

A transcriptional network in polycystic kidney disease

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Mutations in cystic kidney disease genes represent a major genetic cause of end-stage renal disease. However, the molecular cascades controlling the expression of these genes are still poorly understood. Hepatocyte Nuclear Factor 1 β (HNF1 β) is a homeoprotein predominantly expressed in renal, pancreatic and hepatic epithelia. We report here that mice with renal-specific inactivation of HNF1 β develop polycystic kidney disease. We show that renal cyst formation is accompanied by a drastic defect in the transcriptional activation of *Umod*, *Pkhd1* and *Pkd2* genes, whose mutations are responsible for distinct cystic kidney syndromes. *In vivo* chromatin immunoprecipitation experiments demonstrated that HNF1 β binds to several DNA elements in murine *Umod*, *Pkhd1*, *Pkd2* and *Tg737/Polaris* genomic sequences. Our results uncover a direct transcriptional hierarchy between HNF1 β and cystic disease genes. Interestingly, most of the identified HNF1 β target gene products colocalize to the primary cilium, a crucial organelle that plays an important role in controlling the proliferation of tubular cells. This may explain the increased proliferation of cystic cells in MODY5 patients carrying autosomal dominant mutations in HNF1 β .

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Introduction

The development of the kidney depends on reciprocal interactions between the ureteric bud and the metanephric mesenchyme. Factors secreted by the ureteric bud induce the aggregation of the mesenchyme and subsequent conversion

into the epithelium. The induced mesenchyme in turn sends signals to the ureteric bud to promote its branching. This reciprocal induction proceeds in an ordered manner to give rise to the collecting duct system and simultaneously to the nephrons (see Vainio and Lin, 2002).

Failure of the differentiation program during nephrogenesis can lead to diverse pathological conditions that can perturb renal structure and function. Mutations in cystic kidney disease genes constitute the most common genetic cause of chronic renal failure in both children and adults (see Igarashi and Somlo, 2002). Despite their genetic, clinical and histopathological heterogeneity, these renal diseases result in a similar outcome: dilation of tubules and cystogenesis. These observations have led to the hypothesis that the gene defects underlying these disorders might disrupt a common pathway. However, the mechanisms of cyst formation are still not completely elucidated. They are postulated to involve dysfunctions in the control of cell proliferation, apoptosis, cell polarity, the localization of ion channels, fluid secretion and cell–cell adhesion or cell–matrix interactions (see Igarashi and Somlo, 2002). During the past decade, positional cloning strategies have identified several genes responsible for cystic kidney diseases. Genes mutated in autosomal dominant polycystic kidney disease (ADPKD) (*PKD1* and *PKD2*) (Hughes *et al.*, 1995; Mochizuki *et al.*, 1996), autosomal recessive polycystic kidney disease (ARPKD) (*PKHD1*) (Ward *et al.*, 2002), nephronophthisis (*NPHP1 to 4*) (Hildebrandt *et al.*, 1997; Otto *et al.*, 2002; Olbrich *et al.*, 2003; Otto *et al.*, 2003) and medullary cystic kidney disease (MCKD) type 2 (*UMOD/THP*) (Hart *et al.*, 2002) have been identified. Recently, ADPKD, ARPKD and nephronophthisis gene products have been localized in the primary cilium, a solitary non-motile organelle that extends from the apical surface of renal epithelial cells (see Watnick and Germino, 2003). *Pkd1*- and *Pkd2*-encoded proteins may be part of a mechanosensitive Ca²⁺-signaling pathway in renal cilia that inhibits renal tubular cell growth (Nauli *et al.*, 2003). If ciliary structure or function is perturbed, tubular cell growth may continue, leading to cyst formation. The role of the proteins involved in ARPKD and nephronophthisis in the cilium is still not clear. Polaris, a protein involved in a mouse polycystic kidney disease model, is also localized in the cilium, and plays a role in the assembly of this organelle (Pazour *et al.*, 2000). Mutations in the human *UMOD* gene are responsible for MCKD type 2, but the mechanisms underlying the cyst formation in this disease also remain elusive.

Relatively little is known about the transcriptional networks that control epithelial differentiation of renal tubules. Hepatocyte Nuclear Factor 1 β (HNF1 β) is a homeodomain-containing transcription factor that binds DNA and transactivates transcription as homodimer, or heterodimers with the closely related factor, HNF1 α (see Cereghini, 1996). Both proteins were initially described as liver-enriched transcription factors, but their expression pattern is not restricted to hepatocytes (Blumenfeld *et al.*, 1991; see

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Cereghini, 1996). In particular, these proteins are expressed in the tubular epithelial cells of the kidney (Lazzaro *et al*, 1992; Pontoglio *et al*, 1996). HNF1 β is expressed throughout the entire nephron, from proximal tubules to collecting ducts, whereas HNF1 α expression is restricted to proximal tubules. Interestingly, HNF1 β is also highly expressed in other tubular epithelia such as biliary and pancreatic ducts (Coffinier *et al*, 1999a).

Autosomal dominant mutations in the *HNF1 β* gene are associated with a particular form of diabetes mellitus called Maturity Onset Diabetes of the Young type 5 (MODY5) (Horikawa *et al*, 1997). Affected patients also present diverse forms of non-diabetic nephropathies and, with less penetrant occurrence, genital malformations and liver dysfunction (Nishigori *et al*, 1998; Lindner *et al*, 1999; Bingham *et al*, 2000). The most common and penetrant trait of these patients is the presence of renal cysts and diabetes (RCAD). The renal manifestations of the disease also include cystic renal dysplasia, probably due to a defective nephrogenesis, oligomegane-phronia and hypoplastic Glomerulocystic kidney disease (GCKD) (Bingham *et al*, 2001). In zebrafish, mutations of *HNF1 β* affect pronephros development leading to cystic pronephric tubules (Sun and Hopkins, 2001). The expression of HNF1 β mutants in *Xenopus laevis* affects the normal development of pronephric structures (Bohn *et al*, 2003). Furthermore, *HNF1 β* plays an essential role during mouse early embryogenesis. *HNF1 β* -null mice die around embryonic day 7 (E7) due to a defect in visceral endoderm differentiation (Barbacci *et al*, 1999; Coffinier *et al*, 1999b). To assess the roles of HNF1 β during late embryogenesis, we made use of a Cre-LoxP strategy. We demonstrate here that kidney-specific inactivation of *HNF1 β* leads to a polycystic phenotype. We found that the expression of several genes involved in cystic diseases is severely affected in the conditionally inactivated mice. By chromatin immunoprecipitation, we show that three of them (*Umod*, *Pkhd1* and *Pkd2*) are directly controlled by HNF1 β . These results describe a direct transcriptional hierarchy between HNF1 β and these genes, and establish the role of this transcription factor in regulating the terminal differentiation of renal tubular cells.

Results

Kidney-specific inactivation of the HNF1 β gene

The early lethality of HNF1 β -deficient embryos prevented the analysis of its function at later developmental stages. To circumvent this problem, we inactivated the *HNF1 β* gene using the Cre-LoxP strategy. To restrict the inactivation to renal cells, we made use of a KspCre transgenic mouse strain that expresses the Cre recombinase under the control of the Ksp-cadherin (*Cadherin 16*) promoter. This transgene drives efficient Cre expression and Cre-mediated recombination in collecting ducts, loops of Henle and only occasionally (less than 5%) in proximal tubular cells (Shao *et al*, 2002).

Inactivation of the *HNF1 β* gene was achieved by generating mice carrying a homozygous floxed *HNF1 β* gene (Coffinier *et al*, 2002) along with the KspCre transgene (*KspCre*; *HNF1 β ^{lox/lox}*). In a parallel way, we also generated compound heterozygous animals carrying a null *HNF1 β* *lacZ* knock-in (Coffinier *et al*, 1999b) and a floxed allele along with the KspCre transgene (*KspCre*; *HNF1 β ^{lox/LacZ}*). These two genotypes involved a renal-specific inactivation of

HNF1 β and gave rise to the same phenotype. For these reasons, we described these animals as 'mutants'. Conversely, littermates carrying either (*KspCre*; *HNF1 β ^{lox/+}*) or (*HNF1 β ^{lox/lox}*) or (*HNF1 β ^{lox/+}*) or (*HNF1 β ^{lox/LacZ}*) were phenotypically indistinguishable from the wild-type mice. For this reason, we termed these animals collectively as 'controls'.

Kidney-specific inactivation of HNF1 β leads to postnatal lethality and renal failure

Pups carrying all possible genotypes were born in normal Mendelian ratios ($N = 159$, $P > 0.05$). No lethality was observed up to postnatal day 8 (P8). Between days P10 and P21, 75% of mutants died ($N = 8$). Generally, mutant animals were growth retarded by P8. At this stage, the average weight of mutant pups was 11% lower than that of controls ($N_{\text{control}} = 75$, $N_{\text{mutant}} = 22$, $P < 0.05$). Growth retardation increased with age, and mutants that survived weaning exhibited an approximately 25% decrease in body weight.

To investigate renal function, we determined seric urea and creatinine concentrations. Measurements were performed at different ages (P8 and on the few surviving animals at P17 and P30) (Figure 1). Both urea and creatinine levels were increased in mutant mice at all time points. These data indicated that mutants suffered, and died from, a severe impairment of renal function. Most measurements required euthanasia of the pups, therefore these data do not represent an average time-course progression of renal disease.

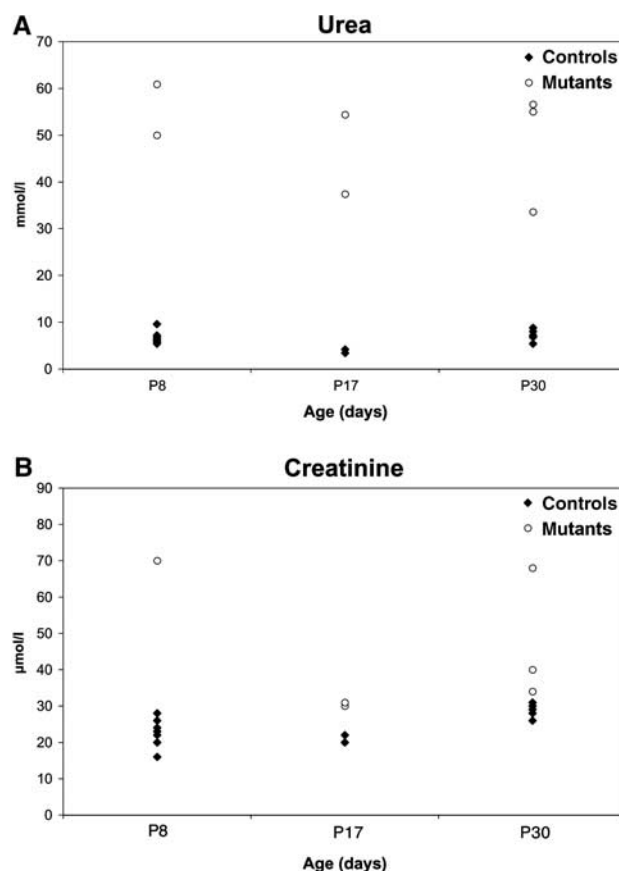


Figure 1 Impaired renal function in mutant mice. Serum concentrations of urea (A) and creatinine (B) at P8, P17 and P30.

Inactivation of HNF1 β results in a renal polycystic phenotype

We observed bilateral ureteral dilation, also involving the renal pelvis, in most mutants at P8 (92%, $N = 25$) (Figure 2). No signs of ureter obstruction were seen. As HNF1 β is expressed in the ureteral epithelium, and as KspCre had driven recombination in the precursors of these cells (data not shown), we concluded that this abnormality was a direct consequence of HNF1 β inactivation in ureter-epithelial cells.

The kidneys of mutants were much paler than the controls but were apparently normally sized at P8 (Figure 2). Gross abnormalities of mature nephrons suggested that terminal morphogenesis did not occur normally at P1. In particular, several tubules located in the medullary region of mutant kidneys were dilated and contained more cells than in controls (Figure 3A and B). Histological sections at P8 revealed that the kidneys of all mutant mice exhibited numerous cysts (Figure 3C and D). These cysts were mostly located in the medulla, but a few could also be observed in the cortex. The average size of cysts increased with age (Figure 3D and F). It is noteworthy that dilation of the urinary space in some glomeruli could be clearly observed at all stages (Figure 3F).

Renal cysts are lined with Cre recombined HNF1 β ^{-/-} cells

The KspCre transgene drives efficient recombination in the renal medulla. Histological abnormalities in HNF1 β -inactivated mice mainly affected this region, suggesting that the renal cysts were a direct consequence of HNF1 β inactivation. To confirm this hypothesis, we monitored the recombination status of cystic cells using the ROSA26R reporter allele (Soriano, 1999). We generated mice with kidney-specific inactivated HNF1 β combined with the ROSA26R reporter allele (KspCre; HNF1 β ^{lox/lox}; ROSA26R). This allele contains a lacZ gene whose activity is induced by the Cre-driven recombination (Soriano, 1999). These mice had the same ureteral and cystic phenotype as KspCre; HNF1 β ^{lox/lacZ} animals (Figure 4B, 5F and data not shown). X-gal stained kidney sections revealed that the renal cysts were lined with β -galactosidase-positive cells (Figure 4B). As expected, control mice (KspCre; HNF1 β ^{lox/+}; ROSA26R) showed

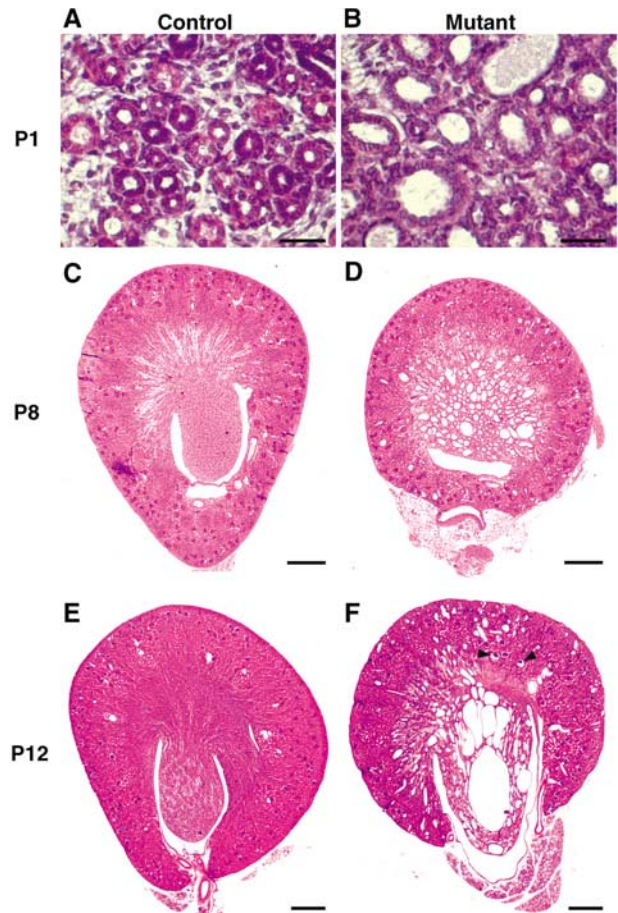


Figure 3 Inactivation of HNF1 β in kidney cells leads to polycystic kidney disease. Hematoxylin-eosin-stained kidney sections from control and mutant mice at P1 (A, B), P8 (C, D) and P12 (E, F). Tubular dilations were visible in the medullary region of mutants at P1, with an increased number of nuclei per tubule section compared to controls (B versus A). In mutants, the medulla was completely disrupted by large cysts by P8 (D). The size of cysts increased with age (F). A few glomerular cysts were observed in the deep cortex (F, arrowheads). Scale bars: A, B: 100 μ m, C–F: 400 μ m.

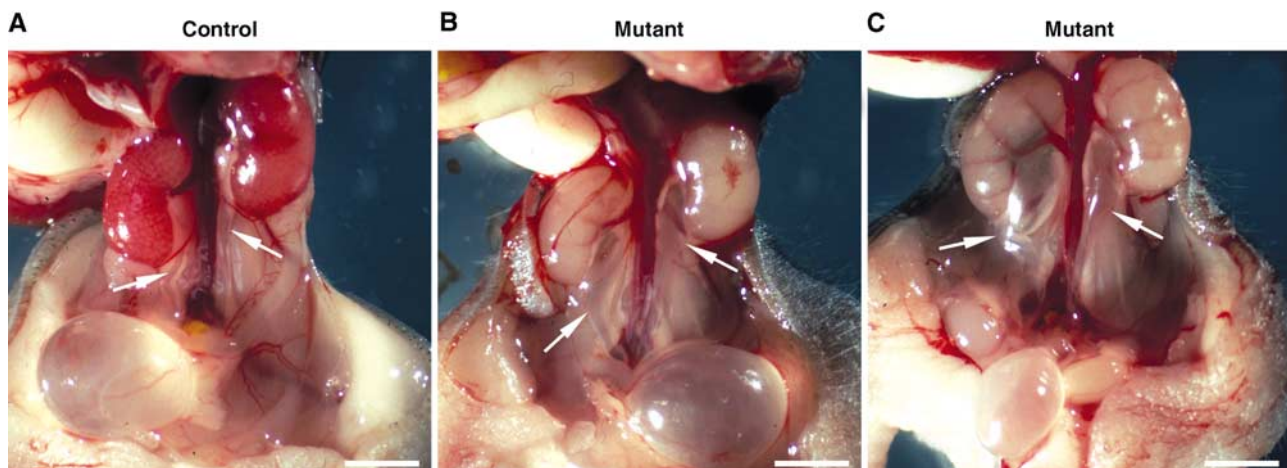


Figure 2 HNF1 β inactivation results in bilateral ureteral dilation. Macroscopic view of urinary tract in control (A) and mutant (B, C) mice at P8. Some mutants exhibit a mild ureter dilation (arrows) (compare B versus A), while others present a more severe phenotype (compare C versus A). Mutant kidneys had a normal size, but were paler than controls. Scale bars: 0.25 cm.

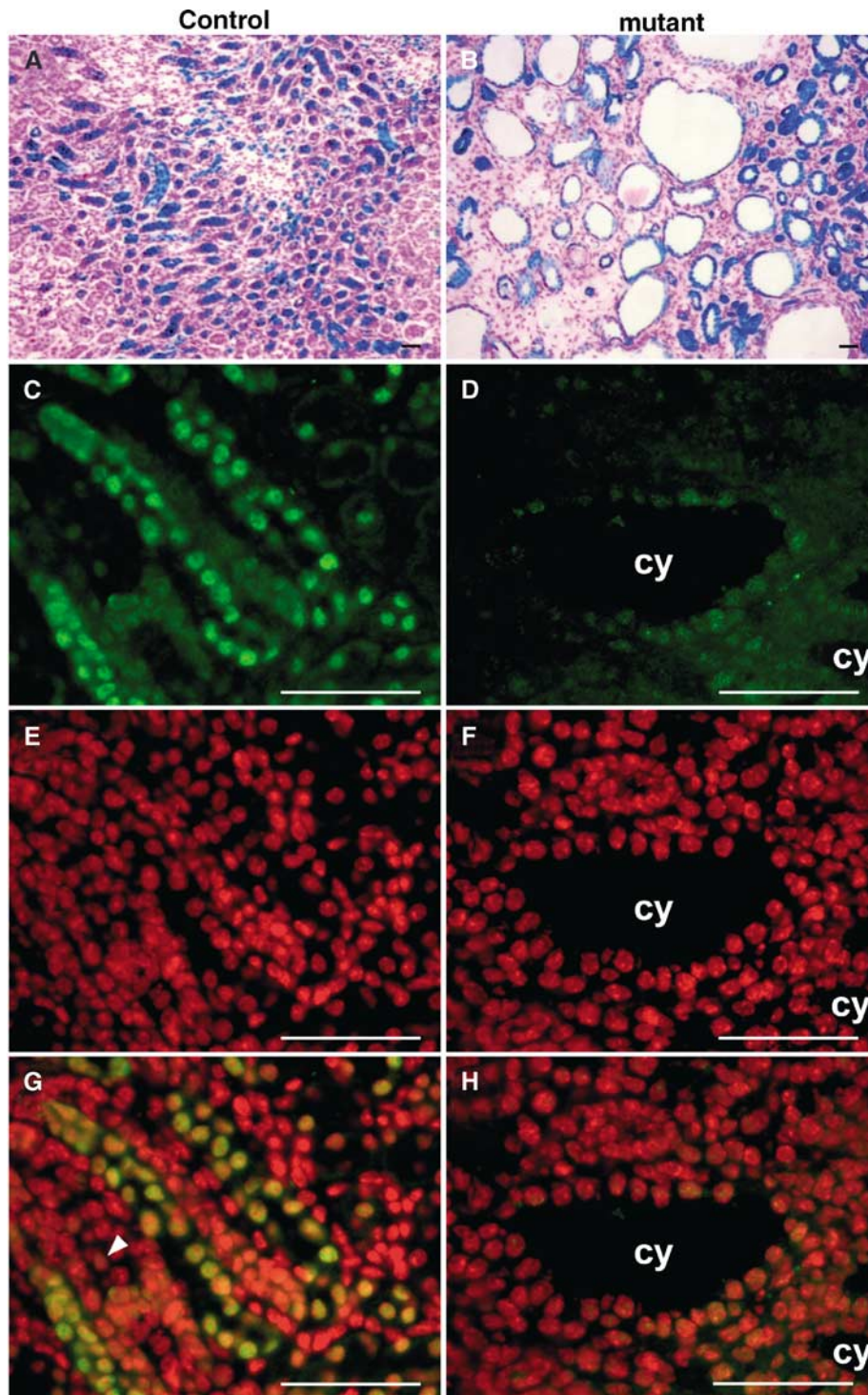


Figure 4 Cystic cells underwent recombination and lack HNF1 β expression. (A, B) X-gal staining on kidney sections of control *KspCre; HNF1 $\beta^{lox/+}; ROSA26R$* (A) and mutant *KspCre; HNF1 $\beta^{lox/lox}; ROSA26R$* (B) at P14. β -gal activity is an indicator of Cre-driven recombination on the *ROSA26R* locus. (A) The medulla of control mice showed recombination in the tubular epithelium. (B) In mutants, all cysts were lined with recombined cells. (C–H) Kidney sections of control *HNF1 $\beta^{lox/lacZ}$* and mutant *KspCre; HNF1 $\beta^{lox/lacZ}$* at P8. (C, D) HNF1 β staining (fluorescein/green). (E, F) Nuclear staining of the same section (DAPI/red). (G, H) Merging. (G) In controls, HNF1 β is expressed in tubular but not in mesenchymal cells (arrowhead). (H) Mutants showed no expression of HNF1 β in cysts. cy: cyst. Scale bars: 75 μ m.

β -gal-positive cells lining most medullary segments of the kidney tubules without any cyst formation (Figure 4A). These data indicate that all cystic cells underwent Cre-mediated recombination or were derived from recombined progenitor cells.

To further prove that Cre activity had inactivated the endogenous *HNF1 β* gene, we performed immunofluorescent detection using a novel polyclonal antibody (HNF1 β -NC-AR) (Figure 4C–H). This analysis revealed that the HNF1 β protein was indeed absent from cystic epithelium (Figure 4D and H),

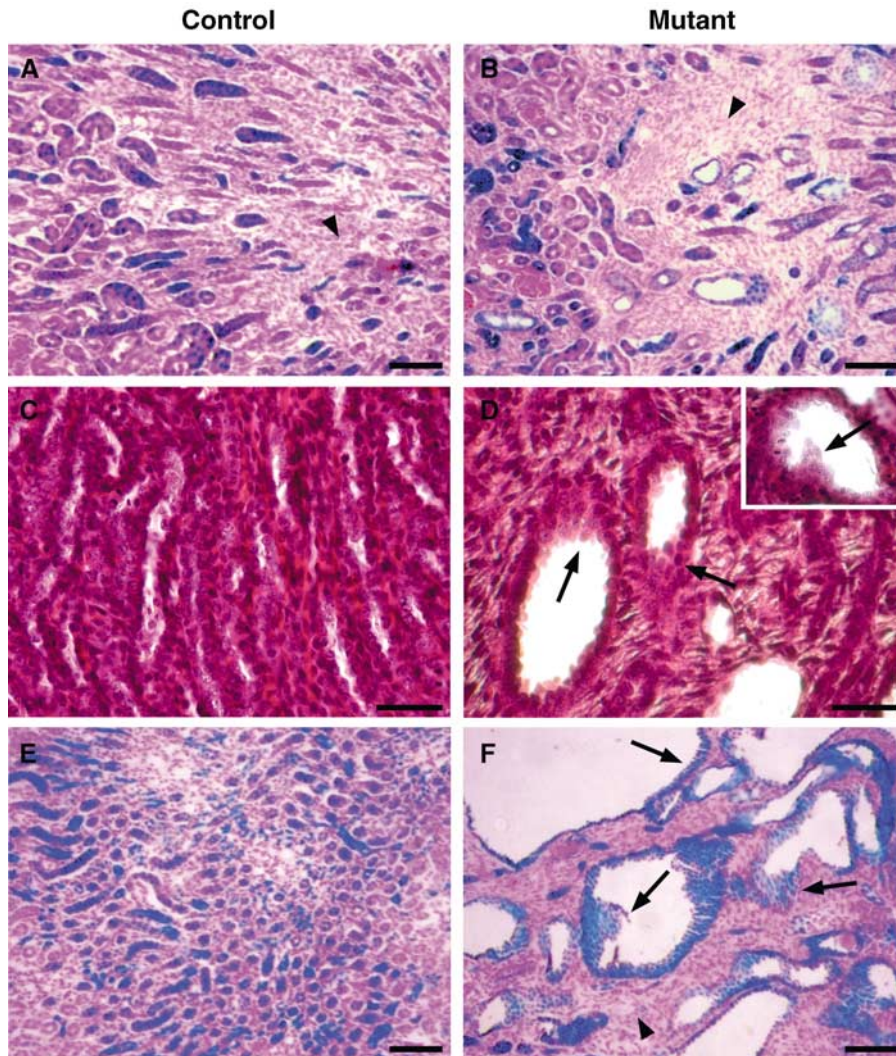


Figure 5 *HNF1* β inactivation leads to multilayered tubular epithelia. (A, B) X-gal staining on kidney sections of control *HNF1* $\beta^{flox/lacZ}$ (A) and mutant *KspCre; HNF1* $\beta^{flox/lacZ}$ (B) at P8. β -gal activity is an indicator of the endogenous *HNF1* β promoter activity. (A) *HNF1* β was expressed in all tubular cells but not in mesenchyme (arrowhead). (B) Mesenchyme is β -gal negative (arrowhead), indicating that it was not programmed to express *HNF1* β . (C, D) Hematoxylin–eosin-stained sections of control (C) and mutant (D) at P8. Multilayered tubular epithelial cells were observed (arrows), as well as polyps growing into the cyst lumen (D and insert). (E, F) X-gal staining on kidney sections of control *KspCre; HNF1* $\beta^{flox/+}; ROSA26R$ (E) and mutant *KspCre; HNF1* $\beta^{flox/flox}; ROSA26R$ (F) at P14. β -gal activity is an indicator of Cre-driven recombination. (E) *KspCre*-driven recombination was seen in a large proportion of medullary tubular cells. (F) Several cysts lack the typical monolayered epithelial structure (arrows). All epithelial cells are β -gal-positive, demonstrating that they underwent Cre recombination. The mesenchyme in mutants was not affected by recombination (arrowhead). Scale bars: 200 μ m.

whereas its expression was conserved in proximal tubules (data not shown). Thus, renal cysts were lined with recombinant HNF1 β -null cells.

To establish the cellular origin of cysts, we analyzed specific markers of collecting ducts and Henle's loops. Most cysts expressed Aquaporin2 (Aqp2) protein, a collecting duct cell marker (Figure 7G). Some cysts expressed the sodium–potassium–chloride cotransporter isoform 2 (Slc12a1), a specific marker of thick ascending limb of Henle's loop (Figure 7C and K) (see Delpire *et al*, 1996). Taken together, these data indicate that the cysts were mainly derived from two nephron segments where *KspCre* is expressed.

Analysis of kidneys from P8 animals also showed that mesenchymal cells were present in both control and mutant mice, but they were more abundant in mutants (Figure 5A and B). From P12 onwards, the presence of mesenchymal cells was visible in mutants, but not in controls at the same age

(Figure 3E and F). We investigated whether these mesenchymal cells expressed *HNF1* β . We took advantage of the *HNF1* β^{lacZ} allele in heterozygous control mice (*HNF1* $\beta^{flox/lacZ}$). Mesenchymal cells were β -gal-negative, indicating that they do not express *HNF1* β (Figure 5A). In addition, immunofluorescence experiments showed that these cells do not express the HNF1 β protein (Figure 4G). Similarly, mesenchymal cells present in mutants (*KspCre; HNF1* $\beta^{flox/lacZ}$) were also β -gal-negative, indicating that these cells were not programmed to express the endogenous *HNF1* β gene (Figure 5B). In a parallel manner, we investigated whether these mesenchymal cells had undergone *KspCre*-driven recombination using the *KspCre; HNF1* $\beta^{flox/flox}; ROSA26R$ mice. X-gal staining of kidney sections from these animals showed that all mesenchymal cells were β -gal-negative, whereas cells lining cysts were β -gal-positive (Figure 5F). Thus, these mesenchymal cells did not express *KspCre* and were not

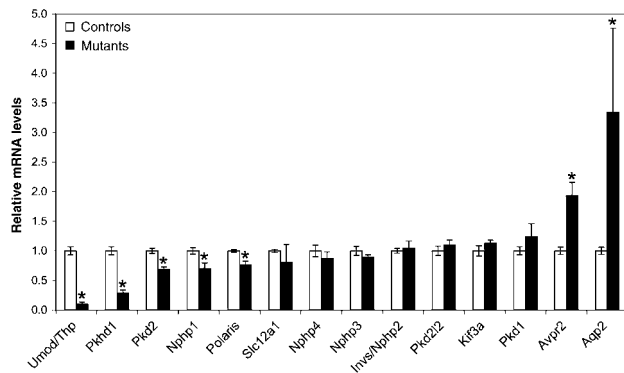


Figure 6 Defective transcriptional activation of cystic disease genes in mutant mice. Quantitative RT-PCR analysis of cystic disease genes and cell-specific markers. *Umod/Thp*, *Pkhd1*, *Pkd2*, *Nphp1* and *Tg737/Polaris* were downregulated in mutant kidneys. Expression levels in mutants are indicated relative to controls. Results were normalized with β -2 microglobulin expression level (except for *Umod*, which was normalized to *Slc12a1* expression). Significant differences between mutants and controls ($P < 0.05$) are indicated (*). Error bars represent standard error of the mean ($N_{\text{control}} = 5$, $N_{\text{mutant}} = 3$).

derived from cells where the KspCre had been expressed. Both experiments demonstrate that the increase in mesenchymal cells in mutant kidneys is a secondary consequence of *HNF1 β* inactivation.

Increased proliferation in mutant renal cysts is cell-autonomous

Increased proliferation is one of the major mechanisms underlying cyst formation in cystic kidney diseases. Notwithstanding this propensity to proliferate, renal cystic cells are usually organized in a monolayered epithelium (see Igarashi and Somlo, 2002). This is actually the case for most of the cysts that we found in mutants at P8. However, about 15% of cysts showed multilayered epithelia at this stage (Figure 5D). Similar to what has been observed in human polycystic kidney disease (see Bernstein *et al*, 1987), some polyps developed into the cyst lumen (insert in Figure 5D). Using the *ROSA26R* reporter allele, we have investigated the lineage of these proliferating cells. Interestingly, in mutant *KspCre; HNF1 β ^{lox/lox}; ROSA26R* animals, all cells in multilayered structures were positively stained with X-gal, indicating that all of them had recombined (Figure 5F). Therefore, the presence of multilayered cysts is probably due to a cell-autonomous defect. This observation indicates that *HNF1 β* inactivation in tubular epithelial cells results in increased proliferation.

Mutant mice show specific transcriptional defects of several cystic disease genes

To unravel the molecular mechanisms underlying cyst formation in our mouse model, we monitored the expression levels of genes known to be involved in cystogenesis in mice and humans. We performed quantitative RT-PCR experiments using kidney RNA from mutant and control animals. We found that the expression level of *Umod* (Uromodulin/Tamm-Horsfall glycoprotein) was decreased 10-fold in mutant kidney. This result could be due to a direct defective transcriptional activation of the *Umod* gene by *HNF1 β* . Alternatively, this deficiency could be due to the absence of

cells that normally express this gene (Bachmann *et al*, 1990) because of a developmental or differentiation defect. The grossly normal expression of a marker of the thick ascending limb of Henle’s loops (TAL), the sodium-potassium-chloride cotransporter isoform 2 (*Slc12a1/Nkcc2*) (Figure 6), argued against this possibility. In addition, when the expression of *Umod* was normalized for the expression of *Slc12a1*, downregulation of *Umod* expression was still calculated to be 8.5-fold (Figure 6). This normalization allowed us to demonstrate that the *Umod* transcriptional defect is not linked to the absence of TAL cells in mutant mice.

Interestingly, two other key genes of cyst formation, *Pkhd1* and *Pkd2*, were also downregulated (3.4- and 1.5-fold, respectively). In contrast, *Pkd1* and *Pkd2l2* (a *Pkd2*-related gene) (Guo *et al*, 2000) expression levels were normal. Among the genes involved in nephronophthisis, only *Nphp1* was significantly downregulated in mutant mice (1.4-fold), whereas *Invs/Nphp2*, *Nphp3* and *Nphp4* were normally expressed (Figure 6).

We also investigated the expression of two genes involved in cystogenesis in mice, *Tg737/Polaris* (Moyer *et al*, 1994) and *Kif3a* (Lin *et al*, 2003). The former was mildly, but significantly, downregulated (1.3-fold) in mutant mice, whereas *Kif3a* was normally expressed (Figure 6).

We analyzed the expression of *Aqp2* and *arginine-vasopressin-receptor type 2* (*Avpr2*). These two genes are expressed in collecting ducts, a structure that contributes to cyst formation. Similar to what has been described in different polycystic rodent models (Gattone *et al*, 2003), both of these genes were upregulated in mutant kidneys (Figure 6).

Our results show that renal *HNF1 β* inactivation results in a reduced expression of several genes known to play crucial roles in cystogenesis. In fact, at least five of these genes (*Umod*, *Pkhd1*, *Pkd2*, *Nphp1* and *Tg737/Polaris*) were specifically affected by *HNF1 β* inactivation.

Umod-, Pkhd1- and Pkd2-encoded proteins are dramatically downregulated in cystic epithelia

In order to assess the expression of *Umod*, *Pkhd1* and *Pkd2* proteins in *HNF1 β ^{-/-}* cystic cells, we carried out immunohistochemical stainings. Uromodulin is known to be exclusively expressed in the cortical and medullary TAL (Bachmann *et al*, 1990). Our results show that in mutant kidney, the expression of this protein is drastically reduced in the medulla (Figure 7A), a compartment where a large fraction of the positive cells is normally detected (compare Figure 7A versus B). A residual expression of uromodulin is detected in rare non-dilated segments of cortical TAL (arrow, Figure 7A). As previously mentioned, the lack of this marker could underlie a defective differentiation of TAL. However, our results have shown that another marker of TAL, the *Slc12a1* protein (see Delpire *et al*, 1996), is normally expressed in mutant kidneys (compare Figure 7C versus D). In addition, serial histological sections have shown that the same TAL-derived cysts, positively stained for *Slc12a1*, were negative for uromodulin (compare arrowheads in Figure 7A and C). In a similar way, polyductin (*Pkhd1*) expression was dramatically reduced in cystic epithelia (compare Figure 7E versus F). As this protein is known to be predominantly expressed in collecting ducts (Ward *et al*, 2003), we assessed the presence of this cell type in the cystic epithelium by the detection of *Aqp2*, a specific marker of collecting ducts. Our

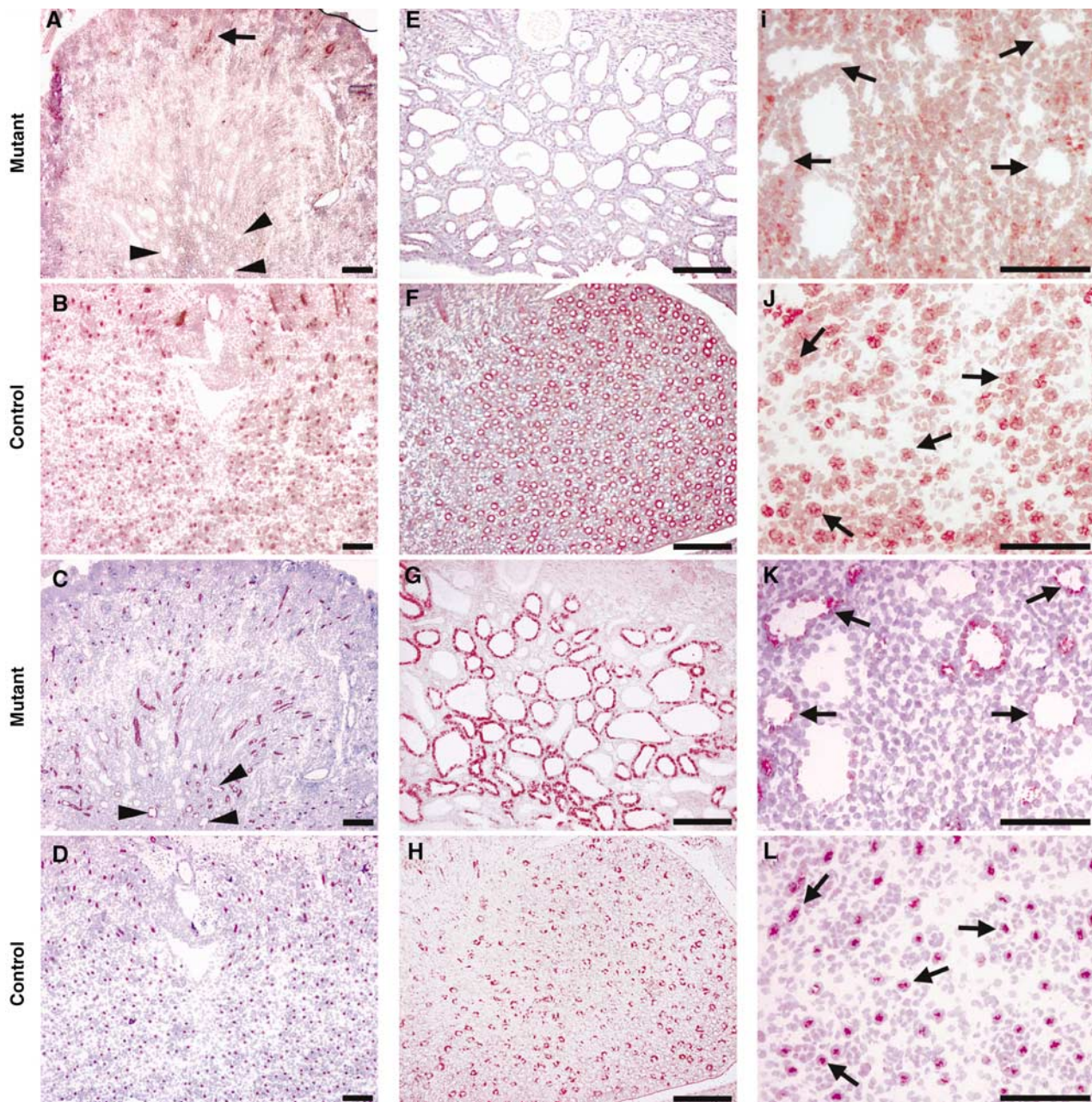


Figure 7 HNF1 β inactivation leads to a strong decrease of Umod, Pkhd1 and Pkd2 protein levels in cystic cells. Immunostainings for uromodulin (A, B), Pkhd1 (E, F), Pkd2 (I, J), Slc12a1 (C, D, K, L), and Aqp2 (G, H) in mutant and control mice at P8. (A–D) Uromodulin expression pattern. In mutants, no uromodulin staining was detected in the medullary cysts (compare A versus B). Some cysts were clearly shown to be thick ascending limb (TAL)-derived (see the arrowheaded positive staining with α -Slc12a1 antibody in C). Arrowheads in A indicate the position of the corresponding cysts in the adjacent section. In controls, both uromodulin and Slc12a1 proteins colocalized (compare adjacent sections B and D, respectively). In mutants, only few cortical nondilated TAL segments expressed uromodulin (see arrow in A). (E–H) Pkhd1 expression pattern. In mutants, Pkhd1 expression was strongly decreased in the vast majority of medullary cystic cells (compare E versus F). Pkhd1 downregulation is not due to the absence of collecting duct cells, as most cysts still expressed aquaporin2 (compare G versus E). (I–L) Pkd2 expression pattern. Pkd2 expression is absent in cystic epithelia (compare I versus J). Again, some cysts were clearly shown to be TAL-derived (see the arrowed positive staining with α -Slc12a1 antibody in K). Arrows in I indicate the position of the corresponding cysts, in the adjacent section. In control animals, both Pkd2 and Slc12a1 proteins colocalized (compare arrows in serial sections B and D, respectively). Scale bars: 300 μ m.

results showed that this cell type was indeed present, as a large proportion of cysts was positive for Aqp2 (compare Figure 7G versus H). Notwithstanding a high background signal detected in mesenchymal cells, we showed that the expression of polycystin2 (Pkd2) was absent in cystic cells (compare arrowed epithelia in Figure 7I versus 7J). Again,

since Pkd2 in the medulla is predominantly expressed in TAL (Foggensteiner *et al*, 2000), we monitored the expression of Slc12a1 in adjacent sections and showed that corresponding cysts expressed this marker but not Pkd2 (see arrows in Figure 7I versus 7K). Remarkably, Pkd2 was normally expressed throughout the whole cortex (Supplementary Figure 1). In

this region of the kidney, KspCre is not expressed and therefore HNF1 β is not deleted. As a result of this residual cortical expression, RT-PCR carried out on whole kidney RNAs might strongly underestimate the actual downregulation of Pkd2 expression. Taken together, these results clearly indicate that uromodulin, Pkhd1 and Pkd2 are drastically downregulated in HNF1 β -deficient cells.

An important fraction of Pkhd1 and Pkd2 proteins is localized to the primary cilium of tubular cells (Pazour *et al*, 2002; Ward *et al*, 2003). To assess the morphological integrity of this organelle, we performed immunostaining of acetylated tubulin, a specific component of the primary cilium. We found that mutant cells had normal cilia (Supplementary Figure 2). Moreover, morphometric analysis showed that the cilium length, specifically measured in collecting duct cells (costained with α -Aqp2 antibodies), was normal.

HNF1 β binds to cis-acting control elements in several genes involved in cystogenesis

To investigate whether the affected genes were directly controlled by HNF1 β we made use of the chromatin immuno-

precipitation (ChIP) technique (Orlando *et al*, 1997; Hecht *et al*, 1996). This technique can monitor direct *in vivo* interactions between proteins, such as transcription factors, and their chromatin DNA target sites. To this end, we first identified the position of putative HNF1 binding sites with an *in silico* approach based on a previously described position-weight matrix (Tronche *et al*, 1997, MP and SG, unpublished results). Subsequently, we tested whether these sites were actually bound *in vivo*. The ChIP assays were performed with novel antibodies raised against HNF1 β and α proteins (see Supplementary Method). Western blot analyses proved that both antibodies were highly specific and did not cross-react (data not shown). In the following, all given site positions will be based on the Ensembl Mouse release 15.30, which is built around the NCBI 30 composite assembly (see Supplementary Table IA).

Our results showed that the two sites detected close to the *Umod* gene (at -1.1 and -0.58 kb with respect to the putative transcriptional start site) were significantly bound by HNF1 β *in vivo* (Figure 8). Given the very large size of the *Pkhd1* gene (spanning more than 550 kb), we only considered 15 sites

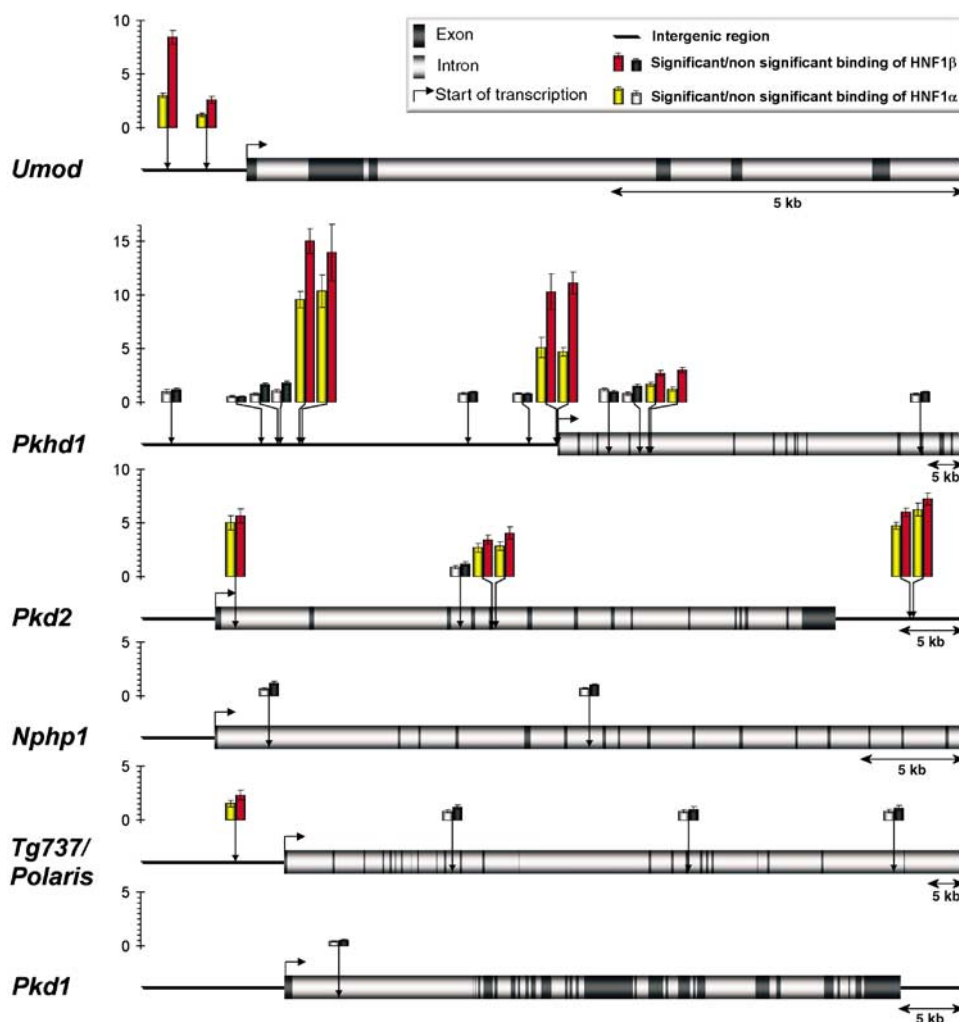


Figure 8 *In vivo* binding of HNF1 proteins to their chromatin target sites in cystic kidney disease genes. Predicted *in silico* HNF1 binding sites (vertical arrows) in *Umod*, *Pkhd1*, *Pkd2*, *Nphp1*, *Tg737/Polaris* and *Pkd1* genes were tested in ChIP experiments for *in vivo* HNF1 α and β binding. The relative enrichment for each DNA fragment upon immunoprecipitation of HNF1 α or β is illustrated as histograms. Colored bars represent HNF1 binding sites with enrichments significantly higher than background (gray bars). PCR experiments were performed in triplicate and the standard errors of these quantifications are shown as error bars.

identified in a window of 100 kb around the transcriptional start site. Six of them showed a significant interaction with HNF1 β *in vivo*. Interestingly, strongly enriched sites were located either in the proximal promoter (within ~200 bp) or much further upstream (-35 kb). All *in vivo*-enriched HNF1 sites in this gene occurred as doublets with a maximal interdistance of 400 bp. The *in silico* search identified four intronic HNF1 sites in the *Pkd2* gene and a doublet downstream of the 3' end of the gene (at around +51 kb). Among the four intronic sites, only one was not enriched *in vivo*, whereas the 3' doublet showed the highest enrichment (~six-fold). The *in silico* search revealed four potential HNF1 binding sites around the *Tg737/Polaris* gene. In this case only the closest site (-6.64 kb) showed a weak, but significant, enrichment for HNF1 β . *Nphp1* was the only candidate gene for which we could not observe any *in vivo* interaction, notwithstanding the *in silico* prediction of 2 sites. In order to assess the specificity of our results, we also monitored the potential binding of HNF1 β to *Pkd1*, another cystic disease gene, whose expression is not altered in our mice. We could predict a single HNF1 binding site positioned at +4 kb, but this site was not bound *in vivo* by HNF1 β or HNF1 α (Figure 8).

In vitro experiments have shown that HNF1 α and HNF1 β bind to their target sites with similar affinities, either as homo- or heterodimers (De Simone *et al*, 1991; Rey-Campos *et al*, 1991). Our results indicated that the enrichment obtained with the α -HNF1 α -specific antibody was systematically weaker than that of HNF1 β . This observation could be explained by the fact that HNF1 β has a more widespread expression pattern than HNF1 α in the kidney. However, the ratio of enrichment in the *Umod* gene was particularly striking. In this case, HNF1 α binding was much lower, compared to that of HNF1 β . Interestingly, the *Umod* gene is the only gene among the analyzed candidates that is not coexpressed with HNF1 α . This observation suggests that HNF1 α is unable to bind these sites efficiently in the proximal tubular cell, a cell type that does not express the *Umod* gene.

In summary, our ChIP experiments demonstrate that HNF1 β clearly interacts with several HNF1 binding sites in the *Umod*, *Pkhd1* and *Pkd2* genes. These cystic disease genes can be considered as direct transcriptional targets of HNF1 β . Moreover, interaction with *Tg737/Polaris* could also be shown, but to a weaker extent.

Discussion

Cystic kidney diseases represent a major cause of end-stage renal failure. Several genes have recently been identified to be involved in these disorders. However, little is known about the transcriptional networks that regulate the expression of these genes. HNF1 β plays a crucial role in the development of extraembryonic visceral endoderm and in the morphogenesis of hepatic bile ducts. Here we show that mice lacking HNF1 β specifically in the kidney suffer from severe polycystic kidney disease.

HNF1 β and cystic kidney diseases

Our results show that the lack of HNF1 β is directly responsible for cyst formation via a cell-autonomous mechanism. In humans, heterozygous mutations of *HNF1 β* are associated with the Renal Cysts and Diabetes (RCAD) syndrome. This syndrome combines a form of type II diabetes called maturity

onset diabetes of the young type 5 (MODY5) with a non-diabetic renal disease (Horikawa *et al*, 1997; Nishigori *et al*, 1998; Lindner *et al*, 1999; Bingham *et al*, 2000, 2001). The manifestations of renal dysfunction are variable, ranging from isolated cysts to multicystic kidneys. Little is known about the molecular mechanisms underlying this phenotype. Here we show that HNF1 β directly controls the transcription of several genes expressed in tubular epithelial cells, namely *Umod*, *Pkhd1* and *Pkd2*. In addition, HNF1 β might contribute to the transcriptional activation of *Tg737/Polaris*. All these genes are known to be mutated in distinct cystic kidney diseases: MCKD type 2 (*UMOD*) (Hart *et al*, 2002), ARPKD (*PKHD1*) (Ward *et al*, 2002), ADPKD (*PKD2*) (Mochizuki *et al*, 1996) and a murine model of ARPKD (*Tg737/Polaris*) (Moyer *et al*, 1994). Strikingly, although a defect in any of these genes in humans is sufficient to elicit renal cyst formation, we observed a concomitant downregulation of a specific subset of cystic disease genes in mutant mice. The combined defects in the expression of these genes could be the origin of the massive cyst formation observed in our mouse model. These results reveal a transcriptional hierarchy between HNF1 β and different cystic disease genes, and could explain why carriers of autosomal dominant mutations in *HNF1 β* develop renal cysts.

Interestingly, MODY5 patients do not develop a strict polycystic disease. They actually present a smaller number of cysts in comparison to ADPKD or ARPKD patients. This difference could be explained by the fact that the human disease occurs in heterozygous carriers, whereas our model involves homozygous *HNF1 β* deletion. In order to understand dominant inheritance in humans, it would be interesting to test whether renal cysts in MODY5 patients develop upon loss of the residual wild-type *HNF1 β* allele. This 'two-hit' mechanism has already been described for genes involved in ADPKD (*Pkd1* and *Pkd2*) (Watnick *et al*, 1998) and for tumor suppressor genes.

Polyductin (*Pkhd1*) and polycystin2 (*Pkd2*) proteins are preferentially localized in a specialized cellular organelle: the primary cilium (Pazour *et al*, 2002; Ward *et al*, 2003). Several studies suggest a link between a defect in cilium assembly, or function, and renal cyst formation (see Watnick and Germino, 2003). We show that HNF1 β is not necessary for cilium formation. However, our data demonstrate that the genetic program driven by HNF1 β involves the expression of genes that play a crucial role in cilium function.

HNF1 β inactivation leads to increased proliferation of epithelial cells

The presence of renal cysts and the appearance of multi-layered epithelia in mutant mice strongly suggest that the inactivation of *HNF1 β* leads to an increased rate of cellular proliferation. Mice with liver-specific *HNF1 β* inactivation have dysplastic epithelia in both the gallbladder and in the few remaining intrahepatic bile ducts. Multilayered epithelial structures, reminiscent of those observed upon kidney-specific inactivation, are present in the biliary tract of these animals (Coffinier *et al*, 2002). Recently, HNF1 α was shown to be involved in the control of cell proliferation. Indeed, biallelic inactivation of *HNF1 α* is frequently observed in liver adenomas and in some hepatocellular carcinomas (Bluteau *et al*, 2002). For this reason, *HNF1 α* can be considered as a tumor suppressor gene. We postulate that *HNF1 β* could have

the same properties, and that the increased proliferation of HNF1 β ^{-/-} cells upon kidney- and liver-specific inactivation of HNF1 β is the result of defective regulation of cell cycle in the absence of HNF1 β . To validate this hypothesis, the incidence of biallelic inactivation of HNF1 β in renal cancers and cholangiocarcinomas should be assessed. The antiproliferative effects of HNF1 β could be mediated by the biological function of the Pkd1/Pkd2 complex, which has been recently shown to control *p21^{Cip1}* transcription via the JAK/STAT pathway (Bhunja *et al*, 2002). In addition, Pkd2 has been shown to inhibit the Pkd1-dependent G protein activation (Delmas *et al*, 2002). However, we cannot rule out that defective expression of additional HNF1 β target genes could result in a nonfully differentiated state that, in turn, could render growth arrest less efficient.

HNF1 β chromatin targets

We show that the defective expression of the cystic disease genes *Umod*, *Pkhd1* and *Pkd2* directly correlates with the presence of several HNF1 binding sites that are bound by HNF1 β *in vivo*. This strongly suggests that HNF1 β directly controls the expression of these genes. Interestingly, the weak interaction of HNF1 β with its target site in *Tg737/Polaris* gene is reflected by its marginally decreased transcription in the mutant kidney.

The binding of HNF1 β was not only restricted to proximal promoter sites but also involved far upstream and downstream sites. In the case of *Pkhd1*, HNF1 binding sites were identified at 35 kb upstream of the transcriptional start site. These sites occur as doublet and are conserved in both human and mouse genomes (data not shown). Interestingly, this doublet is particularly enriched, suggesting a strong interaction with HNF1 β . Similarly, doublets of HNF1 binding sites in the *Umod* and *Pkd2* genes were also strongly bound, whereas isolated binding sites were weakly bound or not bound at all. These data could suggest that HNF1 β occupancy is enhanced by the presence of closely spaced sites on chromatin. Alternatively, a higher relative enrichment could also be explained by the increased probability for a DNA fragment encompassing two HNF1 sites to be immunoprecipitated.

The liver-specific inactivation of HNF1 β leads to defective morphogenesis of intrahepatic bile ducts (IHBD) (ductal plate malformation) (Coffinier *et al*, 2002). Interestingly, polycystin2/Pkd2 and polyductin/Pkhd1 proteins are coexpressed with HNF1 β in biliary epithelial cells (Ong *et al*, 1999; Ward *et al*, 2003). HNF1 β could drive the expression of these genes in both renal tubules and bile ducts. Notably, patients with ARPKD/PKHD1 suffer from ductal plate malformations giving rise to biliary dysgenesis, whereas patients with ADPKD/PKD2 develop biliary cysts (see Igarashi and Somlo, 2002). Although these patients do not strictly phenocopy the hepatic HNF1 β deficiency, concomitant transcriptional defects affecting both *Pkhd1* and *Pkd2* genes could explain why IHBD fail to differentiate in HNF1 β ^{-/-} livers.

Our study provides novel insights into the molecular mechanisms underlying HNF1 β function in normal kidney and in cystic disorders. Our results demonstrate that HNF1 β is a critical regulator of a genetic cascade that is essential for controlling the proliferation and differentiation of renal tubular epithelial cells.

Materials and methods

Animals

Kidney-specific inactivation of HNF1 β was obtained using a Cre-LoxP strategy. KspCre (Shao *et al*), HNFs β ^{lacZ/+} (Coffinier *et al*, 1999b) HNFs β ^{lox/lox} (Coffinier *et al*, 2002) and ROSA26R (Soriano) mice were previously described. All animals received humane care, and the institutional review committee approved the study protocol.

Biochemical analysis

Serum urea and creatinine levels were determined by the Centre d'Explorations Fonctionnelles Intégré (Faculté de Médecine X. Bichat).

Production of HNF1 α and β antibodies

We constructed GST fusion polypeptides of HNF1 α and β by excluding regions of high homology between both proteins using gene splicing by overlap extension PCR (SOE PCR) (Horton, 1995). All SOE PCRs were carried out with corresponding rat cDNAs. See Supplementary method 1 for more details.

Immunohistochemical procedures

Kidney cryosections were fixed in 4% PFA and treated as previously described (Foggensteiner *et al*, 2000) with the following antibodies: rabbit α -HNF1 β -NC-AR, 1:150; rabbit α -NKCC2 (a gift from M Knepper), 1:1000; rabbit α -Pkd2 (a gift from R Sandford), 1:200; α -THP (Biogenesis), 1:50. Aqp2 (rabbit α -Aqp2 antibody (a gift from M Knepper), 1:400) and pkhd1 (mouse α -Pkhd1 antibody (a gift from C Ward), 1:200) were detected on paraffin sections according to Ward *et al*, (2003). X-gal stainings were performed as described (Pontoglio *et al*, 1996). For histological analysis, paraffin sections were stained with hematoxylin and eosin.

Quantitative RT-PCR

cDNAs were generated from total kidney RNAs using the Invitrogen Superscript II reagents. Quantitative RT-PCR was carried out on an ABI PRISM[®] 7000 sequence detection system using SYBR[®] green. Primers were designed using PrimerExpress 2.0[®] software (Applied Biosystems). β -2 *microglobulin* was used as the control gene for normalization. All genes have been tested in P8 control ($N=5$) and mutant ($N=3$) mice. For primer sequences, see Supplementary Table II.

Chromatin immunoprecipitation (ChIP)

Nuclei were prepared from pooled kidneys (Cereghini *et al*, 1987). Chromatin preparation and ChIP were performed as described in Supplementary method 2. Three independent ChIP experiments were performed with α -HNF1 α -NC-AR or α -HNF1 β -NC-AR antibodies, and corresponding eluates were pooled. Primers were designed using PrimerExpress 2.0[®] software. For primer sequences, see Supplementary Table IB. Quantification of precipitated DNA fragments was carried out on an ABI PRISM[®] 7000 sequence detection system using SYBR[®] green in triplicate. Relative fold *in vivo* enrichment of DNA fragments was calculated using the following formula: (ChIP_{target}/ChIP_{normalizer})/(input_{target}/input_{normalizer}). As normalizer, we used a DNA fragment lacking any HNF1 site, located in the first intron of aortic smooth muscle α -actin 2 gene.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Note added in proof

Supporting our results, HNF1 β has been shown to regulate the activity of the *pkhd1* promoter in cultured cells (Hiesberger T, Bai Y, Shao X, McNally BT, Sinclair AM, Tian X, Somlo S, Igarashi P (2004) *J Clin Invest* (in press).

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