{2+} reabsorption in DCT may account for the rapamycin-induced renal Mg^{2+} wasting.[124, 125] The findings in **chapter 6** indicate that chronic treatment with rapamycin in mice lead to hypermagnesuria accompanied by downregulation of the renal mRNA levels of the epithelial Mg^{2+} channel TRPM6, that is responsible for the fine-tuning of Mg^{2+} reabsorption in DCT. Investigation of the mRNA expression levels of other genes that regulate TRPM6 transport ability revealed that HNF1B and epidermal growth factor (EGF) were downregulated in the rapamycin-treated group. On the contrary, the mRNA expression levels of two major players in paracellular Mg^{2+} reabsorption in TAL, the tight junctions proteins claudin-16 (CLDN16) and claudin-19 (CLDN19), were upregulated upon rapamicin treatment. The expression of the TAL marker gene Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) in the rapamycin-treated group did not significantly differ from the control group. Overall, these data indicate that the downregulation of TRPM6 expression in DCT is probably the main molecular mechanism at the basis of the rapamycin-induced hyper-magnesuria. Yet, mRNA data can give an incorrect view of the protein expression. For this reason it is essential to perform additional Western blotting and/or immunohistochemistry experiments. Whether the downregulation of the transcription factor HNF1B contributes to the decrease in *TRPM6* gene transcription remains to be tested. Surprisingly, the increased expression of the heteromeric complex CLDN16/19 should augment the paracellular cation permeability given that a *CLDN16* or *CLDN19* deficiency or inactivation decreases permeability and leads to renal Ca^{2+} and Mg^{2+} wasting.[126, 127] Recently, it was shown that claudin-14 (CLDN14) directly interacts with CLDN16 to act as a negative regulator of paracellular cation permeability.[128] Therefore, upregulation of CLDN16 and CLDN19 could be a compensatory response to changes in CLDN14 expression.

In the kidney of mice treated with rapamycin, EGF mRNA levels were significantly decreased. EGF, together with insulin, are two well-known upstream activators of the mTOR pathway and have been implicated as magnesiotropic hormones.[129, 130] Stimulation of the EGF receptor (EGFR) and insulin receptor (IR) lead to an intracellular cascade involving Rac1 that promotes trafficking of TRPM6 to the plasma membrane.[130, 131] By use of the patch clamp technique in HEK293 cells overexpressing TRPM6, a rapid disturbance in EGF-stimulated TRPM6 activity upon acute exposure to rapamycin was observed. Importantly, mTOR is a serine/ threonine kinase that exists in two separate complexes, mTORC1 and mTORC2. mTORC1 is involved in nutrient sensing and growth factor signaling, whereas mTORC2, primarily regulates cytoskeleton dynamics via a network of small GTP-binding proteins.[132] The inhibitory effect of rapamycin on EGF-stimulated TRPM6

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activity is most probably mediated by block of the mTORC2-Akt axis rather than by inhibition of the mTORC1 signaling.[133] Gene silencing experiments by use of siRNA against the mTORC1 and mTORC2 specific proteins, raptor and rictor respectively, could provide insights into the molecular mechanism responsible for this inhibitory effect of rapamycin on the EGF-stimulated TRPM6. Although insulin and EGF initiate signaling cascades that ultimately increase the cell membrane abundance of TRPM6, rapamycin failed to inhibit the stimulatory effect of insulin on TRPM6. This suggests the insulin and EGF-mediated pathways are not identical and/or that they are subjected to different feedback mechanisms mediated by mTOR. Of note, the expression levels of hormone and growth factor receptors may vary among different cell lines and the observed rapamycin effect on the stimulated TRPM6 activity may be specific to HEK293 cells.[134] Thus, a comparative biochemical study should be performed in order to investigate the mTOR-pathway responsiveness downstream IR and EGFR in the presence or absence of rapamycin in different renal cell lines.

In addition to a stimulatory effect on TRPM6 activity, long-term treatment with EGF increased *TRPM6* gene expression via the ERK-AP1 (c-Fos/c-Jun) pathway *in vitro*.[135, 136] Furthermore, administration of human EGF in rats significantly upregulated the TRPM6 mRNA levels in kidney accompanied by an amelioration of the renal ability to reabsorbed Mg^{2+}.[137] In summary, rapamycin may lead to renal Mg^{2+} wasting according to several mechanisms: *i)* direct inhibition of a signaling cascade responsible for *TRPM6* gene transcription; *ii)* indirect inhibition of *TRPM6* transcriptional regulation by decreasing EGF levels; *iii)* blockade of EGF-dependent stimulation of TRPM6 activity.

Future perspectives

Ion transport across the plasma membrane of epithelial cells depends on the abundance and activity of ion channels and transporters at the cell surface as well as proper (post) transcriptional control by hormones and transcription factors. The studies reported in this thesis illustrated new regulatory mechanisms that modulate gene expression or trafficking of some key proteins involved in Ca^{2+} and Mg^{2+} handling by renal cells, namely the γ-subunit of the Na^+-K^+-ATPase, PTH, TRPC6 and TRPM6. In particular, this research has increased our knowledge on the (patho)physiological role of one major regulator of gene expression in kidney, pancreas and liver, the transcription factor HNF1B. Nevertheless, the presented data also raise new intriguing questions that future investigations should address. Firstly, dysregulation of FXYD2 expression has been pinpointed as the underlying cause of hypomagnesemia observed in almost 50% of the HNF1B patients, even if the exact molecular mechanisms remain elusive. To date, targeted screening for HNF1B binding sites in the promoter region of other genes known to be involved in renal Mg^{2+} reabsorption failed to identify alternative causative genes. A genome-wide analysis

combining chromatin immunoprecipitation with next-generation sequencing technology (ChIP-Seq) would enable the profiling of HNF1B binding sites along the kidney genome. This high-throughput screening could lead to: *i*) the identification of HNF1B binding sites in promoters of other genes directly involved in electrolyte homeostasis; *ii*) the identification of HNF1B binding sites in promoters of genes encoding for other transcription factors or small RNAs that could represent novel (post)transcriptional networks in kidney physiology; *iii*) the understanding of the role of HNF1B in PT where HNF1A and the interacting protein PCBD1 are highly expressed (**Figure 3**). Furthermore, future investigations should elucidate how extracellular stimuli, like hormones and growth factors, can influence HNF1B-mediated transcription and its binding to regulatory proteins (e.g. PCBD1) or other transcription factors (e.g. VDR). Identification of HNF1B upstream regulators will extend our knowledge on the functional role of this transcription factor in the (patho)physiology of multiple organs. Moreover, drug therapies, like rapamycin treatment, target key cell signaling pathways that control downstream transcriptional events. Interestingly, mutations in *HNF1B* associate with pathological conditions whose onset and progression are known to involve the deregulation of mTOR, i.e. cystic kidney diseases and hypomagnesemia.[138] Studying the crosstalk between the mTOR pathway and gene transcription by HNF1B in the kidney may provide new molecular insights into the role of rapamycin as therapeutic treatment in many renal diseases. Furthermore, subsequent studies should aim to the understanding of Ca^{2+} signaling in podocytes. Increased TRPC6 activity or expression at the podocyte cell membrane mediates Ca^{2+} influx with consequent detrimental increase in the intracellular free Ca^{2+} concentrations, cell injury, proteinuria and, ultimately, kidney failure. It would be of great interest to further explore the regulation of TRPC6 expression and activity. Many hormones (PTH and PTH-related peptide),[139, 140] growth factors (EGF and VEGF)[141, 142] and signaling pathways (CaSR signaling cascade and mTOR pathway)[138, 143] have been found to affect glomerular dynamics. These regulatory mechanisms could potentially have an effect on TRPC6 regulation. Of note, a novel emerging paradigm suggests that the antagonistic effects of TRPC6 and TRPC5-mediated Ca^{2+} currents are relevant for cytoskeletal remodeling and cell motility in podocytes.[110] These events in podocytes have been correlated with proteinuric kidney disease.[111] Therefore, agents targeting TRPC6 and/or TRPC5 channels may be a novel therapeutic approach to glomerular diseases. Overall, understanding the transcriptional regulation of ion channels, transporters and hormones involved in epithelial transport and unrevealing the responsiveness of these regulatory pathways to extracellular stimuli will help to design new therapeutic strategies for the treatment of inherited and acquired diseases associated with glomerular and tubular defects in the kidney.

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7

Summary

Samenvatting

Riassunto

8

Summary

Chapter 1: Introduction

The maintenance of Ca^{2+} and Mg^{2+} homeostasis is essential for many physiological functions including intracellular signaling processes, enzymatic reactions, neuronal excitability, muscle contraction and bone formation. The human body preserves plasma Ca^{2+} and Mg^{2+} concentrations within a narrow range due to an efficient homeostatic system encompassing parathyroid gland, intestine, bone and kidney. The latter determines the final amount of Ca^{2+} and Mg^{2+} excreted in the urine by controlling the reabsorption of these cations from the pro-urine into the blood. After filtration in the glomeruli, the bulk of Ca^{2+} and Mg^{2+} in the pro-urine is reabsorbed in the proximal tubule (PT) and thick ascending limb of Henle (TAL) by a passive transport route. Fine-tuning of divalent cation reabsorption occurs in the distal convoluted tubule (DCT) and connecting tubule (CNT) where approximately 10-15% of the filtered Mg^{2+} and 10% of the filtered Ca^{2+} are reabsorbed. As a consequence, only 1-2% of the filtered Ca^{2+} and 5% of the filtered Mg^{2+} are excreted into the urine. Mutations in key regulators of Ca^{2+} and Mg^{2+} transport in epithelial renal cells cause inherited forms of disturbances in these cations. Moreover, perturbations of Ca^{2+} and Mg^{2+} handling can be secondary to other medical conditions or to certain drug therapies, such as immunosuppressive treatment. Many intracellular events regulate the abundance of ion channels and transporters at the plasma membrane at multiple levels, including transcription, post-transcription, translation, post-translation and trafficking. This thesis is mainly directed at elucidating novel transcriptional networks that control the gene expression of ion channels, transporters and hormones, relevant to the handling of Ca^{2+} and Mg^{2+} by the glomerular podocyte and tubular cells of the kidney.

Chapter 2: HNF1B regulates the expression of the γa-subunit of the Na^+-K^+-ATPase

The transcription factor hepatocyte nuclear factor 1 homeobox B (HNF1B) plays an important role in the embryonic kidney development, but also in the regulation of tubular transport in the postnatal kidney. Mutations in *HNF1B* are responsible for an autosomal dominantly inherited syndrome with renal and extra-renal consequences. The HNF1B-related nephropathy includes renal hypomagnesemia, suggested to be due to the dysregulation of FXYD2 expression in DCT. The *FXYD2* gene encodes two main isoforms of the γ-subunit of the Na^+-K^+-ATPase, the γa- and γb-subunits. In this thesis, by use of two different gene reporter assays, it was demonstrated that HNF1B specifically acts as an activator of *FXYD2a* promoter activity, whereas *FXYD2b* expression was not affected. Moreover, the HNF1B mutations p.His69fsdelAC, p.His324Ser325fsdelCA, p.Tyr352finsA and p.Lys156Glu prevented transcription activation of the *FXYD2a* promoter via a dominant negative effect on wild-type HNF1B. Immunohistochemical analysis on mouse kidney sections showed that the γa-subunit is expressed at the basolateral membrane of the DCT

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where it co-localizes with the γb-subunit. In conclusion, abnormalities in HNF1B impair the relative abundance of γa- and γb-subunits at the cell surface, and thereby affect the transcellular Mg^{2+} reabsorption in DCT.

Chapter 3: HNF1B regulates *PTH* gene transcription

The parathyroid hormone (PTH) is released into the circulation by the parathyroid glands in response to changes in the extracellular Ca^{2+} and PO_4^{3-} concentrations. Secondary hyperparathyroidism (HPT) classically appears during the course of chronic kidney failure. In a cohort of eleven patients with *HNF1B* mutations and/or deletions, PTH levels were inappropriately high compared to the level of kidney function. Immunohistochemical analysis demonstrated that HNF1B is expressed in the nuclei of PTH-positive cells of human parathyroid gland and in the rat parathyroid PT-r cell line. Chromatin immunoprecipitation (ChIP) analysis demonstrated that HNF1B directly binds responsive elements within the human *PTH* promoter. *In vitro* luciferase-assays showed that wild-type HNF1B inhibits *PTH* promoter activity with a maximal reduction of 30%, while the HNF1B mutants p. His69fsdelAC, p.His324Ser325fsdelCA, p.Tyr352finsA and p.Lys156Glu lacked this inhibitory property. Serial deletions in the *PTH* promoter revealed that the inhibitory effect of HNF1B resides -200/-70 bp from the transcription initiation site. Taken together, these data suggested that HNF1B is a transcriptional repressor of *PTH* gene expression in the parathyroid gland, which could explain the early development of HPT in patients with *HNF1B* mutations or deletions.

Chapter 4: Mutations in *PCBD1* cause hypomagnesemia and MODY

The *PCBD1* gene encodes the enzyme pterin-4 alpha-carbinolamine dehydratase, that is also known to co-activate HNF1-mediated transcription in the cell nucleus. Mutations in *PCBD1* were previously shown to impair the catalytic function of the protein leading to an autosomal recessively inherited disease, characterized by hyperphenylalaninemia with high urinary levels of primapterin (HPABH4D, or primapterinuria). In this thesis, a long-term follow-up study of two HPABH4D patients revealed that homozygous mutations c.312C>T (p.Gln97Ter) and c.99G>T/283G>A (p.Glu26Ter/p.Arg87Gln) in *PCBD1* associate with hypomagnesemia and renal Mg^{2+} wasting. The renal manifestation is a common finding in patients with mutations in *HNF1B*. Of note, the patient with a p.Gln97Ter mutation was also diagnosed with maturity-onset diabetes of the young (MODY), whose underlying defects include mutations in *HNF1A* or *HNF1B*. Gene expression data revealed that PCBD1 is present with HNF1B in DCT, where its transcript levels are modulated by changes in dietary Mg^{2+}. Overexpression studies in a human kidney cell line demonstrated that wild-type PCBD1 binds HNF1B to co-stimulate the *FXYD2* promoter, whose activity is instrumental in Mg^{2+} reabsorption in DCT. However, the PCBD1 p.Gln97Ter and p.Glu26Ter mutations, as well as four other PCBD1 mutations previously reported in HPABH4D patients, caused proteolytic instability of PCBD1. This consequently abolished the

co-stimulatory effect on the *FXYD2*-promoter activity. Of note, HNF1B p.Gln253Pro and p.His324Ser325fsdelCA mutants showed binding to PCBD1, but lead to an accumulation of PCBD1 in the cytosol compared to the nuclear localization observed upon co-expression with wild-type HNF1B. The reduced abundance of PCBD1 in the nucleus will indirectly result in a decreased co-activation of HNF1B that could in part contribute to the hypomagnesemia observed in *HNF1B* patients. Overall, our findings indicate that PCBD1 is an important co-activator of the HNF1B-mediated transcription, necessary to fine-tune Mg^{2+} reabsorption in the DCT.

Chapter 5: Vitamin D downregulates TRPC6 overexpression in podocyte injury

Beyond a homeostatic role, pre-clinical and clinical studies demonstrated that treatment with vitamin D analogs reduces proteinuria and podocyte loss in glomerular disease. The antiproteinuric activity of 1α,25-dihydroxy-vitamin D_3 (1,25(OH)$_2$D$_3$) is associated with the transcriptional regulation of nephrin and podocin, two podocyte proteins that preserve glomerular filtration by maintaining a functional glomerular slit diaphragm complex. This complex also includes TRPC6, a receptor-mediated signaling protein mediating Ca^{2+} influx in podocytes. *TRPC6* gain-of-function mutations are associated with a hereditary form of focal segmental glomerulosclerosis (FSGS). Furthermore, several other inherited and acquired proteinuric diseases associate with an increased glomerular TRPC6 expression and activity. When investigating the transcriptional regulation of TRPC6 by 1,25(OH)$_2$D$_3$, it was demonstrated that 1,25(OH)$_2$D$_3$ was able to reduce the enhanced TRPC6 expression in both *in vitro* podocyte injury and the *in vivo* adriamycin-induced nephropathy model for FSGS. ChIP experiments in combination with luciferase assays showed that the inhibition of TRPC6 transcription upon 1,25(OH)$_2$D$_3$ treatment occurred via direct binding of the liganded VDR/RXR complex to the TRPC6 promoter. The 1,25(OH)$_2$D$_3$ deficiency in 25-hydroxy-1α-hydroxylase knockout mice increased TRPC6 expression and induced proteinuria, together with partial podocyte foot process effacement. Furthermore, 1,25(OH)$_2$D$_3$ supplementation in 25-hydroxy-1α-hydroxylase knockout mice reversed both the increased TRPC6 expression as well as the proteinuria, and normalized podocyte morphology. In conclusion, TRPC6 downregulation could contribute to the antiprotein-uric effect of vitamin D.

Chapter 6: Molecular mechanisms of rapamycin-induced renal Mg^{2+} wasting

Rapamycin is an immunosuppressive drug primarily used as antirejection therapy for recipients of organ transplants. Inhibition of the mammalian target of rapamycin (mTOR) with rapamycin-based regimens associates with inappropriately high urinary fractional excretion of Mg^{2+}, hypothetically due to impaired Mg^{2+} reabsorption in DCT. In this regard, the chronic treatment with rapamycin in mice decreased renal mRNA levels of the

epithelial Mg^{2+} channel TRPM6, whose expression in the kidney is restricted to the apical membrane of DCT. Among other genes known to affect the transcellular Mg^{2+} reabsorption in DCT, the gene encoding the EGF precursor protein pro-EGF, involved in the hormonal regulation of TRPM6, and HNF1B were downregulated by rapamycin. In contrast, the mRNA levels of two major players in paracellular Mg^{2+} reabsorption in TAL, the tight junction proteins claudin-16 (CLDN16) and claudin-19 (CLDN19), were increased upon rapamycin treatment. This could be due to regulation of claudin-14 (CLDN14) that controls the CLD16-19 complex. Electrophysiological analysis showed that rapamycin rapidly inhibited the stimulatory effect of EGF on TRPM6 currents *in vitro*, while it did not interfere with the pathway that stimulates TRPM6 activity upon exposure to insulin, another magnesiotropic hormone. Additional experiments should aim to characterize the signaling cascades downstream of insulin and EGF receptors that ultimately affect mTOR activation and, thus, TRPM6 activity. Overall, these observations suggest that the downregulation of TRPM6 expression and inhibition of its hormonal regulation in the DCT contribute to the renal Mg^{2+} wasting observed upon chronic treatment with rapamycin.

Chapter 7: Discussion and future perspectives

Ca^{2+} and Mg^{2+} transport across the plasma membrane of epithelial renal cells depends on the abundance and activity of ion channels and transporters at the cell surface, as well as proper hormonal control, as also evidenced by the studies reported in this thesis. A scientific question that remains unsolved, is how changes in the expression of the γ-subunit of the Na^+-K^+-ATPase at the basolateral membrane of the DCT cause hypomagnesemia. Additionally, a complete profiling of HNF1B binding sites along the kidney genome is currently missing. A genome-wide analysis combining ChIP for HNF1B with next-generation sequencing technology (ChIP-Seq) may unravel new transcriptional pathways in renal Ca^{2+} and Mg^{2+} handling. Currently, little is known about the hormonal control of the HNF1B-mediated transcription and of the HNF1B binding to regulatory proteins, like PCBD1. Finally, future investigations should elucidate how the renal transcriptional network that includes HNF1B is targeted by Mg^{2+}-wasting drug therapies like rapamycin. Moreover, great interest resides in the characterization of Ca^{2+} fluxes and Ca^{2+}-mediated signaling cascades in podocyte (patho)physiology. In particular, understanding how the expression and activity of the Ca^{2+} channel TRPC6 are modulated may lead to the development of novel therapeutic approaches to proteinuric kidney diseases. In conclusion, dissecting the transcriptional regulation of proteins involved in renal Ca^{2+} and Mg^{2+} handling, and the responsiveness of these regulatory pathways to extracellular stimuli, can ultimately help to design new therapeutic strategies for the treatment of inherited and acquired diseases associated with impaired Ca^{2+} and Mg^{2+} handling by glomerular or tubular cells in the kidney.

Samenvatting

Hoofdstuk 1: Inleiding

Het behoud van Ca^{2+} en Mg^{2+} homeostase is essentieel voor vele fysiologische functies, waaronder intracellulaire signaaltransductieprocessen, enzymatische reacties, neuronale exciteerbaarheid, spiercontractie en botvorming. Het menselijk lichaam houdt de plasma Ca^{2+} en Mg^{2+} concentraties binnen een nauwe marge door een efficiënt homeostase systeem, welke de bijschildklier (glandula parathyreoïdea), darm, nier en bot omvat. De nieren bepalen de uiteindelijke hoeveelheid Ca^{2+} en Mg^{2+} die wordt uitgescheiden in de urine, door resorptie van deze kationen vanuit de pro-urine naar het bloed. Na filtratie in de glomeruli wordt het grootste deel van Ca^{2+} en Mg^{2+} in de pro-urine geresorbeerd in de proximale tubulus (PT) en het dikke opstijgende been van de lis van Henle (TAL) middels passief paracellulair transport. De fijnafstemming van de resorptie van deze divalente kationen gebeurt in de het distaal convoluut (DCT) en de verbindingsbuizen (CNT), waarin circa 10-15% van het gefilterde Mg^{2+} en 10% van het gefilterde Ca^{2+} weer wordt heropgenomen. Hierdoor wordt slechts 1-2% van het gefilterde Ca^{2+} en 5% van het gefilterde Mg^{2+} uitgescheiden in de urine. Mutaties in cruciale regulatoren van het Ca^{2+} en Mg^{2+} transport in epitheliale niercellen veroorzaken erfelijke stoornissen in de homeostase van deze kationen. Bovendien kunnen verstoringen van Ca^{2+} en Mg^{2+} uitscheiding secundair aan andere aandoeningen of therapieën, zoals bepaalde immuno-suppressiva, ontstaan.

Verscheidene intracellulaire processen reguleren de ionkanalen en transporteurs op de plasmamembraan op o.a. transcriptioneel, post-transcriptioneel, translationeel, post-translationeel niveau, alsmede via beïnvloeding van het transport van en naar de plasmamembraan. Dit proefschrift is vooral gericht op het ophelderen van nieuwe transcriptionele netwerken, welke de genexpressie van ionkanalen, transporteurs en hormonen betrokken bij transport van Ca^{2+} en Mg^{2+} in de glomerulaire podocyt en tubulaire cellen van de nier reguleren.

Hoofdstuk 2: HNFB1 reguleert de expressie van de ya-subunit van het Na^+-K^+-ATPase

De transcriptiefactor hepatocyte nuclear factor 1 homeobox B (HNF1B) speelt een belangrijke rol in de embryonale ontwikkeling van de nier, maar ook in de regulatie van tubulaire transportprocessen in de postnatale nier. Mutaties in *HNF1B* zijn verantwoordelijk voor een autosomaal dominant erfelijk syndroom met renale en niet-renale symptomen. De HNF1B-gerelateerde nefropathie omvat o.a. renale hypomagnesiëmie door een veronderstelde dysregulatie van FXYD2 expressie in DCT. Het *FXYD2* gen codeert voor twee isovormen van de γ-subunit van Na^+-K^+-ATPase, de γa en de γb-subunit. In dit proefschrift werd door middel van van twee verschillende reporter assays aangetoond dat HNF1B fungeert als een specifieke activator van de *FXYD2a* promotor activiteit, terwijl

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FXYD2b transcriptie niet werd beïnvloed. Bovendien verhinderden de HNF1B mutaties p.His69fsdelAC, p.His324Ser325fsdelCA, p.Tyr352finsA en p.Lys156Glu de activatie van de *FXYD2a* promotor via een dominant negatief effect op wild-type HNF1B. Immunohisto-chemische analyse van muizen nier coupes toonde aan dat de γa-subeenheid tot expressie komt in de basolaterale membraan van de DCT, waar het co-lokaliseert met de γb-subeenheid. Concluderend kan worden gesteld dat HNF1B mutaties het evenwicht in de expressie van γa- en γb-subeenheden op het celoppervlak reduceren, waardoor de transcellulaire Mg^{2+} resorptie in DCT wordt beïnvloed.

Hoofdstuk 3: HNF1B reguleert *PTH* gen transcription

Het paraathormoon (PTH) wordt door de bijschildklier in de circulatie uitgescheiden ten gevolge van veranderingen in de extracellulaire Ca^{2+} en PO_4^{3-} concentraties. Secundaire hyperparathyreoïdie (HPT) treedt klassiek op tijdens de progressie van chronisch nierfalen. In een cohort van elf patiënten met *HNF1B* mutaties en/of deleties bleken de PTH-concen-traties discrepant hoog in relatie tot de mate van nierfunctieverlies. Immunohistochemi-sche analyse toonde aan dat HNF1B tot expressie komt in de celkern van PTH-positieve cellen in de humane bijschildklier en in de rat PT-r bijschildkliercellijn. Chromatine immu-noprecipitatie (ChIP) analyse toonde aan dat HNF1B bindt aan regulatoire elementen binnen de humane *PTH* promotor. *In vitro* luciferase-assays toonden aan dat wild-type HNF1B de *PTH* promotor activiteit remt met een maximale reductie van 30%, terwijl bij de HNF1B mutanten p.His69fsdelAC, p.His324Ser325fsdelCA, p.Tyr352finsA en p.Lys156Glu deze remmende eigenschap ontbrak. Uit seriële deleties in de *PTH* promotor blijkt dat de remmende werking van HNF1B tussen -200 en -70 bp van de transcriptie initiatieplaats ligt. Samen suggereren deze data dat HNF1B een repressor is van *PTH* gentranscriptie in de bijschildklier. Dit zou de vroege ontwikkeling van HPT bij patiënten met *HNF1B* mutaties of deleties kunnen verklaren.

Hoofdstuk 4: Mutaties in *PCBD1* veroorzaken hypomagnesiëmie en MODY

Het *PCBD1* gen codeert voor het enzym pterine-4 alpha-carbinolamine dehydratase, een co-activator van de HNF1-gemedieerde transcriptie in de celkern. Eerder werd aangetoond dat mutaties in *PCBD1* de katalytische functie van het eiwit remmen, hetgeen leidt tot een autosomaal recessief erfelijke ziekte, gekarakteriseerd door hyperphenylalaninemie met hoge concentraties van primapterine in de urine (HPABH4D oftewel primapterinurie). In dit proefschrift werd in een lange-termijn follow-up studie van twee HPABH4D patiënten aangetoond dat de homozygote mutaties c.312C>T (p.Gln97Ter) en c.99G>T/283G>A (p.Glu26Ter/p.Arg87Gln) in *PCBD1* geassocieerd worden met hypomagnesiëmie en renaal Mg^{2+} verlies. Zoals hierboven beschreven is een renale hypomagnesiëmie een veel voorkomende bevinding bij patiënten met mutaties in *HNF1B*. Bij de patiënt met een p.Gln97Ter mutatie werd ook maturity-onset diabetes of the young (MODY) gediagnosti-ceerd, een ziektebeeld dat veroorzaakt kan worden door onder meer mutaties in *HNF1A*

of *HNF1B*. PCBD1 komt tot co-expressie met HNF1B in DCT, waarvan het transcriptie niveau wordt gemoduleerd door veranderingen in Mg^{2+} in de voeding. Middels overexpressie experimenten in een humane niercellijn werd aangetoond dat wild-type PCBD1 aan HNF1B bindt en de *FXYD2* promotor co-stimuleert. FXYD2 activiteit is van essentieel belang bij de Mg^{2+} resorptie in DCT. Daarentegen veroorzaakten de PCBD1 p.Gln97Ter en p.Glu26Ter mutaties, zowel als vier andere PCBD1 mutaties die eerder werden beschreven bij HPABH4D patiënten, proteolytische instabiliteit van PCBD1. Hierdoor werd het co-stimulerend effect op de *FXYD2*-promotoractiviteit opgeheven. Van belang is dat HNF1B p.Gln253Pro en p.His324Ser325fsdelCA mutanten weliswaar binding aan PCBD1 vertoonden, maar in tegenstelling tot de nucleaire lokalisatie waargenomen bij co-expressie met wild-type HNF1B, leidden tot een accumulatie van PCBD1 in het cytosol. De afname van PCBD1 in de kern zal indirect resulteren in een verminderde co-activatie van HNF1B, hetgeen mogelijk bijdraagt aan de hypomagnesiëmie bij HNF1B patiënten. Kortom, onze bevindingen wijzen PCBD1 aan als een belangrijke co-activator van de HNF1B-gemedieerde transcriptie, noodzakelijk voor de fijn afstemming van Mg^{2+} resorptie in de DCT.

Hoofdstuk 5: Vitamine D downreguleert TRPC6 overexpressie bij podocytschade

Klinische en pre-klinische studies hebben aangetoond dat, naast een homeostatische rol, behandeling met vitamine D en haar analogen proteïnurie en podocytschade verminderen bij glomerulaire ziekten. De antiproteïnurische activiteit van 1α,25-dihydroxy-vitamine D_3 (1,25(OH)$_2$D$_3$) is geassocieerd met regulatie van nephrine en podocine, twee podocyteiwitten die onderdeel uitmaken van het glomerulaire slit diafragma, complex welke van cruciaal belang is bij de glomerulaire filtratie. Dit complex bevat ook TRPC6, een receptor-gemedieerd ionkanaal betrokken bij Ca^{2+} influx in podocyten. *TRPC6* gain-of-function mutaties veroorzaken een erfelijke vorm van focale segmentale glomeruloscle-rose (FSGS). Bovendien zijn diverse andere erfelijke en verworven proteïnurische ziekten geassocieerd met een verhoogde glomerulaire TRPC6 expressie en activiteit. De transcrip-tionele regulatie van TRPC6 door 1,25(OH)$_2$D$_3$ werd onderzocht in *in vitro* podocytschade-modellen, in een rat adriamycine-geïnduceerde nefropathie model voor FSGS en in 1,25(OH)$_2$D$_3$-deficiënte 25-hydroxy-1α-hydroxylase knockoutmuizen. Er werd aangetoond dat 1,25(OH)$_2$D$_3$ de verhoogde expressie van TRPC6 zowel in deze *in vitro* als in *in vivo* modellen van podocytschade vermindert. ChIP experimenten in combinatie met luciferase assays toonden aan dat de downregulatie van TRPC6 transcriptie na 1,25(OH)$_2$D$_3$ behandeling plaatsvindt via directe binding van het VDR/RXR complex aan de TRPC6 promoter. De 1,25(OH)$_2$D$_3$ deficiëntie in 25-hydroxy-1α-hydroxylase knockout muizen verhoogde de TRPC6 expressie, proteinurie en podocytvoetjesversmelting. Behandeling met 1,25(OH)$_2$D$_3$ voorkwam zowel de verhoogde TRPC6 expressie alsmede de proteïnurie en normaliseerde de morfologie van de podocyten. Concluderend kan TRPC6 down-regulatie bijdragen aan het antiproteïnurisch effect van vitamine D.

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Hoofdstuk 6: Moleculaire mechanismen van rapamycine-geïnduceerd Mg^{2+} verlies

Rapamycine, een remmer van de zoogdier target of rapamycin (mTOR), is een immuno-suppressief medicijn dat o.a. wordt gebruikt als anti-afstotingstherapie voor ontvangers van orgaantransplantaten. Rapamycine-gebaseerde behandelingen zijn geassocieerd met een toegenomen fractionele excretie van Mg^{2+} in de urine, mogelijk door verminderde Mg^{2+} resorptie in DCT. Chronische behandeling met rapamycine verminderde bij muizen de renale mRNA niveaus van het epitheliale Mg^{2+} kanaal TRPM6, waarvan de expressie in de nier beperkt is tot de apicale membraan van DCT. Naast andere genen die betrokken zijn bij de transcellulaire Mg^{2+} resorptie in DCT, was het gen dat codeert voor het EGF precursor eiwit pro-EGF gedownreguleerd. Dit eiwit is betrokken bij de hormonale regulatie van TRPM6 en HNF1B. Daarentegen werden de mRNA niveaus van twee belangrijke, bij het paracellulaire Mg^{2+} resorptie in TAL betrokken, tight junction eiwitten claudin-16 (CLDN16) en claudin-19 (CLDN19) opgereguleerd na rapamycine behandeling. Dit zou mogelijk een effect kunnen zijn van beïnvloeding door claudin-14 (CLDN-14), welke het CLDN16-19 complex reguleert. Elektrofysiologische analyse toonde aan dat rapamycine snel het stimulerende effect van EGF op TRPM6 functie remde, terwijl het geen invloed had op de TRPM6 activatie na blootstelling aan insuline, een ander magnesiotroop hormoon. Aanvullende experimenten zouden gericht moeten zijn op de karakterisering van de signaaltransductie cascades van insuline en EGF-receptoren, die uiteindelijk van invloed zijn op mTOR activatie en daarmee op TRPM6 activiteit. Samengevattend kan geconcludeerd worden dat deze resultaten suggereren dat de downregulatie van TRPM6 en de remming van de hormonale regulatie daarvan bijdragen aan het renale Mg^{2+} verlies bij chronische behandeling met rapamycine.

Hoofdstuk 7: Discussie en toekomstperspectieven

Ca^{2+} en Mg^{2+} transport over de plasmamembraan van epitheliale niercellen hangt af van expressie en activiteit van ionkanalen en transporteurs op het celoppervlak, zowel als van een goede hormonale controle daarvan, zoals ook blijkt uit de studies beschreven in dit proefschrift. Een wetenschappelijke vraag die onopgelost blijft, is hoe veranderingen in de expressie van de γ-subeenheid van het Na$^+$-K$^+$-ATPase in de basolaterale membraan van de DCT hypomagnesiëmie kan veroorzaken. Daarnaast ontbreekt momenteel een volledig overzicht van de HNF1B bindingsplaatsen in genen die tot expressie komen in de nier. Een genoom-brede analyse die ChIP voor HNF1B combineert met next-generation sequencing technologie (ChIP-Seq) zou nieuwe transcriptionele cascades betrokken bij renale Ca^{2+} en Mg^{2+} verwerking kunnen ontrafelen. Verder is er weinig bekend over de hormonale regulatie van HNF1B-gemedieerde transcriptie en van HNF1B binding aan regulerende eiwitten zoals PCBD1. Tenslotte zal toekomstig onderzoek moeten uitwijzen op welke wijze het transcriptionele netwerk in de nier, waar o.a. HNF1B onderdeel van uitmaakt, het doelwit is van Mg^{2+} verlagend therapieën zoals rapamycine. Bovendien is er

grote belangstelling voor de karakterisering van Ca^{2+} influx en Ca^{2+}-gemedieerde signaal-transductiecascades in de (patho)fysiologie van de podocyt. In het bijzonder begrip van de modulatie van TRPC6 expressie en activiteit in podocyten kan leiden tot de ontwikkeling van nieuwe therapeutische aangrijpingspunten bij de behandeling van proteïnurische nierziekten. Het ontleden van de transcriptionele regulatie van eiwitten betrokken bij Ca^{2+} en Mg^{2+} transport in de nier, en de reactie van deze regulerende cascades op extracellulaire stimuli, zal uiteindelijk bij kunnen dragen aan ontwikkeling van nieuwe therapeutische strategieën. Dit zou belangrijke consequenties kunnen hebben voor de behandeling van erfelijke en verworven ziekten die geassocieerd zijn met verminderde Ca^{2+} en Mg^{2+} verwerking door glomerulaire of tubulaire cellen in de nier.

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Riassunto

Capitolo 1: Introduzione

Il mantenimento dell'omeostasi di Ca^{2+} e Mg^{2+} è essenziale per molte funzioni fisiologiche, quali i processi di segnalazione intracellulare, numerose reazioni enzimatiche, la trasmissione neuronale, la contrazione muscolare e la formazione ossea. Il corpo umano mantiene le concentrazioni plasmatiche di Ca^{2+} e Mg^{2+} in uno stretto intervallo di valori grazie ad un efficiente sistema omeostatico che comprende le paratiroidi, l'intestino, le ossa e i reni.

I reni determinano le quantità finali di Ca^{2+} e Mg^{2+} eliminate nelle urine controllando il riassorbimento di questi cationi dalla pro-urina al sangue. Dopo la filtrazione nei glomeruli renali, la maggior parte di Ca^{2+} e Mg^{2+} nella pro-urina è riassorbita tramite un processo di trasporto passivo nel tubulo prossimale e nel tratto ascendente spesso dell'ansa di Henle del nefrone. Il trasporto attivo di Ca^{2+} e Mg^{2+} avviene nel tubulo distorto distale e nel tubulo reuniente, dove circa il 10-15% del Mg^{2+} filtrato e il 10% del Ca^{2+} filtrato sono rispettivamente riassorbiti. In conclusione, solo l'1-2% del Ca^{2+} e il 5% del Mg^{2+} filtrati sono eliminati nelle urine.

Mutazioni in proteine coinvolte nella regolazione del trasporto di Ca^{2+} e Mg^{2+} nelle cellule epiteliali renali possono causare disturbi ereditari del trasporto di questi cationi. Inoltre, alterazioni nell'omeostasi di Ca^{2+} e Mg^{2+} possono originare da altre condizioni mediche o dall'uso di determinate terapie farmacologiche, come il trattamento con im-munosoppressori.

L'abbondanza di canali ionici e trasportatori nella membrana plasmatica dipende da molti eventi intracellulari, come trascrizione, modifiche post-trascrizionali, sintesi proteica, modifiche post-traduzionali e traffico di membrana. Questa tesi è principalmente diretta a delucidare nuovi network trascrizionali che controllano l'espressione genica di canali ionici, trasportatori e ormoni importanti per il trasporto di Ca^{2+} e Mg^{2+} nei podociti dei glomeruli renali e nelle cellule tubulari del nefrone.

Capitolo 2: HNF1B regola l'espressione della subunità γa della Na⁺-K⁺-ATPasi

Il fattore nucleare epatocitario 1B (HNF1B) è un fattore trascrizionale coinvolto nello sviluppo embrionale del rene, ma anche nella regolazione del trasporto degli elettroliti nel rene in età adulta. Mutazioni nel gene *HNF1B* sono responsabili di una sindrome a trasmissione autosomica dominante con conseguenze renali ed extra-renali. Tra le patologie renali connesse a mutazioni in *HNF1B* si annovera l'ipomagnesemia renale, dovuta all'insufficiente trascrizione genica di *FXYD2* nel tubulo distorto distale. Il gene *FXYD2* codifica le due principali isoforme della subunità γ della Na^+-K^+-ATPasi, denominate γa e γb.

In questa tesi sono stati utilizzati due saggi con geni reporter per dimostrare che HNF1B attiva specificamente il promotore *FXYD2a*, mentre l'espressione di *FXYD2b* non è influenzata. È stato inoltre provato che le seguenti mutazioni in HNF1B p.His69fsdelAC,

p.His324Ser325fsdelCA, p.Tyr352fsinsA e p.Lys156Glu impediscono l'attivazione trascrizionale del promotore *FXYD2a* tramite un effetto dominante negativo su HNF1B wild-type. L'analisi immunoistochimica di sezioni renali di topo ha infine dimostrato che la subunità γa è espressa insieme a γb nella membrana basolaterale del tubulo distorto distale.

In conclusione, anomalie in HNF1B alterano l'abbondanza relativa delle subunità γa e γb della Na^+-K^+-ATPasi alla superficie cellulare, influenzando il riassorbimento transcellulare di Mg^{2+} nel tubulo distorto distale.

Capitolo 3: HNF1B regola la trascrizione del gene *PTH*

L'ormone paratiroideo (PTH) è rilasciato in circolo dalle ghiandole paratiroidee in risposta ai cambiamenti nelle concentrazioni extracellulari di Ca^{2+} e PO_4^{3-}. L'insorgenza di iperparatiroidismo secondario è frequente nei casi di insufficienza renale. In una coorte di undici pazienti con mutazioni e/o delezioni in *HNF1B*, i valori di PTH nel sangue erano troppo elevati rispetto ai livelli di attività renale.

In questo capitolo è riportata la dimostrazione, mediante analisi immunochimica, che HNF1B è presente nel nucleo di cellule paratiroidee umane positive all'espressione di PTH e nel nucleo della linea cellulare paratiroidea di ratto, PT-r. Esperimenti di immunoprecipitazione della cromatina (ChIP) hanno inoltre evidenziato che HNF1B lega specifici elementi responsivi presenti nel promotore del gene umano *PTH*. Saggi *in vitro* con la luciferasi hanno in particolare provato che la forma wild-type di HNF1B inibisce del 30% l'attività trascrizionale del promotore di *PTH*, mentre le mutazioni p.His69fsdelAC, p.His-324Ser325fsdelCA, p.Tyr352fsinsA and p.Lys156Glu in HNF1B annullano tale effetto. Delezioni seriali del promotore di *PTH* dimostrano infine che la regione minima necessaria per osservare l'effetto inibitorio di HNF1B risiede tra -200 e -70 paia di basi dal sito di inizio della trascrizione.

Questi dati suggeriscono, dunque, che HNF1B è un repressore della trascrizione del gene *PTH* nelle ghiandole paratiroidee. Tale dimostrazione potrebbe spiegare il precoce sviluppo di iperparatiroidismo in pazienti con mutazioni e/o delezioni in *HNF1B*.

Capitolo 4: Mutazioni nel gene *PCBD1* causano ipomagnesemia e MODY

Il gene *PCBD1* codifica l'enzima pterin-4 alpha-carbinolamine deidratasi, una proteina che è anche conosciuta come co-attivatore della trascrizione genica mediata da HNF1B. È stato precedentemente dimostrato che mutazioni in *PCBD1* alterano l'attività catalitica della proteina, causando una malattia a trasmissione autosomica recessiva (HPABH4D o primapterinuria) caratterizzata da iperfenilalaninemia e alto contenuto urinario di primapterina.

In questa tesi è riportato uno studio a lungo termine di due pazienti affetti da HPABH4D, che attesta che le mutazioni in omozigosi c.312C>T (p.Gln97Ter) e c.99G>T/283G>A (p.Glu26Ter/p.Arg87Gln) in *PCBD1* sono associate ad ipomagnesemia e perdita di Mg^{2+} nelle urine. È interessante notare che queste manifestazioni renali sono comuni in pazienti con mutazioni in *HNF1B*. Inoltre, al paziente con la mutazione p.Gln97Ter è stato anche

diagnosticato maturity-onset diabetes of the young (MODY), una forma monogenica di diabete causata da mutazioni in *HNF1A* o *HNF1B*. Dati di espressione genica hanno evidenziato che PCBD1 è presente con HNF1B nel tubulo distale contorto di topo. L'espressione di PCBD1, ma non di HNF1B, in questo segmento del nefrone è modulata da cambiamenti nel contenuto di Mg^{2+} nella dieta. Esperimenti condotti in cellule renali umane hanno dimostrato che PCBD1 normalmente lega HNF1B nel nucleo e quindi co-stimola il promotore del gene *FXYD2a*, a sua volta coinvolto nel processo di riassorbimento del Mg^{2+} nel tubulo distale contorto. Le mutazioni p.Gln97Ter e p.Glu26Ter, così come altre quattro mutazioni in PCBD1 precedentemente identificate in pazienti affetti da HPABH4D, causano instabilità proteolitica di PCBD1 e quindi aboliscono la co-stimolazione del promotore di *FXYD2a*. Invece le mutazioni p.Gln253Pro e p.His-324Ser325fsdelCA in HNF1B non influiscono sull'abilità di HNF1B di legare PCBD1, ma causano una localizzazione citosolica di PCBD1, diversa dalla localizzazione nucleare osservata in presenza di HNF1B wild-type. La ridotta quantità di PCBD1 nel nucleo potrebbe indirettamente avere come effetto una diminuzione della trascrizione genica da parte di HNF1B, e ciò potrebbe contribuire all'ipomagnesemia osservata nei pazienti con mutazioni in *HNF1B*.

In conclusione, i risultati riportati in questo capitolo indicano PCBD1 come un importante co-attivatore della trascrizione genica attivata da HNF1B, necessario per il controllo del riassorbimento di Mg^{2+} nel tubulo distale contorto.

Capitolo 5: La vitamina D diminuisce l'espressione del gene *TRPC6* in cellule podocitarie lesionate

Studi clinici e pre-clinici dimostrano che gli analoghi della vitamina D, oltre ad avere un ruolo omeostatico, riducono la proteinuria e la perdita di cellule podocitarie nel corso di malattie glomerulari. L'attività antiproteinurica della forma attiva della vitamina D, nota come $1,25(OH)_2D_3$, si basa sulla regolazione trascrizionale di nefrina e podocina, due proteine podocitarie che preservano la filtrazione glomerulare mantenendo intatto il complesso proteico del diaframma di filtrazione. Questo complesso include anche TRPC6, un canale ionico coinvolto nell'influsso di Ca^{2+} nei podociti. Mutazioni gain-of-function in *TRPC6* sono associate ad una forma ereditaria di glomerulosclerosi focale e segmentaria (FSGS). Inoltre, altre malattie ereditarie o acquisite che causano proteinuria sono associate ad un aumento dell'espressione e dell'attività glomerulare di TRPC6.

In questo capitolo, è stato dimostrato che la somministrazione di $1,25(OH)_2D_3$ riduce l'aumentata espressione di TRPC6 in un modello di lesioni podocitarie *in vitro*, ma anche in ratti affetti da nefropatia indotta dopo trattamento con adriamicina e usati come modello animale di FSGS. Esperimenti di ChIP e saggi di luciferasi hanno mostrato che la diminuzione della trascrizione di TRPC6 dopo trattamento con $1,25(OH)_2D_3$ si verifica tramite il legame del complesso recettoriale $1,25(OH)_2D_3$/VDR/RXR al promotore del gene *TRPC6*. La carenza di $1,25(OH)_2D_3$ in topi knockout per il gene 25-idrossi-1 alfa-idrossilasi è

associata ad un aumento dell'espressione glomerulare di TRPC6, ad una parziale riduzione delle digitazioni tra i podociti e a proteinuria. La somministrazione di $1,25(OH)_2D_3$ in questo modello animale riduce l'aumentata espressione di TRPC6 e la proteinuria, e normalizza la morfologia dei podociti.

In conclusione, la diminuzione dell'espressione di TRPC6 è uno dei meccanismi che contribuisce all'effetto anti-proteinurico della vitamina D.

Capitolo 6: Meccanismi molecolari alla base della perdita di Mg^{2+} nelle urine dopo trattamento con rapamicina

La rapamicina è un farmaco immunosoppressivo usato principalmente come terapia anti-rigetto in pazienti sottoposti a trapianto d'organo. Il trattamento con rapamicina inibisce la molecola di segnale intracellulare mTOR e causa un'elevata frazione di escrezione renale di Mg^{2+}, forse a causa di un difetto nel riassorbimento di Mg^{2+} nel tubulo distale contorto.

A tale proposito, in questo capitolo viene dimostrato che il trattamento cronico con rapamicina riduce nei topi l'espressione genica renale di TRPM6, il canale ionico permeabile al Mg^{2+}, localizzato sulla membrana apicale del tubulo distale contorto. Tra gli altri geni noti per essere importanti nel riassorbimento transcellulare di Mg^{2+} nel medesimo tratto del nefrone, anche il fattore di crescita epiteliale (EGF), coinvolto nella regolazione ormonale di TRPM6, e HNF1B mostrano una diminuzione dei livelli di mRNA in seguito al trattamento con rapamicina. Al contrario, l'espressione genica di due proteine delle giunzioni occludenti, la claudina-16 (CLDN16) e la claudina-19 (CLDN19), essenziali per il riassorbimento paracellulare di Mg^{2+} nel tratto ascendente spesso dell'ansa di Henle, è maggiore negli animali trattati con rapamicina rispetto ai controlli. Tale risultato potrebbe essere causato da variazioni nei livelli di mRNA della claudina-14 (CLDN14), che regola il complesso CLDN16-19. Esperimenti di elettrofisiologia hanno dimostrato che la rapamicina inibisce rapidamente la stimolazione da parte di EGF delle correnti ioniche mediate da TRPM6 *in vitro*, mentre non interferisce con l'effetto stimolatorio che l'insulina esercita sull'attività di TRPM6. In futuro, ulteriori esperimenti saranno finalizzati alla caratterizzazione dei processi di segnalazione intracellulare a valle dei recettori per l'EGF e per l'insulina che influenzano l'attivazione di mTOR e quindi l'attività di TRPM6.

In breve, lo studio riportato in questo capitolo suggerisce che la diminuzione dell'espressione di TRPM6 e della sua regolazione ormonale nel tubulo distale contorto del rene contribuisce alla perdita di Mg^{2+} nelle urine dopo trattamento cronico con rapamicina.

Capitolo 7: Discussione e prospettive future

Il trasporto di Ca^{2+} e Mg^{2+} attraverso la membrana plasmatica delle cellule epiteliali renali è condizionato dall'abbondanza e dall'attività di canali ionici e di trasportatori sulla superficie cellulare, ma anche da un appropriato controllo ormonale, come dimostrato in questa tesi. Una domanda che rimane irrisolta è come cambiamenti nell'espressione della

subunità γ della Na^+-K^+-ATPasi sulla membrana basolaterale del tubulo distale contorto possano causare ipomagnesemia. Sarebbe inoltre interessante conoscere il profilo completo dei siti di legame di HNF1B nel genoma renale. Esperimenti di ChIP per HNF1B associati a tecnologie di sequenziamento di nuova generazione (ChIP-Seq) potrebbero rivelare nuovi network trascrizionali coinvolti nel trasporto di Ca^{2+} e Mg^{2+} nel rene. Inoltre, la nostra conoscenza circa il controllo ormonale della trascrizione mediata da HNF1B e del legame di HNF1B a proteine accessorie è ancora limitata. Studi futuri dovrebbero infine investigare come il network di fattori trascrizionali che include HNF1B è influenzato dal trattamento con terapie farmacologiche che causano uno stato di deficienza di Mg^{2+}, come ad esempio la rapamicina.

Di grande rilevanza clinica è la caratterizzazione del ruolo dei flussi di Ca^{2+} nella (pato) fisiologia dei podociti. In particolare, comprendere come l'espressione e l'attività del canale ionico per il Ca^{2+}, TRPC6, sono modulate nei podociti potrebbe portare allo sviluppo di nuovi approcci terapeutici per il trattamento di malattie renali associate a proteinuria.

In conclusione, lo studio dei meccanismi molecolari che influenzano la trascrizione di proteine coinvolte nel trasporto renale di Ca^{2+} e Mg^{2+}, e l'analisi della regolazione ormonale di tali eventi rappresentano le basi per lo sviluppo di nuove strategie farmacologiche per la cura delle malattie ereditarie e acquisite associate a disturbi del trasporto di Ca^{2+} e Mg^{2+} nelle cellule podocitarie e tubulari del rene.

8

Curriculum Vitae

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Acknowledgements – Ringraziamenti

Curriculum vitae

Silvia Ferrè was born in Busto Arsizio, Italy, on 24 November 1983. After her studies in accounting, she decided to pursue her interest for science by studying Biotechnology at the University of Insubria, Varese, where in 2005 she obtained her bachelor degree *cum laude*. She then attended the master course in Medical Biotechnologies and Molecular Medicine at the University of Milan. In 2007, she graduated *cum laude* with her thesis entitled: "Hypomagnesemia and tumor growth inhibition: an *in vivo* and *in vitro* study", as a result of her research conducted at the Department of Clinical Sciences LITA Vialba, under the supervision of Prof. dr. Jeanette Maier. In the same laboratory, Silvia continued her research for one year as post-graduated fellow on a project about the effect of hypomagnesemia on endothelial cells. In 2008, she moved to the Netherlands to start a PhD project in renal physiology at the Department of Physiology, Radboud University Nijmegen Medical Centre. Under the supervision of Prof. dr. René J.M. Bindels and Prof. dr. J.G. Hoenderop, Silvia mainly investigated new regulatory pathways of Ca^{2+} and Mg^{2+} transport in renal cells. She successfully followed the Nijmegen Centre for Molecular Life Sciences (NCMLS) PhD Research Training Programme. In June 2011 she received the Young Investigator Award at the European meeting by the International Society for the Development of Research on Mg^{2+}. In September 2012 she won the best poster award by Nature Reviews Nephrology at the ISN Nexus Symposium in Copenhagen, Denmark. From December 2010 to January 2012 she participated in the NCMLS PhD Committee as organizer of the NCMLS Technical Forums. From September 2013, she will work as a postdoctoral researcher in the laboratory of Prof. Peter Igarashi in the Department of Internal Medicine at UT Southwestern Medical Center in Dallas, USA. Her new project will be directed at understanding the role of the transcription factor HNF1B in polycystic kidney disease.

List of publications

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List of abbreviations

$1,25(OH)_2D_3$	1,25-dihydroxy-vitamin-D_3/ calcitriol
1α-OHase	25-hydroxyvitamin-D_3-1α-hydroxylase
AFM	α-albumin
ALP	alkaline phosphatase
ALT	alanine transaminase
AST	aspartate transaminase
CaSR	Ca^{2+}- sensing receptor
CD	collecting duct
CDH16	gene encoding kidney-specific cadherin
ChIP	chromatin-immunoprecipitation
CLC-Kb	chloride channel protein CLC-Kb
CLDN14	claudin-14
CLDN16	gene encoding claudin-16
CLDN19	gene encoding claudin-19
CNNM2	cyclin M2
CNT	connecting tubule
D_{28K}	calbindin-D_{28K}
DCoH	dimerization cofactor of hepatocyte nuclear factor 1-alpha
DCT	distal convoluted tubule
DCT1	early distal convoluted tubule
DCT2	late distal convoluted tubule
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FGA/FGB	genes encoding α/β-fibrinogen respectively
FXYD2	gene encoding Na^+-K^+-ATPase pump, subunit γ
FSGS	focal segmental glomerulosclerosis
GBM	glomerular basement membrane
GLUT2	gene encoding glucose transporter type 2
GPCR	G protein-coupled receptor
HNF1A	hepatocyte nuclear factor 1 homeobox A
HNF1B	hepatocyte nuclear factor 1 homeobox B
HPABH4D	hyperphenylalaninemia, BH4-deficient, D
HPT	hyperparathyroidism
hs-CRP	high-sensitivity C reactive protein
IL-2	interleukin-2
INS-1	gene encoding insulin-1
Kif12	Kinesin-like protein Kif12
Kir4.1	K^+ channel, inwardly rectifying subfamily J, member 10
Kv1.1	K^+ voltage-gated channel subfamily A, member 1
L-PK	gene encoding L-type pyruvate kinase
MODY	maturity-onset diabetes of the young
mTOR	mammalian target of rapamycin
NCC	Na^+-Cl^- cotransporter
NCX1	Na^+-Ca^{2+} exchanger
NFATc	nuclear factor of activated T-cells, cytoplasmic
NFATn	nuclear factor of activated T-cells, nuclear
NKCC2	Na^+-K^+-Cl^- cotransporter 2
NODAT	new-onset diabetes after transplantation
NPT1-4	gene encoding Na^+-dependent Pi transport protein 1-4
OAT1-4	gene encoding organic anion transporter 1-4

OATP1B1-3	gene encoding solute carrier organic anion transporter member 1B1-3
PAH	phenylalanine-4-hydroxylase
PCBD1	pterin-4 alpha-carbinolamine dehydratase
PDX-1	gene encoding pancreas/duodenum homeobox protein 1
Pkd2	polycystin-2
PkhD1	gene encoding polycystic kidney and hepatic disease 1 protein
PMCA1b	plasma membrane Ca^{2+}-ATPase
PT	proximal tubule
PTH	parathyroid hormone
PTHrp	parathyroid hormone- related peptide
Rac1	ras-related C3 botulinum toxin substrate 1
RCAD	renal cysts and diabetes syndrome
ROMK	inward rectifying ATP-sensitive K^+ channel
RXR	retinoid X receptor
SGLT2	gene encoding Na^+-glucose co-transporter 2
SOCS3	gene encoding suppressor of cytokine signaling 3
TAL	thick ascending loop of Henle
TMEM27	gene encoding collectrin
TRPC6	transient receptor potential channel, subfamily C, member 6
TRPM6	transient receptor potential channel, subfamily M, member 6
TRPV5	transient receptor potential channel, subfamily V, member 5
TRPV6	transient receptor potential channel, subfamily V, member 6
UMOD	gene encoding uromodulin
URAT1	gene encoding urate anion exchanger 1
VDR	vitamin D receptor
VDRE	vitamin D-responsive element
VEGFR	vascular endothelial growth factor receptor

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I believe that you get to know yourself, grow and evolve when you're challenged, not when you're circulating in your comfort zone. That's why I decided to move to the Netherlands for my doctorate. In the past four years, I have been living a considerable scientific and human experience that significantly changed me. I owe a lot to many people for making me better through tears and joy.

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Silvia

"I tell young people: do not think of yourself, think of others. Think of the future that awaits you, think about what you can do and do not fear anything."

Rita Levi-Montalcini
1909-2012

Italian neurobiologist

She received the Nobel Prize in Physiology or Medicine 1986, together with the colleague Stanley Cohen, for the discovery of nerve growth factor.