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Transcription factor HNF1β and novel partners affect nephrogenesis

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Heterozygous mutations of the tissue-specific transcription factor hepatocyte nuclear factor (HNF)1β, cause maturity onset diabetes of the young (MODY5) and kidney anomalies including agenesis, hypoplasia, dysplasia and cysts. Because of these renal anomalies, *HNF1*β is classified as a CAKUT (congenital anomalies of the kidney and urinary tract) gene. We searched for human fetal kidney proteins interacting with the N-terminal region of HNF1β using a bacterial two-hybrid system and identified five novel proteins along with the known partner DCoH. The interactions were confirmed for four of these proteins by GST pull-down assays. Overexpression of two proteins, E4F1 and ZFP36L1, in Xenopus embryos interfered with pronephros formation. Further, *in situ* hybridization showed overlapping expression of HNF1β, E4F1 and ZFP36L1 in the developing pronephros. HNF1β is present largely in the nucleus where it colocalized with E4F1. However, ZFP36L1 was located predominantly in the cytoplasm. A nuclear function for ZFP36L1 was shown as it was able to reduce HNF1β transactivation in a luciferase reporter system. Our studies show novel proteins may cooperate with HNF1β in human metanephric development and propose that E4F1 and ZFP36L1 are CAKUT genes. We searched for mutations in the open reading frame of the *ZFP36L1* gene in 58 patients with renal anomalies but found none.

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In humans, heterozygous mutations of the *TCF2* gene encoding the hepatocyte nuclear factor (HNF)1β result in several kidney abnormalities, including formation of cysts, oligomeganephronia, renal agenesis, renal hypoplasia, and familial juvenile hyperuricemic nephropathy. In addition, mutation carriers have a variety of extrarenal phenotypes, such as maturity onset diabetes of the young (MODY5), malformations of the genital tract, gout, deranged liver function, and pancreatic atrophy.[1] As heterozygous deletions of the *TCF2* gene[2–4] have an equivalent phenotype to mutations, it is likely that an impaired gene dosage is the causative factor for the *HNF1*β-associated diseases.

HNF1β is a homeodomain-containing transcription factor expressed in the liver, pancreas, gut, lung, genital tract, and kidney.[5,6] It also has a function in early embryogenesis, as *HNF1*β-null mice die before gastrulation due to the failure of proper differentiation of the visceral endoderm.[7,8] The rescue of this early lethality by tetraploid aggregation results in pancreas agenesis,[9] whereas by using tissue-restricted knockouts, the differential function of HNF1β in the liver,[10] pancreatic β-cells,[11] and in the kidney[12] could be identified. All these tissue-specific defects in mice reflect the human diseases observed in patients with heterozygous HNF1β mutations. Notably, the kidney-specific knockout of HNF1β revealed the defective activation of the *UMOD*, *PKHD1*, and *PKD2* genes whose mutations are responsible for distinct kidney defects in humans.[12] A link between *HNF1*β and the *PKHD1* gene that is mutated in humans with autosomal-recessive polycystic kidney disease was also established by kidney-specific overexpression of an *HNF1*β mutant leading to renal cysts.[13] Based on studies in mice with a kidney-restricted knockout of *HNF1*β, it has been concluded that the tubular enlargement and cyst formation are triggered by a disruption of the association of mitotic cell orientation along the tubule axis.[14]

As the structure and function of HNF1β is highly conserved in vertebrates, lower vertebrates are informative models for HNF1β function. In fact, mutations in the zebrafish *HNF1*β gene result in phenotypes closely related to the human diseases including kidney cysts as well as underdevelopment of the pancreas and the liver.[15] In the *Xenopus*, we have previously shown that overexpression of

human *HNF1*β mutants leads to a reduction or enlargement of the pronephros size depending on the mutant type injected.[16,17] The pronephros is the functional kidney of *Xenopus* larvae, which is the simplest form of the three types of kidneys in vertebrates. It consists of only one nephron, which is the functional unit in the meso- and metanephros as well. Therefore, the *Xenopus* pronephros is an excellent organ system to identify the nephrogenic gene network, which undoubtedly has applicability to human kidney development.[18–20]

*HNF1*β is a member of the group of genes responsible for congenital anomalies of the kidney and urinary tract (CAKUT), which cause 40% of renal insufficiency or end-stage renal failure of children.[21] However, in most CAKUT patients (75–90%), the defective gene expression has yet to be identified.[1,22,23] Assuming that genes coding for protein interaction partners of HNF1β are potential CAKUT genes, we search in this report for interaction partners of HNF1β using a two-hybrid system. To validate proteins interacting with HNF1β, we tested their function in cell cultures and defined their biological relevance for kidney formation via overexpression in *Xenopus* larvae. Based on these experiments, we propose that *ZFP36L1* and *E4F1* are genes whose dysfunction may lead to kidney malformations in humans.

RESULTS

Identification of interaction partners of HNF1β

To identify interaction partners of HNF1β, we used the bacterial two-hybrid system BacteriomatchII (Stratagene, La Jolla, CA, USA). Identification of interaction is based on transcriptional activation of reporter genes mediated by interaction of the fusion proteins containing either the bait or the target (Figure 1a). The bait is fused to the λ-repressor λcl that recognizes the operator sequence in the promoter, whereas the target sequence is linked to the RNA polymerase. When bait and target interact, RNA polymerase is recruited to the promoter and transcription of the reporter genes is initiated. As bait, we cloned downstream of the λ-repressor λcl the N-terminal region of human *HNF1*β (HNF1bbb in Figure 1b), which contains the region of HNF1β conferring defective pronephros development in *Xenopus*.[24] A control experiment revealed that HNF1bbb linked to λcl did not self-activate the transcription of the reporter genes in the bacterial system. As targets, we used a human fetal kidney cDNA library fused to the DNA encoding the α-subunit of RNA polymerase. By cotransformation of the bait vector with the target cDNA library, we screened 1.5 million transformants for interaction. After validation and exclusion of self-activation by the target proteins, 138 candidates remained for sequence analysis. We obtained eight distinct protein sequences with more than 20 amino acids fused in frame to the RNA polymerase α-subunit (Table 1). Whereas the open reading frame (ORF) of dimerization cofactor of HNF1 (DcoH) was recovered as full length, all the other ORFs represented a C-terminal fragment. This reflects that the kidney cDNA library was oligodT primed and had an average length of only 1.39 kb. Six proteins could be identified as the *bona fide* proteins DAK, PCBD1, E4F1, HADH, TRIM26, and ZFP36L1, whereas C17orf45 represents only a protein predicted by the ORF. As no cDNA was available for C17orf45, the analysis of this potential interaction partner was not further pursued. As PCBD1, also known as DCoH, is a well-known interaction partner of HNF1β ,[25] but plays no role in nephrogenesis,[26,27] we excluded it from further investigation.

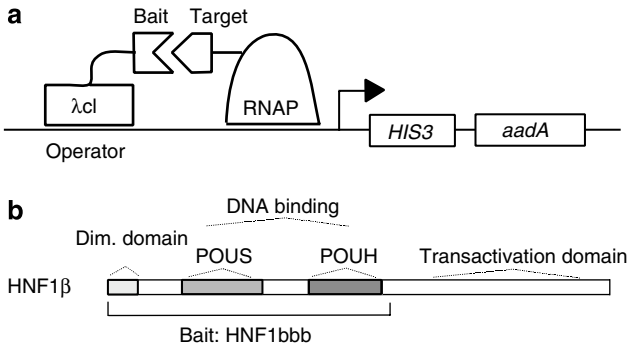

Figure 1 | Two-hybrid system components. (**a**). The reporter genes HIS3 and aadA are driven by a promoter whose activity is dependent on the interaction of RNA polymerase (RNAP) with the λcl repressor bound to the operator. The interaction is mediated by protein–protein contact of the bait and the target linked to the repressor and the polymerase, respectively. The target proteins are encoded by the commercial human oligodT primed cDNA library derived from kidneys of five male fetuses, 17–28 weeks after gestation (Stratagene). (**b**) Schematic drawing of the human HNF1β protein with the dimerization (dim.) domain, the DNA-binding domains POU_S and POU_H as well as the transactivation domain. The bracket indicates the N-terminal portion containing the nephrogenic domains (HNF1bbb) used as bait.

To verify the interaction with HNF1β for the five remaining proteins, we made an *in vitro* GST pull-down assay. We produced the five full-length proteins as myc-tagged versions in a rabbit reticulocyte system and incubated the lysates *in vitro* with the purified GST–HNF1bbb fusion protein produced in *Escherichia coli*. Interaction could be confirmed for the proteins E4F1, HADH, TRIM26, and ZFP36L1, whereas DAK did not interact and thus was not investigated further (Figure 2). TRIM26 seems to be a weak interactor, as interaction was only observed in an incubation buffer with 50 mM NaCl.

E4F1 and ZFP36L1 interfere with nephrogenesis in *Xenopus* embryos

To elucidate a potential nephrogenic role of the HNF1β interaction partners, we expressed the myc-tagged proteins in *Xenopus* embryos. RNAs encoding the human proteins were coinjected with RNA encoding green fluorescent protein (GFP) into one blastomere of *Xenopus laevis* two-cell-stage embryos. Proper translation of the interaction partners in *Xenopus* embryos was proven by Western blots of injected larvae using an anti-myc antibody (data not shown). Using GFP fluorescence, the injected side of the tailbud-stage