

HNF1B Mutations Associate with Hypomagnesemia and Renal Magnesium Wasting

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ABSTRACT

Mutations in *hepatocyte nuclear factor 1B (HNF1B)*, which is a transcription factor expressed in tissues including renal epithelia, associate with abnormal renal development. While studying renal phenotypes of children with *HNF1B* mutations, we identified a teenager who presented with tetany and hypomagnesemia. We retrospectively reviewed radiographic and laboratory data for all patients from a single center who had been screened for an *HNF1B* mutation. We found heterozygous mutations in 21 (23%) of 91 cases of renal malformation. All mutation carriers had abnormal fetal renal ultrasonography. Plasma magnesium levels were available for 66 patients with chronic kidney disease (stages 1 to 3). Strikingly, 44% (eight of 18) of mutation carriers had hypomagnesemia (<1.58 mg/dl) compared with 2% (one of 48) of those without mutations ($P < 0.0001$). The median plasma magnesium was significantly lower among mutation carriers than those without mutations (1.68 versus 2.02 mg/dl; $P < 0.0001$). Because hypermagnesuria and hypocalciuria accompanied the hypomagnesemia, we analyzed genes associated with hypermagnesuria and detected highly conserved HNF1 recognition sites in *FXYD2*, a gene that can cause autosomal dominant hypomagnesemia and hypocalciuria when mutated. Using a luciferase reporter assay, we demonstrated HNF1B-mediated transactivation of *FXYD2*. These results extend the phenotype of *HNF1B* mutations to include hypomagnesemia. HNF1B regulates transcription of *FXYD2*, which participates in the tubular handling of Mg^{2+} , thus describing a role for HNF1B not only in nephrogenesis but also in the maintenance of tubular function.

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Hepatocyte nuclear factor 1B (HNF1B), also known as variant hepatocyte nuclear factor 1 and transcription factor-2, is a POU (Pit-1/Oct-1/2-UNC-86) domain transcription factor closely related to HNF1A.^{1,2} Both are expressed in the pancreas, liver, and kidney.^{1,2} Mutations of either gene can cause maturity-onset diabetes of the young, and *HNF1B* mutation can cause neonatal diabetes.^{1,3}

Hnf1b is expressed in developing mouse ureters and collecting ducts, whereas, postnatally, proximal

and distal tubules express the gene.⁴ HNF1B is ex-

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pressed in both maturing human collecting ducts and nephrons.^{5,6} *Hnf1b* deletion in mouse collecting ducts causes cysts.^{7,8} Furthermore, *Hnf1b* upregulates transcription of *uro-modulin (Umod)*, *polycystic kidney and hepatic disease 1 (Pkd1)*, and *polycystic kidney disease (Pkd2)*, the human homologues of which are respectively mutated in medullary cystic kidney disease type 2, autosomal recessive polycystic kidney disease (PKD), and a subset of autosomal dominant PKD.⁸

After *HNF1B* gene mutations were implicated as causing maturity-onset diabetes of the young 5, reports linking mutations with developmental renal disease appeared.^{9–12} The association of *HNF1B* mutations with cystic dysplasia and a glomerulocystic variant of PKD led to the term renal cysts and diabetes syndrome.¹³ Subsequently, fetal bilateral hyperechogenic kidneys, multicystic dysplastic kidneys (MCDK), and hyperuricemic nephropathy with gout have been associated with *HNF1B* mutations.^{14–16} Other features include pancreatic atrophy and exocrine dysfunction in patients with diabetes.^{17,18} *HNF1B* mutations can occur *de novo* or be inherited and comprise monoallelic changes in the sequence of *HNF1B* or whole gene deletions.^{17,18} Biallelic *HNF1B* inactivation, a combination of monoallelic germline and somatic mutations, occurs in a subset of chromophobe renal carcinomas.¹⁹

We aimed to define phenotypes of children with renal malformations, comparing those with and without *HNF1B* mutations. We assessed the magnesium axis because a teenager with renal malformation and an *HNF1B* mutation presented with tetany and hypomagnesemia.

RESULTS

Index Case

A 13-yr-old boy of Pakistani origin presented with paresthesia and positive Chvostek and Trousseau signs. Initial antenatal

screening had shown echogenic kidneys, and a subsequent fetal scan showed a left MCDK; this involuted in the first few post-natal months, and he received no specialist follow-up. His laboratory investigations revealed hypocalcemia and hypomagnesemia (Table 1). He received oral calcium and magnesium supplements; plasma calcium levels improved and parathyroid hormone (PTH) and phosphate values normalized, but plasma total magnesium levels remained low and fractional magnesium excretion (FEMg) was elevated at 9.5% (normal <4% in the presence of hypomagnesemia). Urinary calcium concentration was below the detection threshold of 1 mg/dl (<0.25 mM), indicating a fractional calcium excretion (FECa) of <0.2%. His sister had already been noted to have two echobright, cystic kidneys, detected upon routine fetal screening. When her GFR was 87 ml/min per 1.73 m², her plasma total magnesium was recurrently low (1.38 to 1.48 mg/dl; 0.57 to 0.61 mM), with a FEMg of 8%. She had normocalcemia and, like her brother, hypocalciuria with a FECa of <0.1%. Both siblings have a heterozygous *HNF1B* deletion. Assessment of the mother was unremarkable, but the father also has a heterozygous *HNF1B* deletion; he was subsequently found to have a kidney cyst and hypomagnesemia (1.1 mg/dl; 0.48 mM).

General Characteristics of Children with *HNF1B* Mutations

Next, we surveyed plasma magnesium values in our cohort of patients with identified *HNF1B* mutations (*mut+*) and compared these data with those of patients with renal malformations but without *HNF1B* mutations (*mut-*). Of 91 patients tested, *HNF1B* mutations were identified in 21 (10 male; ethnicity: 12 white; six Asian; two Afro-Caribbean; one other). All cases presented independently and derived from 18 separate families; three of the families included two *mut+* siblings with renal malformations (including the index family described already). Comprehensive notes and results could be accessed on

Table 1. Pertinent laboratory investigations in the index patient^a

Parameter	Presentation with Tetany	Follow-up 4 Mo Later	Normal Range
Plasma indices			
magnesium (mg/dl [mM])	1.58 (0.620) ^b	1.22 (0.500) ^b	1.58 to 2.43 (0.650 to 1.000)
total calcium (mg/dl [mM])	5.20 (1.300) ^b	9.20 (2.310)	8.80 to 10.65 (2.190 to 2.660)
phosphate (mg/dl [mM])	7.53 (2.430) ^b	4.34 (1.400)	3.40 to 5.40 (1.100 to 1.750)
creatinine (mg/dl [mM])	0.55 (0.049)	0.81 (0.072)	0.40 to 0.90 (0.035 to 0.080)
albumin (g/L)	45	49	37 to 56
PTH (ng/L [pM])	176 (19.0) ^b	55 (5.8)	10 to 65 (0.7 to 5.6)
25-hydroxyvitamin D (nM)	25	Not obtained	15 to 100
Urinary indices			
magnesium (mg/dl [mM])	Not obtained	6.08 (2.50)	
FEMg (%)	Not obtained	9.5 ^b	<4 ^c
calcium (mg/dl [mM])	Not obtained	<1 (<0.25)	
FECa (%)	Not obtained	<0.2	>1 ^c
creatinine (mg/dl [mM])	Not obtained	43 (3.80)	

^aSelected laboratory investigations at presentation of the index case and at follow-up 4 mo later. Note the hypocalcaemia and hypomagnesemia at presentation. While the plasma calcium normalized with time, magnesium remained low.

^bAbnormal value.

^cNo clearly defined normal values exist because urinary calcium and magnesium excretion reflects dietary intake in steady state; however, in the presence of hypomagnesemia, FEMg >4% is considered pathologic hypermagnesuria and hypocalciuria is usually defined as FECa <1% (see the Concise Methods section).

all *mut+* cases and in 66 (39 male) of the 70 *mut-* patients (ethnicity: 49 white; 14 Asian; one Afro-Caribbean; two other), except for fetal ultrasound reports (available in 63 of 70 *mut-*).

Mutation Analyses

Of the 91 patients with renal malformations in which *HNF1B* was screened, 21 (23%) had a heterozygous *HNF1B* mutation, nine of which have previously been reported.^{5,13,14,17,18,20} Twelve cases had a heterozygous *HNF1B* deletion c.1_1674del; p.Met1_Trp557del (four of 12 previously reported)¹⁷ from 10 different families. Nine patients from eight different families had heterozygous mutations detected by direct sequencing; three cases had frame shift mutations (c.1055_1056insA, p.Tyr352fsinsA⁵; c.972_973delCA, His324Ser325fsdelCA¹⁸; c.206_207delAC, His69fsdelAC²⁰), five cases from four families had splice-site mutations (two families with c.544 + 1G→T, IVS2 + 1G→T¹⁴; two siblings with the novel mutation c.544 + 3_544 + 6delAAGT, IVS2 + 3_+6delAAGT and one case with the novel mutation c.810-2A→C, IVS3-2A→C) and one with a missense mutation (c.466A>G, p.Lys156Glu¹⁸). In addition to pathogenic mutations, we identified the previously described c.73G→T, p.Val25Leu variant of unknown pathogenicity.¹⁸ This is the second Pakistani family to be described with this variant. In our family, the child has renal cysts and his father has diabetes and a smaller left kidney with scarring. To establish pathogenicity, we screened 302 chromosomes from control subjects of Pakistani ethnicity and identified the heterozygous Val25Leu variant in three, and we therefore concluded that this is a polymorphism in the Pakistani population, which is unlikely, *per se*, to cause severe renal malformation.

Radiology

Radiologic findings in the *mut+* patients are detailed in Table 2. All had abnormal fetal renal ultrasonography *versus* 26 (41%) of 63 *mut-* ($P < 0.05$). Postnatally, all four MCDKs involuted. Of the 38 remaining kidneys in all 21 patients, kidney lengths were available for 30. On the latest ultrasounds in the *mut+* patients, performed at median age of 6.6 yr (range 2.3 to 16.5 yr), lengths were between the fifth and 95th centiles for age in all but seven kidneys (five were under the fifth centile, and two were above the 95th centile), and none of the four solitary kidneys (*i.e.*, those opposite the involuted MCDKs)

Table 2. Radiologic findings in *HNF1B* mutation carriers^a

Radiologic Abnormality	n (%)
Abnormal antenatal renal scan	21 (100)
First postnatal scan	
bilateral disease	16 (76)
large, echogenic, no cysts	9 (43)
large, echogenic with cysts	5 (24)
unilateral MCDK	4 (19)
bilateral hydronephrosis	3 (14)

^aNote that all mutation carriers had abnormal antenatal scans. On the first postnatal scan, cysts were present in only nine (43%) patients, and abnormalities were unilateral in five (24%).

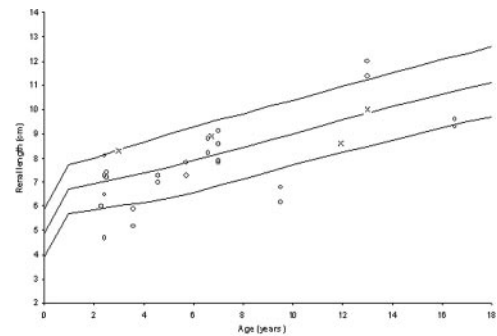


Figure 1. Renal length on latest ultrasound in those with *HNF1B* mutations. Crosses, renal length of the four solitary functioning kidneys after regression of contralateral MCDKs. Circles, all other (26 of 30) kidneys from the *HNF1B* mutation cohort for which lengths were accurately measured. Top, middle, and bottom lines indicate the 95th, 50th, and fifth centiles for age as based on data from our hospital's radiology department (see the Concise Methods section). Note absence of compensatory hypertrophy in single remaining kidneys (crosses).

were larger than the 95th centile (Figure 1). No patient had nephrocalcinosis. One *mut+* patient had persistent postnatal hydronephrosis and hydroureter. Isotope renography showed delay of drainage from the upper tract, but cystography demonstrated neither vesicoureteric reflux nor bladder outflow obstruction. In the *mut-* group, the range of clinically diagnosed renal tract phenotypes was generally similar to *mut+*; however, in the *mut-* group, one had a urogenital sinus and four had posterior urethral valves, whereas none of the *mut+* had these diagnoses. Compared with *mut-* patients ($n = 66$), *mut+* were more likely to have cysts in either kidney on initial postnatal ultrasound scan (57 *versus* 36%; $P = 0.02$) and increased echogenicity of either kidney (76 *versus* 48%; $P = 0.04$).

GFR, Diabetes Mellitus, and Uric Acid Levels

The most recent clinic review (or last review before renal transplant) of the *mut+* patients was performed at 8.0 yr (range 3.1 to 17.1), when GFR was 63 ml/min per 1.73 m² (range 8 to 113 ml/min per 1.73 m²). In the *mut-* cohort, assessed at 8.4 yr (range 0.3 to 17.8 yr), GFR was 46 ml/min per 1.73 m² (range 5 to 123 ml/min per 1.73 m²). Two *mut+* patients had reached chronic kidney disease (CKD) stage 5 *versus* 17 *mut-* ($P = 0.14$), including those who presented in established renal failure. Five (24%) of the 21 *mut+* children developed diabetes during follow-up, at a mean age of 12 yr (range 10 to 14 yr) *versus* five (8%) of the 66 *mut-* children ($P = 0.0567$). Blood uric acid levels, assessed at stage 3 CKD or less, were available for only a subset of patients and were elevated in 71% (10 of 14) of *mut+* patients *versus* 47% (seven of 15) *mut-* patients tested ($P = 0.26$) compared with age-appropriate norm values.

Plasma and Urine Magnesium Levels

Plasma total magnesium values were available for 66 patients with stages 1 through 3 CKD (Table 3, Figure 2). Striking, 44%

Table 3. Laboratory investigations for 66 patients assessed at stages 1 through 3 CKD^a

Parameter	Mutation Status				P
	HNF1B mut+		HNF1B mut–		
	Median	Range	Median	Range	
Plasma magnesium (mg/dl [mM])	1.68 (0.69)	1.16 to 2.09 (0.48 to 0.86)	2.02 (0.83)	1.46 to 2.34 (0.60 to 0.96)	<0.0001
Plasma calcium (mg/dl [mM])	9.64 (2.41)	8.84 to 10.40 (2.21 to 2.60)	9.56 (2.39)	8.16 to 10.88 (2.04 to 2.72)	0.7500
Plasma phosphate (mg/dl [mM])	4.24 (1.37)	2.97 to 6.69 (0.96 to 2.16)	4.57 (1.48)	2.72 to 7.06 (0.88 to 2.28)	0.3500
Blood albumin (g/dl)	42	35 to 49	41	24 to 48	0.2600
Plasma PTH (ng/L [pM])	59.40 (6.30)	22.60 to 101.80 (2.40 to 10.80)	46.63 (4.95)	9.40 to 179.10 (1.00 to 19.00)	0.2000
GFR (ml/min per 1.73 m ²)	73.0	32.0 to 113.0	70.5	30.0 to 123.0	0.8500

^aMedian plasma concentrations of magnesium and corresponding time-matched GFR, calcium, phosphate, albumin, and PTH values. *n* = 18 for *mut+* and *n* = 48 for *mut–*, apart from PTH values, for which only a subset of values was available (*mut+* *n* = 11; *mut–* *n* = 35). Only plasma magnesium was significantly different among groups.

(eight of 18) *mut+* patients had hypomagnesemia (<1.58 mg/dl; 0.65 mM) versus 2% (one of 48) *mut–* patients (*P* < 0.0001). Of the eight *mut+* patients with hypomagnesemia, five had heterozygous whole-gene deletions, two had splice-site mutations, and one had a frame-shift mutation. Thus, there was no clear association of hypomagnesemia with type of mutation. Notably, the upper and lower limits of plasma magnesium values were shifted downward in the mutation carriers. Corresponding GFRs for *mut+* and *mut–* patients with available magnesium values were not significantly different. Using hypomagnesemia as a diagnostic criterion for *HNF1B* mutation in our cohort of children resulted in a calculated sensitivity of 44%, a specificity of 98%, and a positive predictive value of 89%. The likelihood ratio of detecting an *HNF1B* mutation in this cohort was 21 when hypomagnesemia was present. Time-matched values for plasma calcium, phosphate, albumin, and PTH (where available) were also compared (Table 3) and did not demonstrate significant differences between the two groups. In all eight *mut+* patients with hypomagnesemia, there was evidence of renal magnesium wasting (hypermagnesuria) with a median FEMg of 6.5% (4.5 to 14.3%). In all six *mut+* patients who had hypomagnesemia and for whom urinary calcium data were available, hypocalciuria was present, with a median FECa of 0.20% (0.06 to 0.45%). The actual urine calcium value was below the measurement threshold of 1.00 mg/dl (0.25 mM) in three patients, suggesting that true calcium excretion was even lower. Two of the *mut+* children had a history of both hypomagnesemia and diabetes.

HNF1B as a Regulator of Tubular Magnesium Transport

We analyzed putative HNF1-binding sequences of genes associated with renal magnesium wasting and identified four highly homologous such sites arranged as two pairs located in the intron between exons γ -b and exon γ -a of the *FXD2* gene (Figure 3). These exons encode alternative start sites for the *FXD2* splice variants B (NM 021603) and A (NM 001680), respectively. We therefore assessed possible regulation of *FXD2* by HNF1B in two different cell lines, using an expression construct with luciferase as a reporter gene downstream of

the putative HNF1-binding sites (see the Concise Methods section). In both the human embryonic kidney cell line HEK293 and the rat insulinoma cell line INS-1, conditional expression of HNF1B upregulated luciferase activity (Table 4). The combination of renal magnesium wasting with hypocalciuria in *mut+* patients implicates a defect in the distal convoluted tubule (DCT; see the Discussion section). We therefore sought expression of *Hnf1* and *Fxyd2* transcripts in a mouse DCT cell line using reverse transcription-PCR (RT-PCR; Figure 4). We detected both *Hnf1a* and *Hnf1b* mRNA; with regard to *Fxyd2*, the *b* form was detected.

DISCUSSION

Ours is not an epidemiologic study because not all our patients with renal malformations were selected for *HNF1B* analyses (see the Concise Methods section). Nevertheless, the data show that mutations are not uncommon in our clinical department, which assesses pediatric referrals from a total population of approximately five million. *HNF1B* mutations have been found in 8% of children with renal hypodysplasia,²¹ but the detection rate increases up to 30% when restricted to children with early presentation and certain renal abnormalities,¹⁵ comparable to our detection rate of 24%. Our *HNF1B* cohort of 21 index cases is the largest single-center collection reported to date and allows us to integrate clinical data using uniform radiologic and biochemical analyses.

All index cases with *HNF1B* mutations had abnormal fetal ultrasonography, emphasizing the important role of this gene in renal development. The range of phenotypes comprised large bright kidneys, MCDKs, and hydronephroses. Postnatally, although all four MCDKs involuted, the contralateral functioning kidneys were not larger than normal, as might be expected if they had “hypertrophied” as expected.²² Furthermore, although several of the (non-MCDK) kidneys were enlarged antenatally, the same kidneys regressed into age-appropriate normal size ranges during childhood. Thus, *HNF1B* mutations accelerate overall kidney growth in gestation, whereas, postnatally, overgrowth is not maintained. We also

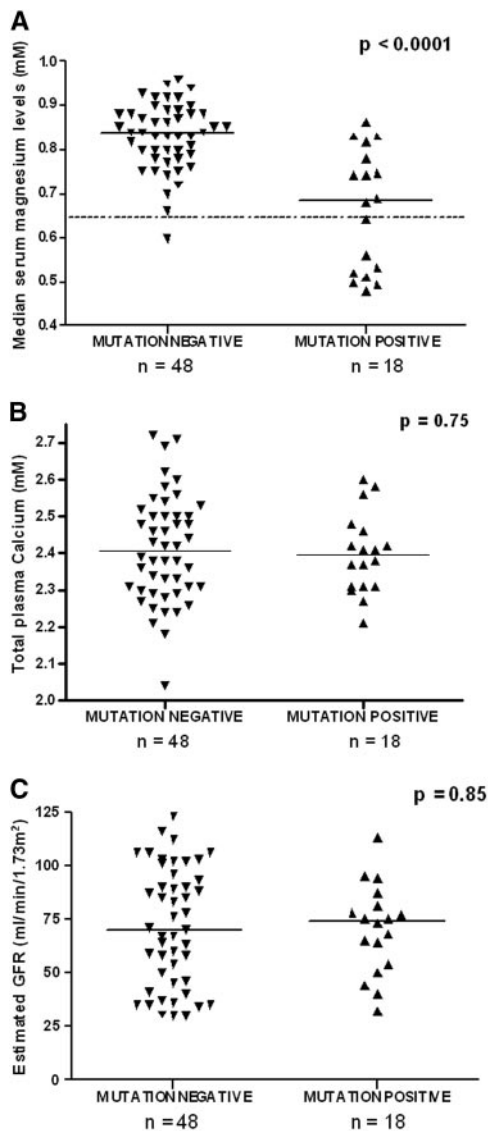


Figure 2. Plasma total magnesium, calcium, and GFR values in *mut+* and *mut-* patients assessed at stages 1 through 3 CKD. (A) Median plasma magnesium value for each patient is shown, together with *mut-* and *mut+* group medians (short horizontal bars). The lower limit of normal is indicated by the dotted horizontal line. Note the lower magnesium values in the *mut+* cohort. Eight of 18 HNF1B mutation carriers had hypomagnesemia versus only one of 48 in the *mut-* group. (B and C) Plasma calcium and GFR values (time matched for the plasma magnesium points shown in A). *P* values refer to comparisons of the median (ranges) between the two groups.

recorded the occurrence of hydronephrosis and hydroureter. Given that *HNF1B* is expressed in ureteric bud derivatives,^{4,5} this is perhaps not surprising, although this clinical observation has not previously been emphasized; however, further evidence comes from a recent report describing a case with mega-bladder associated with *HNF1B* mutation and that mice with genetic downregulation of *Hnf1b* in the bud lineage have

megaureter.^{8,23} *Mut+* patients were also more likely versus *mut-* patients to have kidney cysts on initial postnatal ultrasonography.

Median magnesium levels were lower in the cohort of *mut+* versus *mut-* patients, and almost half had hypomagnesemia. Whereas most other ions are predominantly reabsorbed in the proximal tubule, the key sites for magnesium reabsorption are the thick ascending limb of Henle's loop (TAL) and DCT.²⁴ Consequently, all genes associated with abnormal renal magnesium handling affect transport in these two segments. Clinically, the affected segment can be determined by analysis of urinary calcium excretion; the molecular pathway for magnesium reabsorption in TAL includes Claudins 16 and 19 and is shared with calcium.²⁵ The necessary voltage gradient is generated by the combined actions of SLC12A1 and KCNJ1²⁶; therefore, loss of function in any of these four proteins leads to wasting of both magnesium and calcium. Conversely, defects in DCT lead to hypermagnesuria associated with hypo- or normocalciuria. Hypocalciuria will be present when the defect also impairs sodium reabsorption in the DCT, as occurs with mutations in *SLC12A3*, *CLCKNB* (Gitelman syndrome), or *FXYD2*.²⁷ The resulting volume loss leads to increased proximal sodium transport, which is paralleled by calcium reabsorption.²⁸ In contrast, if the mutation affects a gene primarily involved in magnesium transport in DCT, such as *TRPM6* or *EGF*, then renal calcium handling is unchanged and patients have normocalciuria.²⁹ Our observation of hypocalciuria and hypermagnesuria pinpoints the defect to DCT and specifically to a gene also involved in sodium reabsorption in this segment.

To date, roles of HNF1B in morphogenesis have been emphasized, quite appropriately given the structural kidney anomalies found in mutation carriers. Our study also provides evidence to implicate HNF1B in physiologic functions in the postnatal kidney. *FXYD2* encodes the γ subunit of the $\text{Na}^+ - \text{K}^+ - \text{ATPase}$, which regulates epithelial ion transport.³⁰ An *FXYD2* mutation has been associated with autosomal dominant hypomagnesemia and hypocalciuria.³¹ To date, however, mutation of *FXYD2* has been reported in just one family. The combination of our current observation that increased HNF1B expression upregulates *FXYD2/luciferase* expression, together with the fact that children with *HNF1B* mutations can experience renal magnesium wasting, supports the concept that *FXYD2* plays an important role in renal magnesium handling.

We demonstrated HNF1B-mediated transactivation of a luciferase reporter gene downstream of the putative HNF1-binding sites in *FXYD2* in an embryonic kidney and a pancreatic endocrine line. The more marked stimulation in HEK293 versus INS-1 cells may be explained by the fact that endogenous expression of HNF1B in the embryonic renal cells³² is less than in the insulinoma line.³³ Moreover, in the latter cells, we previously described that HNF1B regulated levels of transcripts of the endogenous *FXYD2* gene.³³ Recently, chromatin immunoprecipitation array experiments were used to seek gene targets for Hnf1b in mouse kidneys.³⁴ Consistent with our observation of HNF1-binding sites in human *FXYD2*, the mouse im-

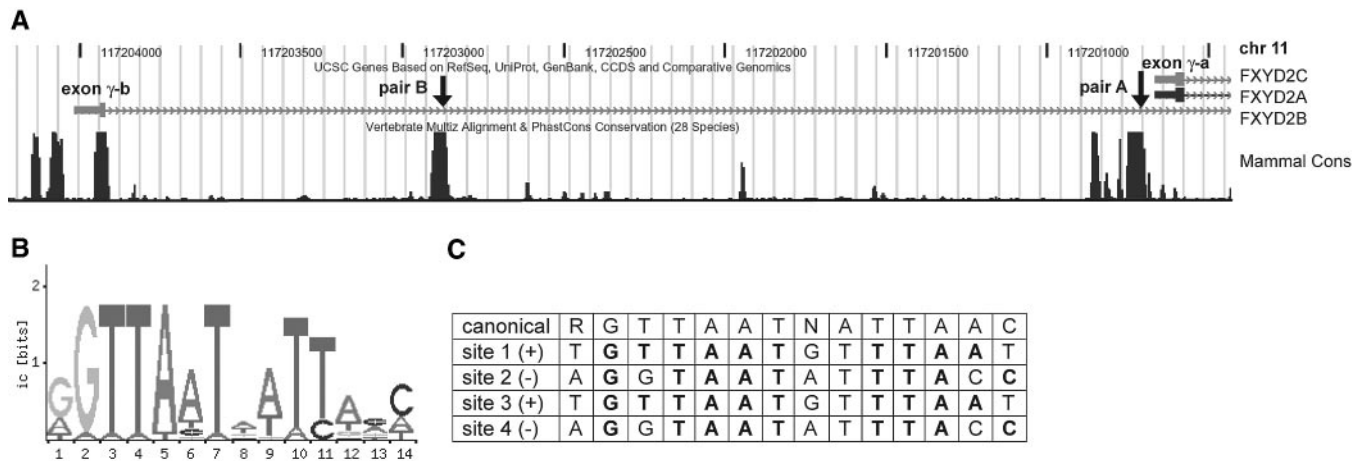


Figure 3. HNF1 recognition sites in the *FXYD2* promoter. (A) Localization of HNF1 recognition sites at the *FXYD2* locus. The *FXYD2* gene has different splice variants, starting with either exon γ -b (*FXYD2b*; NM021603) or exon γ -a (*FXYD2a*; NM001680).³⁵ Two identical pairs of HNF1-binding sites (pair A and pair B) were identified. Pair A is 62 nucleotides (nt) upstream of the transcription initiation site of exon γ -a, whereas pair B is 2128 nt further upstream. In each pair, the HNF1-binding sites are in opposite orientation and separated by 6 nt. Note the high mammalian conservation at both sites (derived from UCSC human genome browser). No HNF1 binding sites were found upstream of exon γ -b. (B) Sequence of established HNF1 recognition sites. The size of each letter is in approximate proportion to the frequency of finding the denoted nt at this position in the recognition site.⁵⁴ (C) Alignment of the putative HNF1 recognition sites with the canonical sequence. Pair A and pair B contain sites 1/2 and sites 3/4, respectively. Sites 1 and 3 are in the upper strand (+), whereas sites 2 and 4 are in the lower strand (-). R denotes puRine (A or G).

Table 4. Activation of the *FXYD2* promoter by HNF1B^a

Cell Line	Fold Activation		
	<i>FXYD2</i>	<i>Ksp-cadherin</i>	<i>Ksp-cadherin-mut</i>
HEK293	149.20 ± 7.70	14.30 ± 3.10	1.30 ± 0.10
INS-1	5.00 ± 0.60	2.30 ± 0.30	1.30 ± 0.03
INS-1 (endogenous) [§]	15.80 ^b	Not expressed	NA

^aThe assay measured expression of firefly luciferase under the control of the *FXYD2* or *Ksp-cadherin* promoter. The *FXYD2*/luciferase contained base pairs -2382 to -1 relative to the ATG of exon γ -a of *FXYD2* (Figure 3). *Ksp-cadherin* contains the promoter of this kidney-specific cadherin gene from -113 to 74. *Ksp-cadherin-mut* is an established mutant (m3) with inactivated HNF1 binding site, as described previously.⁴¹ The fold activation refers to the increase in luciferase activity in cells treated for 24 h with 1 μ g/ml tetracycline to induce HNF1B expression versus untreated cells. Data are means ± SD of six measurements involving two independent plasmid preparations. *FXYD2*/luciferase was activated by HNF1B in both cell lines, and in HEK292 cells the, fold activation was an order of magnitude greater than that found with *Ksp-cadherin*, an established transcriptional target of HNF1B.⁴¹ As expected, expression of HNF1B failed to transactivate the *Ksp-cadherin* with mutated HNF1B binding sites.

^bFor comparison, these data represent activation of the endogenous *FXYD2* gene as assessed by previously published microarray analyses.³³

munoprecipitation study showed that the *Fxyd2* promoter was four-fold enriched in chromatin from kidneys immunoprecipitated with an antibody against HNF1B versus control IgG, providing evidence for direct binding of Hnf1b to the *Fxyd2* gene (Z. Ma and P. Igarashi, University of Texas Southwestern Medical Center, Dallas, TX, personal communication, November 17, 2008).

Three different splice variants are recognized for human *FXYD2* (Figure 3A), the main ones being variants a and b.³⁵ An immunohistochemical study of rat kidneys reported that TALs in the inner medullary stripe expressed both *Fxyd2a* and *b* isoforms, whereas *Fxyd2a* was predominant in proximal tubules and *Fxyd2b* in DCTs, connecting tubules, and other TAL.³⁶ This is in accordance with our current RT-PCR data

showing that immortalized mouse DCT cells express *Fxyd2b* but not *Fxyd2a* transcripts. In the absence of immunohistochemical studies in humans, one might assume similar expression in humans. If we accept that magnesium wasting and hypocalciuria indicate a DCT defect, then we are left with the apparent paradox that HNF1-regulatory regions are directly upstream of the promoter of exon γ -a, which initiates transcription of human *FXYD2a*, while being downstream of exon γ -b, which initiates transcription of variant b (Figure 3). The HNF1-binding sites lie in the intron between the two alternative initiating exons of *FXYD2*, and we speculate

that they are a transcriptional enhancer for both exons. Indeed, intronic HNF1-binding sites exist in the *apolipoprotein B* gene, and, in this context, HNF1 and CCAAT/enhancer-binding protein act synergistically with protein II to enhance gene expression.³⁷ Furthermore, the murine *Pkd2* gene has intronic HNF1-binding sites.⁸

On the basis of our experiments with mouse cells, DCT epithelia express Hnf1a as well as Hnf1b. Although both proteins bind to the same sites on DNA as homo- or heterodimers,^{38–40} we note that HNF-mediated induction of endogenous *Fxyd2* in a pancreatic cell line is specific to HNF1B.³³ We therefore predict that, in our patients with hypomagnesemia, HNF1A cannot compensate for disruption of HNF1B activity. Interestingly, analysis of another gene regulated by

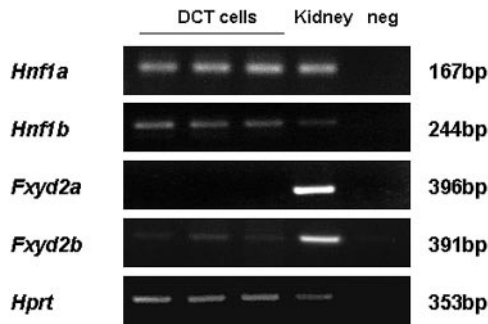


Figure 4. RT-PCR analyses in a DCT cell line. *Hnf1a*, *Hnf1b*, and *Fxyd2b* transcripts are expressed in mouse DCT cells. From left to right, lanes show consistent results from three independent cDNA preparations from DCT cells, mouse whole kidney (kidney), and reaction buffer only (neg). Note that *Fxyd2a* transcripts were not detected in DCT cells but were present in the whole kidney. *Hprt* was included as a housekeeping gene.

HNF1B in the kidney, *Ksp-Cadherin*, showed unaltered expression levels in *Hnf1a* knockout mice, whereas mutations in *Hnf1b* strongly affected *Ksp-Cadherin* expression *in vitro*.⁴¹

CONCISE METHODS

Cohort Details

All patients were children followed by nephrologists and urologists at Great Ormond Street Hospital NHS Trust (London, UK) between 2000 and 2007. Informed consent was obtained from all patients, and the study was conducted in agreement with the Declaration of Helsinki as revised in 2000. From 2006, a clinical geneticist (R.C.H.) joined the team to undertake genetic counseling. All patients' leukocyte DNA was initially screened for mutations in *HNF1B* using direct sequencing.¹⁸ From 2005, a multiplex ligation-dependent probe amplification assay for gene dosage became available and was used retrospectively and prospectively on samples with normal *HNF1B* sequencing.¹⁷ Testing was performed with consent of parents and assent of children, when appropriate. Inclusion criteria for genetic testing were "typical" RCAD phenotype (*e.g.*, renal cysts with diabetes); undiagnosed renal cystic disease (*i.e.*, not autosomal dominant or recessive PKD); or index patients who had kidney malformations and had one or more first- or second-degree relatives with one or more of renal cysts/malformation, diabetes, or acute gout. Renal morphology of nine of the 21 patients with *HNF1B* mutations has been reported previously, but their magnesium axis was not described.^{5,13,14,17,18,20}

Clinical Investigations

We performed a retrospective case note review. Histories were obtained from hospital notes, and we analyzed laboratory and radiologic investigations. We reviewed available reports of fetal ultrasonography and postnatal ultrasound scans and isotope renograms and cystograms. Renal length was assessed according to published percentiles used in our hospital.⁴² GFR was estimated using the Schwartz-Haycock formula, optimized for children with renal malformations as

assessed in our hospital.⁴³ Plasma total magnesium was interrogated only when the renal disease was staged 3 CKD or less severe (GFR \geq 30 ml/min per 1.73 m²) to avoid potentially confounding magnesium retention when GFR is decreased below 25% of normal.⁴⁴ Values were not included once patients received magnesium supplementation. No patient took medication associated with hypomagnesemia (*e.g.*, diuretics, calcineurin inhibitors, corticosteroids). A conservative lower normal limit of plasma total magnesium was defined as 1.58 mg/dl (0.65 mM). In several cases, multiple plasma magnesium values were available; in these cases, to avoid bias, we quote the median value together with time-matched plasma calcium, phosphate, albumin, and, when available, PTH levels. Renal magnesium and calcium excretion were assessed in patients with hypomagnesemia, and hypermagnesuria was defined as a fractional magnesium excretion $>$ 4%^{45,46} and hypocalciuria as a fractional calcium excretion $<$ 1%.⁴⁷ Statistical significance between parameters with discrete values was determined using Fisher exact test and Mann-Whitney *U* test. Fractional excretions were calculated using total plasma values for magnesium and calcium.

Bioinformatic Tools

HNF1A and B recognize a 14-bp consensus sequence, 5-GGTTAAT-NATTAAC-3, initially identified in the promoter of several liver-specific genes but subsequently verified in other tissues, including kidney.^{38–40} Minor sequence variations still allow regulation by HNF1B (Figure 3).⁴⁸ Using the UCSC Human Genome Browser (<http://www.genome.ucsc.edu>), we identified the 500 bp preceding the transcription initiation site as the putative proximal promoter region of genes known to affect renal magnesium transport: *BSND*, *CLCNKB*, *CLDN16*, *CLDN19*, *EGF*, *FXSD2*, *KCNJ1*, *SLC12A1*, *SLC12A3*, and *TRPM6*.^{26,31,49} We searched for the canonical motif using the Web-based tools ConSite (<http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite>) and MAST (<http://meme.sdsc.edu/meme/intro.html>) and the HMR Conserved Transcription Factor Binding Sites track using the UCSC Human Genome Browser.

Luciferase Reporter Assay

The promoter region of *FXSD2* from -2382 to -1 bp relative to the translation initiation site of exon γ -a (Figure 3), including all four putative HNF1 recognition sites, was amplified with the primers 5'-GGAGGTACCTCTGTTGGTGCCTCCCCATGC-3' and 5'-GGAA-GATCTTCCCCAGGTGAATGGGCTGC-3' using PfuUltra II Fusion HS DNA polymerase (Stratagene, La Jolla, CA). The product was cloned into the firefly luciferase vector pGL3-basic (Promega, Southampton, United Kingdom) using KpnI and BgIII sites. In a 96-well plate, 17 ng of the promoter firefly luciferase plasmids were transfected into the HEK293 β #1a³² or INS-1(HNF1B-1a)³³ cell lines containing tetracycline-inducible HNF1B. For standardization of the transfection efficiency, 0.08 ng of renilla luciferase plasmid pRL-Con⁵⁰ was used as a reference. Twenty-four hours after transfection using FuGene HD (Roche, Burgess Hill, United Kingdom), firefly and renilla luciferase activity was measured with the Dual-Luciferase Reporter Assay (Promega). As controls, we used the *Ksp-cadherin* promoter, which is known to be regulated by HNF1B and the same construct but with the HNF1 site mutated, as provided by Dr. Igarashi.⁴¹

PCR for HNF1A and B and FXYD2 in Mouse Distal Tubule

Immortalized mouse DCT cells⁵¹ (a gift from Dr. Peter Friedman, University of Pittsburgh, Pittsburgh, PA) were cultured at 37°C in a 5% CO₂ incubator in DMEM/F-12 (Invitrogen, Paisley, United Kingdom), 5% FCS (Invitrogen), and antibiotics (Sigma Chemical Co., Poole, United Kingdom). RNA was extracted from confluent dishes using Tri-Reagent (Sigma), and 1 μg was used to prepare cDNA (kit from Bio-Rad Laboratories; Bio-Rad Hemel-Hempstead, United Kingdom). PCR was performed using the general methods previously described⁵² using the following primer sets either designed by Primer 3 software or obtained from published articles⁵³ for Hnf1a, Hnf1b, Fxyd2a, Fxyd2b, and hypoxanthine-guanine phosphoribosyltransferase (Hprt; a housekeeping gene): Fxyd2a sense 5'-GATCTGTCAGCGAACAGTG-3', Fxyd2b sense 5'-CTACCATGGACAGGTG-GTA-3', Fxyd2a and Fxyd2b antisense 5'-GGAGTGGGGTGT-AGAGAA-3' (amplicon sizes 396 and 391 bp for Fxyd2a and Fxyd2b, respectively); Hnf1a sense 5'-ACTTGCAGCAGCACAAACATC-3', Hnf1a antisense 5'-GAATTGCTGAGCCACCTCTC-3' (amplicon size 167 bp) Hnf1b sense 5'-TGGTACGTCAGAAAGCAACG-3', Hnf1b antisense 5'-GGTCGTAGGCCTGGTACAAA-3' (amplicon size 344 bp); Hprt sense 5'-AAGCTTGCTGGTAAAAAGGA-3', Hprt antisense 5'-GCAAATCAAAAAGTCTGGGGA-3' (amplicon size 354 bp). RNA made from adult mouse kidneys was used as a positive control, and negative controls without cDNA were included in each reaction.

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DISCLOSURES

None.

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