The transcription factor HNF1β has several domains involved in nephrogenesis and partially rescues Pax8/lim1 induced kidney malformations

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Guizhi Wu

aus Shandong, China

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1. Gutachter: Gerhart U. Ryffel

2. Gutachter: Helmut Esche

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Abbreviations used in the text

aa	amino acid
BSA	bovine serum albumine
cDNA	complementary DNA
d.	day, days
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DN	dominant negative
DTT	1,4-Dithiothreitol
EDTA	ethylene-diamine tetra-acetate
EGTA	ethylene-glycol-bis(2-aminoethylether)-N,N'-tetra-acetate
Fw	forward
GFP	green fluorescent protein
h	hour, hours
hCG	human chorionic gonadotropin
HEPES	*N*-(2-Hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid
HNF	hepatocyte nuclear factor
min	minute, minutes
MMR	Marcs modified Ringer's solution
MODY	maturity onset diabetes of the young
MOPS	3-(N-morpholino)propanesulfonic acid
MS-222	tricaine methanesulfonate
OFD1	Oral Facial Digital Syndrome Type 1
OPN	osteopontin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PTB	PBS containing 2mg/ml BSA
p.c.	post coital
PCR	polymerase chain reaction
PMSF	phenylmethyl sulfonylfluoride
Re	reverse
RPM	round per minute
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecylsulfate
TEMED	N,N,N,N-Tetramethylethylendiamid
Tris	Tris-(hydroxymethyl-)aminoethan
VE	visceral endoderm
WT	wild type

The tissue-specific transcription factors, HNF1α and HNF1β, are two closely related homeodomain proteins that are conserved throughout vertebrate evolution. Heterozygous mutations in the human HNF1β and HNF1α genes are linked to maturity onset diabetes of the young (MODY), but mutated HNF1β is also associated with kidney malformations. Consistent with this, it has been demonstrated that overexpression of HNF1β in *Xenopus* embryos leads to defective pronephric development and agenesis of the pronephros, while HNF1α has no effect on kidney development. The regions in the HNF1β protein responsible for this functional difference were defined in transfected HeLa cells as well as in injected *Xenopus* embryos. Using domain swapping experiments a nuclear localization signal was localized in the POU_H domain of HNF1β. The POU_S and POU_H domains of HNF1β also were responsible for the most of the transactivation activity in transfected cells. In injected *Xenopus* embryos, three HNF1β domains are involved in nephrogenesis. These include the dimerization domain, the 26 aa segment specific for splice variant A as well as the POU_H domain. HNF1β together with Pax8 and lim1 constitute the earliest regulators in the pronephric anlage. Overexpression of lim1 together with Pax8 in *Xenopus* embryos led to an enlarged pronephros with ectopic pronephric structures. In an effort to evaluate whether HNFβ antagonizes the nephrogenic effect of lim1 and Pax8, all three transcription factors were coinjected into *Xenopus* embryos. The data shown here that HNF1β can overcome the enlargement and the induction of an ectopic pronephros mediated by overexpression of Pax8 and lim1. But the phenotype induced by Pax8 and lim1 overexpression and characterized by cyst-like structures and thickening of the pronephric tubules was not altered by HNF1β overexpression. Taken together, HNF1β acts antagonistically to Pax8 and lim1 in only some processes during nephrogenesis, and a simple antagonistic relationship does not completely describe the functions of these genes. I conclude that HNF1β has some distinct morphogenetic properties during nephrogenesis.

I. Introduction

1. Transcription factors

Eukaryotes employ diverse mechanisms to regulate gene expression, including chromatin condensation, DNA methylation, transcriptional initiation, alternative splicing of RNA, mRNA stability, translational controls, several forms of post-translational modification, intracellular trafficking, and protein degradation. The rate of transcriptional initiation (when and how often a given gene is transcribed) is the most important point of control. The transcription of each gene is controlled by gene regulatory proteins known as transcription factors. Eukaryotic genes that encode proteins are transcribed by the RNA polymerase II with the help of general transcription factors to position the RNA polymerase correctly at the basal promoter and pull apart the two strands of DNA to allow transcription to begin. The basal promoter is a 100-bp region whose function is to provide a docking site for the transcription complex and position the start of transcription relative to coding sequences (Reinberg et al., 1998; Lee and Young, 2000). General transcription factors are termed general because they assemble on all promoters used by polymerase II. This group includes 10 to 12 proteins, most of them being ubiquitously expressed, and therefore, providing little regulatory specificity (Orphanides et al., 1996; Lee and Young, 2000).

Only some of the genes in an eukaryotic cell are expressed at any given moment. The proportion and composition of transcribed genes change considerably during the life cycle, among cell types, and in response to fluctuating physiological and environmental conditions (White et al., 1999; Iyer et al., 2001; Kayo et al., 2001; Mody et al., 2001; Arbeitman et al., 2002). Producing functionally significant levels of mRNA requires the sequence-specific association of transcription factors with DNA sequences outside the basal promoter (Lemon and Tjian, 2000). These specific transcription factors bind their specific DNA sequences and recruit cofactors to attract, position and modify the general transcription factors and RNA polymerase II at the promoter so that transcription can begin (Roeder, 1998; Lee and Young, 2000; Orphanides and Reinberg, 2002).

Most transcription factors that activate gene transcription have a modular design consisting of at least two distinct domains. The DNA binding domain usually contains one of the structural motifs that recognizes a specific regulatory DNA sequence. A second domain, sometimes called an activation domain, accelerates the rate of transcription initiation.

Some of the known DNA binding proteins are restricted to a cell lineage. They interact with DNA sequences necessary for tissue-specific activation or repression of genes, and cause the correct spatial and temporal pattern of gene expression. For example, the sequence encoding the muscle determination factor (MyoD) belongs to a superfamily of basic helix-loop-helix (bHLH) transcription factors (Rudnicki and Jaenisch, 1995). It plays a dominant role in myogenesis, and has the ability to convert fibroblasts into myogenic cells. However, only few of the tissue-specific transcription factors are sufficient to confer phenotype to a cell. Most of the transcription factors cannot be considered as a 'master gene' for the phenotype. For example, the hepatocyte nuclear factor 1 family (HNF1), containing HNF1α (HNF1α, also named HNF1 or TCF1) and HNF1β (also named vHNF1 or TCF2), were initially identified as transcription factors enriched in the liver (Bach et al., 1991). In addition to the liver, HNF1 is also expressed in the kidney, pancreas and intestine, whereas, HNF1β in addition is expressed in the lung and testis. HNF1α regulates numerous liver specific genes, and its expression appears linked to the hepatic phenotype. In addition to the HNF1 family, four other families of transcription factors have been isolated that are involved in the liver-specific regulation of different genes: C/EBP, HNF3, DBP and HNF4. None of these factors can singly account for the determination of the liver differentiation program. It is most likely that they participate a network of regulators in hepatocytes to define the hepatic phenotype.

2. The tissue-specific transcription factors, HNF1α and HNF1β

Duplication of developmental control genes is thought to be an evolutionary mechanism for the generation of novel functions allowing for increased diversity in complex organisms. It provides an organism with a cornucopia of spare gene copies, which are free to mutate to serve divergent purposes. The related duplicated genes often remain functional while changes in their sequence and expression pattern occur and they take on different functional

roles. In many cases, the most obvious functional differences between the duplicated genes is that they are expressed in different tissues or at different stages of development (Amores et al., 1998; Kawazoe et al., 2002). The genes encoding the tissue-specific transcription factors, HNF1α and HNF1β, have arisen from an ancestral gene at the onset of vertebrate evolution (Deryckere et al., 1995). They are highly conserved in vertebrates with homologs in fish (Deryckere et al., 1995; Sun and Hopkins, 2001), frog (Bartkowski et al., 1993; Demartis et al., 1994) and mammals (Frain et al., 1989). They are encoded as distinct genes on separate chromosomes. In humans, HNF1α and HNF1β are located on chromosomes 12 and 17, respectively. The exon-intron pattern in genome encoding for HNF1α and HNF1β is conserved in vertebrate species, and is essentially the same between *Xenopus* and mammals (Zapp et al., 1993).

2.1 Functional regions of the HNF1 proteins

The transcription factors, HNF1α and HNF1β, are two related tissue-specific transcription factors of the homeodomain family (Gehring et al., 1994). They are two unique members of this gene family as they contain an extra 21 amino acid loop between helices 2 and 3 (Cereghini, 1996). Both HNF1 proteins contain a highly conserved N-terminal dimerization domain, a bipartite DNA binding region and a more divergent C-terminal transactivation domain (Figure 1). Based on the crystal structure, the dimerization domain in the dimer has been identified as an intertwined four-helix bundle that allows the formation of homo- or heterodimers of the HNF1 proteins (Rose et al., 2000; Narayana et al., 2001). A dimerization cofactor termed DCoH binds to the dimerization domain of HNF1 factors, and stabilizes the active dimeric form. (Rose et al., 2000).

The DNA binding domain is composed of a POU-A related domain (POU_S) and the divergent homeodomain, POU_H. The POU_S domain is strictly conserved between the α and β proteins (98% identity of the amino acid sequence), indicating that it is crucial for the overall DNA binding activity of HNF1. Only nine out of 90 residues within POU_H differ between HNF1α and HNF1β, six of which are conservative changes. Together POU_S and POU_H bind to the palindromic 13 bp consensus sequence, GTTAATNATTANC (Courtois et al., 1988). Recent three-dimensional structural analysis of the HNF1α protein indicates that the POU_S domain interacts with the 21 aa loop of the POU_H domain to create a stable

interface between the two DNA binding domains, and that this rigidity is necessary for normal function. This feature distinguishes HNF1α from other more flexible POU-homeodomain factors including Pit1 and Oct1, where flexibility is critical for their DNA binding (Chi et al., 2002). Since the primary structures of HNF1α and HNF1β within the dimerization as well as the DNA binding regions are very similar, it is not surprising that HNF1α and HNF1β bind DNA as homo- or heterodimers and display indistinguishable DNA binding sequence specificity (De Simone et al., 1991; Rey-Campos et al., 1991; Bach et al., 1991; Cereghini, 1996).

The transactivation domains of the HNF1α and HNF1β proteins are the most divergent regions of the primary structure. The glycine/proline-rich stretch downstream to the homeodomain in HNF1α (288-308 aa in HNF1α) is absent in HNF1β. This region has been proposed to function as a potential hinge structure in the HNF1α protein (Chouard et al., 1990), and to be essential for transcriptional activation by HNF1α (Nicosia et al., 1990). This may also explain why HNF1α is a more powerful activator than HNF1β (Rey-Campos et al., 1991).

The N-terminal region of HNF1β differs from that of HNF1α, in that it contains a 26 aa insertion between the POU_S and POU_H domains (Figure 1). Two isoforms of HNF1β designated HNF1β-A and -B, resulting from alternative exon 2 usage, are always present, albeit at different levels, in all tissues where HNF1β is expressed (Ringeisen et al., 1993; Cereghini et al., 1992; Bach and Yaniv, 1993). This 26 aa segment is only present in isoform HNF1β-A, and absent in splice variant HNF1β-B. Moreover, this segment is found in the mammalian as well as in *Xenopus* HNF1β proteins, and the sequence is highly conserved from *Xenopus* to humans (Figure 1). Taken together, these findings indicate that this 26 aa segment plays a specific role for HNF1β function.

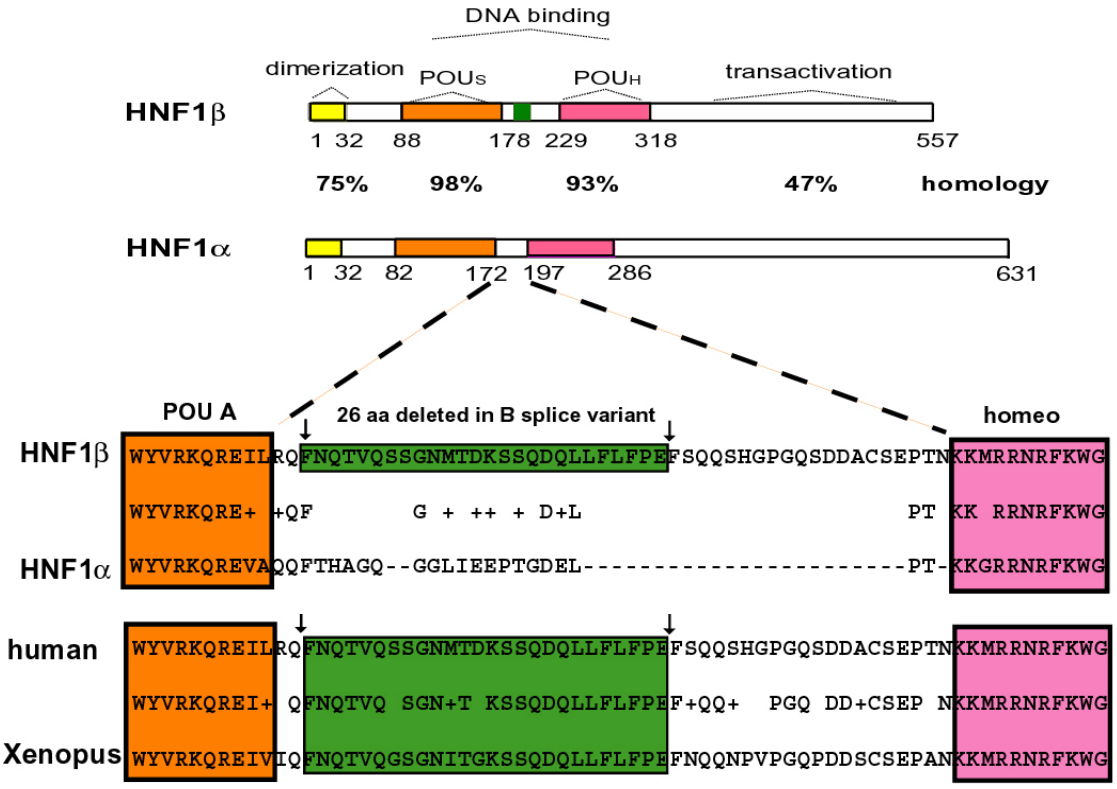

Figure 1: The related human transcription factors, HNF1α and HNF1β. Above, HNF1β and HNF1α are represented schematically. The domains are indicated and numbers refer to the amino acid positions. Amino acid identity of the domains between HNF1α and HNF1β is shown in bold face. Below, the 26 aa segment between the POU_S and POU_H domains of the human HNF1α and HNF1β proteins as well as of the human and *Xenopus* HNF1β protein are aligned. The 26 aa segment deleted in the B splice variant of the HNF1β is indicated. Identical amino acids between β and α or human β and *Xenopus* β sequences are shown and conserved amino acid changes are indicated by +.

2.2 HNF1 expression during development

In murine embryogenesis, HNF1α and HNF1β are coexpressed in the yolk sac endoderm as well as in developing liver, kidney, and pancreas, although with different spatio-temporal patterns (Lazzaro et al., 1992; Ott et al., 1991; Cereghini et al., 1992; Barbacci et al., 1999; Coffinier et al., 1999a). Transcription of HNF1β precedes that of HNF1α, and starts during the earliest stages of organogenesis. HNF1α appears in the visceral endoderm of the yolk sac at approximately 8.5 d. p.c., and in the liver and pancreatic buds at 10.5 d. p.c. (Cereghini et al., 1992; Nammo et al., 2002). In contrast, HNF1β is uniquely expressed in the primitive and visceral endoderm from 4.5 to 7.5 d. p.c. and in the liver, pancreatic, and ureteric buds at 9.5-11 d. p.c.. (Barbacci et al., 1999). In addition, HNF1β is expressed in the forming neural tube, lungs, and genital tract, where HNF1α is absent (Barbacci et al., 1999; Coffinier et al., 1999b; Reber and Cereghini, 2001).

The embryonic expression pattern of the HNF1 proteins is evolutionarily conserved in the vertebrates. The expression of HNF1β occurs prior to HNF1α in *Xenopus* embryo (Weber et al., 1996; Pogge v.Strandmann et al., 1997), and only HNF1β is expressed in the developing brain in *Xenopus* as well as in Zebrafish (Demartis et al., 1994; Sun and Hopkins, 2001). In *Xenopus*, HNF1β protein is detectable in the blastula (stage 9), whereas HNF1α protein is not detected until the hatched larvae (stage 35), even though HNF1α transcription starts in the gastrula (stage 11) (Pogge v.Strandmann et al., 1997). It seems likely that HNF1β acts initially through the HNF1 binding site of HNF1α promoter to affect the accumulation of HNF1α transcripts in *Xenopus* (Weber et al., 1996; Pogge v.Strandmann et al., 1997).

2.3 Phenotype of HNF1 homozygous knockout mice

In agreement with the differential embryonic expression patterns of the two HNF1 proteins, inactivation of the corresponding genes in the mouse has different effects. Homozygous knockout of the HNF1β gene leads to early embryonic lethality at day 7.5 due to abnormal extraembryonic development, including poorly organized extraembryonic ectoderm as well as defective differentiation of the parietal and visceral endoderm (VE) of the yolk sac. (Barbacci et al., 1999; Coffinier et al., 1999b). The VE serves as a source of nutrients and multiple signals essential for normal development of the pregastrulating mouse embryo. In addition to its nutritional and histotrophic role, the VE participates in other embryonic developmental processes including early anterior neural patterning (Beddington and Robertson, 1998), cavitation of the ectoderm (Coucouvanis and Martin, 1999), and specification of hematopoietic and endothelial cell fates (Belaoussoff et al., 1998). HNF1β homozygous mutant embryos lack a distinct extraembryonic VE, and as a consequence, the extraembryonic ectodermal cells are severely disorganized. The early embryonic lethality in mice with homologous inactivation of the HNF1β gene has precluded further analysis of the functions of this gene later in development.

To circumvent early lethality, site-specific inactivation and knock-down strategies have been used for HNF1β in two recent studies. HNF1β was specifically inactivated in renal cells using the Cre-loxP strategy (Gresh et al., 2004). The KspCre transgenic mouse expressing the Cre recombinase under the control of the Ksp-cadherin (Cadherin 16)

promoter was used to restrict the inactivation to renal cells. Inactivation of the HNF1β gene was achieved by generating mice carrying a homozygous floxed HNF1β gene together with the KspCre transgene (Coffinier et al., 2002). Mice with renal-specific inactivation of HNF1β developed polycystic kidney disease via a drastic reduction of Umod, Pkhd1 and Pkd2 gene expression. Mutations in these genes have also been shown to cause other distinct cystic kidney syndromes (Kudo et al., 2004; Bergmann et al., 2004; Wu et al., 2002). The A263insGG mutant of HNF1β functions as a dominant-negative mutant when expressed in liver, pancreatic, and kidney cell lines (Tomura et al., 1999; Bohn et al., 2003). Expression of this mutant in transgenic mice under the control of a kidney-specific promoter led to the development of renal cysts and renal failure, similar to humans carrying the A263insGG mutation. The DN-HNF1β mutant protein dimerizes with WT HNF-1β, inhibiting binding to and activation of the Pkhd1 promoter (Hiesberger et al., 2004). Taken together, these data strongly support a role for HNF1β during nephrogenesis in mice.

In contrast, HNF1α is not required for early embryonic development. Defects caused by knockout of HNF1α in mice only manifested themselves after birth. These defects included hepatomegaly, phenylketonuria and Fanconi syndrome, and the mice die during postnatal life because of hepatic, pancreatic and renal dysfunction (Pontoglio et al., 1996; Pontoglio et al., 1998; Lee et al., 1998). A more detailed analysis revealed defective insulin secretion in the pancreatic β-cells in HNF1α-deficient mice (Dukes et al., 1998; Pontoglio et al., 1998; Pontoglio, 2000). The differences in the phenotypes of HNF1α- and HNF1β-knockout mice clearly establish differential roles for these genes, but do not directly reveal whether this functional difference reflects differential properties of the two transcription factors or rather differential expression patterns. Addressing this issue, a functional equivalence of the α and β proteins has been recently been shown in embryonic stem cells. Expression of HNF1α in HNF1β-deficient stem cells fully restored their ability to differentiate into mature visceral endoderm (Haumaitre et al., 2003).

2.4 Heterozygous mutations in HNF1 genes cause MODY in humans.

In contrast to the mice with heterozygous mutations in HNF1 genes had no phenotype, heterozygous mutations in HNF1 genes cause MODY in humans (Barbacci et al., 1999; Pontoglio et al., 1998). Maturity onset diabetes of the young (MODY) is an autosomal

dominant inherited disease in humans that is characterized by an early onset of non-insulin dependent diabetes mellitus (NIDDM) (recent reviews: (Hattersley, 1998; Froguel and Velho, 1999; Winter et al., 1999; Winter and Silverstein, 2000). This disease manifests itself clinically by the occurrence of diabetes in at least two generations with at least one member affected under the age of 25 years, and is caused by defective insulin secretion of the β-cells of the pancreas. To date, heterozygous mutations in five genes have been associated with this disease in humans. Except for the MODY2 gene, which encodes the glucokinase enzyme expressed in the β-cells, all the other MODY genes identified encode cell-specific transcription factors expressed in pancreatic β-cells. MODY4 represents mutations in the gene encoding the transcription factor IPF-1 (insulin promoter factor-1), also referred to as PDX-1 (pancreatic duodenal homeobox-1), IDX-1 (islet duodenal homeobox-1) or STF-1 (somatostatin transcription factor-1), that was initially identified as a transcription factor necessary for pancreatic development and islet peptide hormone expression (Habener and Stoffers, 1998). In contrast, MODY1, MODY3 and MODY5 are genes for HNF4α, HNF1α and HNF1β, respectively (Huang and Tsai, 2000).

Additionally, HNF1β mutations are associated with severe non-diabetic renal defects, pancreatic atrophy as well as genital malformations in females (Eeckhoute et al., 2003; Briancon et al., 2004; Ryffel, 2001). These renal defects are distinct from the diabetic nephropathy frequently occurring in MODY3 patients due to microvascular complications in the kidney leading to progressive microalbuminuria, macroalbuminuria and renal failure strongly correlated with poor glycemic control (Isomaa *et al.* 1998). Patients with HNF1β mutations are born with renal defects, and a defective kidney has even been observed in a 17-week-old fetus carrying a heterozyous mutation in HNF1β (Bingham et al., 2000), suggesting that HNF1β dysfunction interferes with kidney organogenesis. This assumption was confirmed by overexpression of HNF1β in *Xenopus* embryos (Wild et al., 2000). Overexpression of HNF1β induced severe defects in pronephros, the first type of kidney to develop in vertebrates. This effect was specific, as overexpression of HNF1α did not affect any phase of kidney organogenesis. Together these results indicate that different intrinsic biochemical properties of these two transcription factors mediate the HNF1β-specific effects in nephrogenesis.

3. The *Xenopus* pronephros is a model system for studying kidney development.

3.1 Xenopus laevis as a model system

Xenopus laevis, commonly called the South African clawed frog due to the presence of claws on the three inner toes of the hind feet, has been used extensively as a non-mammalian model not only for vertebrate embryology, but also for research in the areas of cellular biology, physiology and biochemistry. The *Xenopus* embryo is a good system to study embryonic development because early development occurs outside of the mother, the eggs and embryos are relatively large, the blastomeres determined to become specific structures are readily identifiable and the embryo can withstand extensive surgical intervention as well as survive *in vitro* culture. The animals can be housed in a laboratory with minimal infrastructure. Thousands of eggs can be collected after priming egg ripening by injection of chorionic gonatropin into the lymph sac. Finally, development can be synchronized by fertilizing batches of eggs *in vitro*, so that groups of embryos are obtained at a defined stage of embryonic development (Sive et al., 2000; Olive et al., 2003; Ryffel, 2003). *Xenopus* embryos develop rapidly after fertilization, producing tadpoles with fully functional organs within 2-3 days, depending on temperature. The embryos develop well in a simple low-salt solution, and the larvae do not require feeding. Embryogenesis and, especially organogenesis, can easily be monitored *in vivo*, as the larvae are quite transparent. This allows the uninvasive examination of embryos after experimental manipulation.

Surgical manipulations performed in *Xenopus* embryos included the explantation of a defined region of the embryo as well as the injection of RNA or DNA into specific blastomeres. The mRNA for a specific gene can be injected into the fertilized egg or into blastomeres of early cleaving stages (Sive et al., 2000) to produce an efficient overexpression of the protein targeted to a specific region of the embryo. The injection can also be restricted to one side of the embryo by injection into one blastomere at the 2-cell stage, allowing the use of the uninjected side as a control within the same animal. Additionally, the localized expression of an exogenous mRNA can be controlled by

coinjecting mRNA or cDNA for a suitable lineage marker such as β-galactosidase or green fluorescent protein. Alternatively, the expression of a protein can be knocked down through injection of a morpholino antisence oligonucleotides. Native gene expression can be measured via RT-PCR of isolated RNA from whole embryos or embryonic areas. Finally, all of these manipulations can be carried out at the specific developmental stage of interest after embryo manipulation.

3.2 The pronephros as a model system for kidney development

Three distinct types of kidneys develop progressively after one another in vertebrates. The pronephros forms first during early larval development, followed by the mesonephros or middle kidney, and lastly the metanephros which is also the functional kidney in the adult. They have a similar functional organization and differ primarily in their spatial organization and complexity. The functional unit of every kidney type is the nephron. Later kidney types contain more and more complexly organized nephrons.

Similar regulatory genes are expressed during the development of all three kidney types, indicating that the molecular processes controlling the development of the different kidneys are closely related (Jones, 2003; Vize et al., 2003). Many of the gene products identified in the developing *Xenopus* pronephros have also been shown to play eminent roles in the development of the vertebrate meso- and metanephros. The simplicity of the structure as well as the parallels that can be drawn for gene function in the more complex vertebrate kidneys make the *Xenopus* pronephros a good model system for the study of kidney development.

The pronephros is the simplest vertebrate kidney, and consists of a single nephron with an external glomus. The basic structure of the *Xenopus* pronephros is illustrated in figure 2. The glomus freely filters wastes into the nephrocoel or coelom. The fluid of the coelom is taken up by three ciliated funnels (nephrostomes) that are joined into the coiled pronephric tubule (consist of connecting and common tubules). The common tubule is highly convoluted and surrounded by blood vessels into which water and small molecules that are

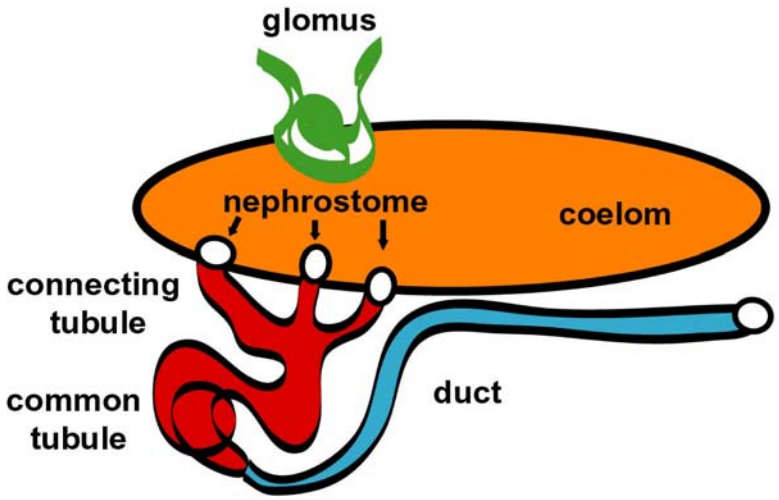

Figure 2. The *Xenopus* pronephros. The basic structure of the pronephros is shown schematically. Glomus, tubule and duct tissues are shown in green, red and blue, respectively. For further details see (Vize et al., 1997; Brändli, 1999).

not to be excreted are collected. The concentrated waste is then disposed of via the pronephric duct through the cloaca.

The pronephros develops from the somatic and splanchnic layers of intermediate mesoderm, and its development is completed within five days. The region determined to become the pronephros can be identified in the early neurula stage (stage 12/13). The first morphological indication of pronephric development is observed in the late neurula stage (stage 20/21) as a thickening of the somatic portion of the lateral mesoderm below somites 3 through 5. This thickening is caused by a cellular shape change as the pronephric precursor cells become columnar. In the tailbud embryo (stage 24), this thickening extends posteriorly to somite 6, and the cells can be seen to be assembling into a compact aggregate, forming the pronephric anlage (Hausen and Ribesel, 1991; Nieuwkoop and Faber, 1975). The anlage is subdivided into the tubules and duct during tadpole development (stages 30 to 38). At this time the third pronephric compartment, the glomus, develops from the splanchnic mesoderm which lines the coelom (Bernardini et al., 1999). The pronephric tubules can be easily identified in the living larvae, but the duct and glomus can only be visualized in the whole embryo after staining for specific markers using either specific antibodies or hybridization probes. The *Xenopus* pronephros has been described in

detail using such techniques, and excellent reviews are available (Carroll et al., 1999; Vize et al., 1997; Brändli, 1999).

3.3 lim1, Pax8 and HNF1β in pronephric development

The transcription factors, lim1, Pax8 and HNF1β play important roles in pronephric induction, pattering and differentiation. The earliest marker of pronephric specification in early development is lim1, which is expressed at the late gastrula stage, concomitant with the time at which the specification of tubules is occurring. Treatment of animal caps derived from blastula stage embryos with either activin or retinoic acid results in the expression of lim1 in the animal cap tissue (Taira et al., 1994; Uchiyama et al., 1996). It has been demonstrated that such treatment of animal caps results in the histological and immunohistological identification of pronephric tubules (Uchiyama et al., 1996; Brennan et al., 1999). lim1 overexpression following the injection of synthetic mRNA into early embryos, however, is insufficient to cause a high frequency of ectopic kidney formation, although there is synergism between lim1 and pax8 in kidney development following coinjection (Taira et al., 1994; Carroll and Vize, 1999). Furthermore, injection of wild-type or constitutively active lim1 in both activin- and RA-treated caps, augmented pronephric formation was observed. In contrast, when a dominant-negative lim1-eng was injected, this inhibited differentiation of the pronephros by 25-75%. Studies of targeted overexpression in whole tadpoles showed that a functional deficiency in lim1 resulted in a failure of the pronephros to undergo tubulogenesis (Chan et al., 2000). These studies suggest that lim1, although an important regular of pronephric tubule development, is unable to induce pronephros independently, suggesting that there are other molecules that are involved in formation of the pronephric primordium.

The Pax8 protein has also been shown to play a role in the development of the pronephros. Pax8 expression is detected at late gastrulation in both the otic vesicles and the presumptive pronephros (Carroll and Vize, 1999). The timing of pronephric expression of Pax8 coincides absolutely with the time at which the pronephros is specified. Injection of synthetic Pax8 mRNA alone targeted into the C2 blastomere of the 32 cell embryo has been shown to lead to the development of either enlarged or ectopic pronephroi. Further experiments have shown dramatically that lim1 can synthegize with Pax8 to generate

ectopic kidney (Carroll and Vize, 1999). Another Pax genes implicated in the control of kidney development, Pax2, was also capable of causing the same effects as Pax8 when coinjected with lim1, showing that Pax8 and Pax2 are functionally redundant in *Xenopus* (Carroll and Vize, 1999). These experiments suggest that lim1 and Pax8 are certainly important regulators of tubulogenesis.

HNF1β is also expressed in this region at the same time as lim1 and Pax8. Overexpression of human HNF1β in *Xenopus* embryos led to defective development and agenesis of the pronephros (Wild et al., 2000). A similar phenotype is seen after the expression of certain mutants of the human HNF1β gene, that have been linked with kidney disease. The expression of other HNF1β mutant proteins resulted in an enlargement of the pronephors (Wild et al., 2000; Bohn et al., 2003). Together, these data indicate that HNF1β, lim1 and Pax8 are the earliest regulators in the pronephric anlage, and may cooperate in the early events of nephrogenesis.

4. Aim of this work

As only the HNF1β protein has an effect on pronephric development, the aim of this work was to identify the protein domains of HNF1β that are specifically involved in pronephric development. To this end, mutant and chimeric HNFα and HNF1β proteins were expressed in *Xenopus* embryos as well as in mammalian cell lines. The functionality of these proteins was analyzed by examining their subcellular localization and transactivation potential in HeLa cells, as well as their effect on pronephric development after expression in *Xenopus* embryos. In an effort to evaluate the cooperation of HNF1β with lim1 and Pax8, all three transcription factors were coinjected into *Xenopus* embryos. Specifically, whether HNF1β could rescue lim1/Pax8-induced malformations of the pronephros was examined.

II. Materials and Methods

1. Chemicals and enzymes

The chemicals used in these experiments were obtained either from Fluka (Neu-Ulm), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) or Sigma-Aldrich (Taufkirchen) when sources are not specifically named in the text. Solutions and buffers were prepared as previously described (Sambrook et al., 1989). Restriction enzymes and enzymes for modifying DNA und RNA were purchased from Biolabs (Schwalbach), Invitrogen (Karlsruhe), Pharmacia (Freiburg) und Roche (Mannheim) when sources are not specifically named. All primers employed for PCR were synthesized by Invitrogen (Karlsruhe).

2. Molecular cloning and expression vectors

DNA techniques including the preparation of competent cells, plasmid DNA, *in vitro* amplification of DNA using the polymerase chain reaction (PCR), and electrophoresis were carried out as previously described in standard molecular biology technique collections (Sambrook et al., 1989). Preparation of large quantities of plasmid DNA was carried out using the Qiagen "Plasmid Maxi Kit" (Hilden) according to the supplied protocol.

2.1 Plasmid Constructions

The expression plasmids pCSGFP2, myc-Rc/CMVHNF1β (HNF1β) and myc-Rc/CMVHNF1α (HNF1α) have been described elsewhere (Wild et al., 2000). The expression construct for *Xenopus* HNF1β (XHNF1β) was kindly provided by Roberto Vignali (Vignali et al., 2000). Expression plasmids for *Xenopus* lim1 and Pax8 were kindly supplied by Peter D.Vize (Carroll and Vize, 1999). The syn(0)4tk-luc reporter plasmid used in transfection assays contains four HNF1 binding sites upstream to a thymidine kinase promoter and the luciferase gene (Drewes et al., 1996). All fragments generated by PCR for cloning were verified by sequencing.

All HNF1β variants listed in Table 1 were inserted into two expression vectors. The Rc/CMVGFP vector was used for transfection in HeLa cells. The pCS2^{+}MT vector, containing six Myc tags at the 5'-end of the first polylinker, was used for overexpression in *Xenopus* embryos (Rupp et al., 1994). The coding region of GFP was amplified by PCR from the plasmid pCSGFP2 plasmid. The GFPFw sense primer contains a *Hind*III restriction site, and the GFPRe antisense primer contains an *EcoR*I restriction site. The PCR fragment was double digested with *Hind*III-*EcoR*I, and cloned into the *Hind*III-*EcoR*I sites of Rc/CMV (Invitrogen).

HNF1aaa was generated by ligating the *EcoR*I-*BamH*I and *BamH*I-*Xba*I PCR fragments encoding 1-69 aa and 70-321 aa of HNF1α amplified with the primers listed in Table 1. HNF1bbb was generated by ligating the *EcoR*I-*BamH*I and *BamH*I-*Xba*I PCR fragments encoding amino acids 1-79 aa and 80-352 aa of HNF1β using the primers listed in Table 1. A *BamH*I site was introduced both at G69 (α) and G79 (β) without changing the predicted amino acid sequence. The *EcoR*I and *Xba*I restriction sites were generated immediately upstream to the translational starts and immediately to the translational 3' stops using PCR, respectively.

The HNF1abb chimera was generated by ligating the *EcoR*I-*BamH*I PCR fragment encoding 1-69 aa of HNF1α and the *BamH*I-*Xba*I fragment encoding 80-352 aa of HNF1β. The HNF1baa chimera was generated by ligating the *EcoR*I-*BamH*I PCR fragment encoding 1-79 aa of HNF1β and the *BamH*I-*Xba*I fragment encoding 70-321 aa of HNF1α.

HNF1bbbD was generated by constructing a primer containing sequence areas complementary to regions upstream and downstream of the cDNA sequence encoding the 26 aa segment to be deleted, but lacking the segment transcribing the 26 aa itself. The PCR fragment generated was consequently lacking the 26 aa segment coding region. The fragment was digested with *BamH*I and *Hinc*II, then inserted into the *BamH*I-*Hinc*II sites of HNF1bbb. HNF1βD was generated by replacing the *Pvu*I fragment coding for 1-251 aa of HNF1β with the corresponding fragment from HNF1bbbD containing the 26 aa deletion.

Table 1. Constructs created for use in expression vectors. Restriction enzyme sites included in the primers are underlined.

Name	Primer sequences (5'-3')	Protein fragment encoded
HNF1aaa	α1-69Fw: CGGAATTCAATGGTTTCTAAACTGAGCC α1-69Re: CGCGGATCCCCGAGTCTCCCCC α70-321Fw: CGCGGATCCGAGGACGAGACGG α70-321Re: GCTCTAGATTAGCGCACACCGTGGAC	HNF1α, 1-321aa
HNF1bbb	β1-79Fw: CGGAATTCAATGGTGTCCAAGCTCACGT β1-79Re: CGCGGATCCCTCGTCGCCGGACAA β80-352Fw: CGCGGATCCGAGGACGGCGACGA β80-352Re: GCTCTAGATTAGCGCACTCCTGACAGC	HNF1β, 1-352aa
HNF1abb	α1-69Fw, α1-69Re; β80-352Fw, β80-352Re	HNF1α, 1-69aa HNF1β, 80-352aa
HNF1baa	β1-79Fw, β1-79Re; α70-321Fw, β70-321Re	HNF1β, 1-79aa HNF1α, 70-321aa
HNF1bbbD	β80-211Fw: CGCGGATCCGAGGACGGCGACGA β80-211del183-208aaRe: GCTCTGTTGACTGAATTGTCGGAGGATCTCTCGT	HNF1β, 1-352aa with a deletion of 183-208aa
HNF1βD		HNF1β, 1-557aa with a deletion of 183-208aa
HNF1βhomeo	β229-352Fw: CGGAATTCAAAGAAGATGCGCCGCAAC, β80-352Re	HNF1β, 229-352aa
HNF1aab	β229-352Fw: GATGAGCTACCAACCAAGAAGATGCGCCGCA β229-352Re: GCCGCTCTAGATTAGCGCACTC	HNF1α, 1-196aa HNF1β, 229-352aa
HNF1aabins26	β183-352Fw: CGAGAGGTGGCGCAGCAGTTCAACCAGACAGTCCAG β229-352Re	HNF1α, 1-176aa HNF1β, 183-352aa
HNF1aaains26	β183-208Fr: CGAGAGGTGGCGCAGCAGTTCAACCAGACAGTCCAG β183-208Re: CTCCCTGCCCTGCATGGGTGAACTCTGGAAAGAGAAAC	HNF1α, 1-321aa with an insertion of HNF1β, 183-208aa at 176 aa of HNF1α
HNF1aabH	α70-196Fw: CGGAATTCAATGGTGTCCAAGCTCACGT β229-319Re: GCTCTAGATTAGCTATAGGCGTCCATGG	HNF1α, 1-196aa HNF1β, 229-319aa
HNF1aabHS	α70-196Fw, β229-311Re: GCTCTAGATTATTGCCGGAATGCCTCCT	HNF1α, 1-196aa HNF1β, 229-311aa
Rc/CMVGFP	GFPFw: GGCAAGCTTCTGGCCACCATGAGTAAAGGA GFPRe: CGGAATTCGTTTTGTATAGTTCATCCATGC	GFP, 1-238aa to create GFP-fusion protein expression vector

The HNF1aab, HNF1aabins26 and HNF1aaains26 chimeras were generated by site-directed mutagenesis using the Quickchange site-directed Mutagenensis Kit (Stratagene, La Jolla,

CA) with pairs of complementary mutagenic primers. These mutagenic primers were the PCR products encoding 229-352 aa, 183-352 aa and 183-208 aa of HNF1β, respectively. The HNF1aaa construct was used as a template for the mutagenesis.

The HNF1aabH and HNF1aabHS constructs were generated by replacing the *BamH*I-*Xba*I fragment from HNF1aab with PCR products encoding either 70-319 aa or 70-311 aa of HNF1abb, respectively, using the primers listed in Table 1.

3. Functional protein studies in HeLa cells

The transactivition activity of HNF1 proteins were assayed in transiently transfected HeLa cells. Expression vectors encoding various HNF1 fusion proteins were cotransfected together with a luciferase reporter plasmid. The HeLa cell line (derived from a human cervical carcinoma) was cultured at 37°C, 7.2% CO_2 and 95% humidity in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100U/ml), streptomycin (100U/ml), 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum (Biochrom).

The day before transfection, 3 x 10^5 HeLa cells were seeded with 3 ml of supplemented culture medium into each 3.3 cm cell culture well, and incubated overnight at 37°C and 5% CO_2. The cells were 50-80% confluent 24 h after seeding. For the luciferase assay, the cells in one well were transiently transfected with 1.3 µg reporter plasmid, 0.3 µg expression vector and 6µl of lipofectamine (Invitrogen, Carlsbad, CA). The total DNA concentration per well was equalized by the addition of Rc/CMV vector where necessary. Additionally, the subcellular localization of GFP-HNF1 fusion proteins was examined after transient transfection with 1µg expression vector into HeLa cells. The cells were rinsed once with Optimem (Gibcol,) and overlayed with the diluted DNA-liposome complex solution, then incubated at 37°C and 5% CO_2 for 4 h. The culture medium was replaced with fresh DMEM after transfection, and the transfected cells were cultured an additional 20 h. The growth medium was removed from the transfected cell wells, and cells were rinsed twice with PBS. For the luciferase assay, cells were lysed in 50µl of lysis buffer (25 mM tris-phosphate (pH 7.8), 2 mM DTT, 2 mM CDTA, 10% glycerol, 1% triton X-100). After incubating 5 minutes at room temperature, the attached cells were scraped free from the

culture dish. The lysed cells were transferred to a microcentrifuge tube, and centrifuged at 4°C for 5 min at 13,000 RPM to pellet the cell debris. The supernatent (cell extract) was transferred to a new tube, and the transactivation activity was measured using the luciferase reporter assay system (Promega, Madison, WI) in a Lumat LB 9501 luminometer (Berthold, Wilbad, Germany) according to the manufacturer's directions. For the subcellular localization, the cells were analyzed using fluorescence microscopy (Leica, Köln)

4. *Xenopus* embryos

4.1 Manipulation of *Xenopus* embryos

Adult *Xenopus laevis* frogs were purchased from distributors *Xenopus* 1 (USA), and maintained in the Institute for Cell Biology (Essen) according to animal care regulations. Adult female frogs were injected in the dorsal lymph sac with 200-250 IU (depending on the individual size) of human chorionic gonadotropin (hCG, Serono Pharma GmbH, Unterschleißheim). Injected frogs were kept overnight at room temperature. The frogs began to lay eggs 9-10 h later. A male frog was first anesthesized by submerging it in 0.5% MS-222 for 20 min before sacrificing it and dissecting out the testes. The testes was kept up to 10 days at 4°C in a humidity chamber. A piece was washed with Holtfreter's solution (60 mM NaCl, 0.6 mM KCl, 0.9 mM $CaCl_2$, 0.2 mM $NaHCO_3$), then rubbed over the *Xenopus* eggs. The fertilized eggs were flooded with water. Within 20 min, the eggs rotated so that the animal pole was up, indicating that fertilization had occurred. The jelly coat was removed from the embryos by swirling gently in 0.1 × Marcs modified Ringer's solution (MMR: 0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES (pH 7.8), 0.1 mM EDTA) containing 2% cysteine (Fluka) at pH 8.0 for 1-2 min until the jelly coats were no longer surrounding the egg. The fertilized eggs were rinsed at least 5 times with water, then incubated in 0.1x MMR at 14-20ºC until the desired stage. The developmental stages were assigned according to the *Xenopus laevis* development table from Nieuwkoop and Faber (Nieuwkoop and Faber, 1975).

4.2 *In vitro* mRNA synthesis

For overexpression in *Xenopus*, mRNA was synthesized *in vitro* following the protocol from Nielsen and Shapiro (1986). The expression vector (3µg) was linearized, then 100 units RNA polymerase were used for each *in vitro* transcription reaction. Expression constructs in the pCS2 vector were digested with *Not*I, and pCSGFP2 plasmid was linearized with *Pv*uII, then purified by extraction with phenol:chloroform using the Phase Lock Gel™ Light (1.5ml, Eppendorf) according to the manufacturer's directions. All expression constructs in the pCS2 vector were transcribed using SP6 RNA polymerase. The expression constructs in the Rc/CMV vector were linearized with *Sma*I (25°C, 1 h), and transcribed using T7 RNA polymerase (Nielsen and Shapiro, 1986). After *in vitro* transcription, Rnase-free DNase I was added to destroy the template DNA (37°C, 15 min). The RNA was purified by extraction with phenol:chloroform using the Phase Lock Gel™ Heavy (1.5ml, Eppendorf) according to the manufacturer's directions. The aqueous phase was transferred to a fresh microfuge tube, and 2.5 volumes of ethanol were added. The precipitated RNA was stored in ethanol in 20µl aliquots at –80°C. The RNA concentration was measured by a photometer (Abs_{260}) and the RNA quality was controlled by gel electrophoresis.

4.3 Microinjection

Prior to injection, mRNA was precipitated and dissolved in DEPC-treated water. Either 250pg mRNA encoding a protein together with 100pg GFP mRNA or 350pg GFP mRNA alone was injected in a volume of 10µl. Capped and tailed mRNA was microinjected into one blastomere of 2-cell stage *Xenopus* embryos using glass microcapillaries (ø 0.59mm, World precision instruments, Sarasota) and a micromanipulator (Gernaral valve corporation, Fairfield). Embryos were kept in 2% Ficoll 400 in 0.1× MMR during microinjection, and for 1 h following the injection to facilitate plasma membrane sealing. Embryos were cultured in 0.1 x MMR at 20ºC until the desired stage was reached.

5. Immunohistochemistry

Xenopus embryos expressing HNF1 proteins were examined for pronephric defects at stage 44-45 after immunohischemical staining for kidney markers. The tadpoles were fixed 1 h in MEMFA (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde), then dehydrated in methanol overnight at 4ºC. Embryos were subsequently rehydrated in PBS, then blocked 15 min with PBT (2 mg/ml BSA (bovine serum albumin, fraction V) and 0.1% Triton X-100 in PBS) at room temperature. The embryos were incubated overnight at 4°C with a 1:2 dilution of the pronephric tubule-specific 3G8 antibody and the duct-specific 4A6 antibody in PTB containing 20% goat serum (Vize et al., 1995). These antibodies were kindly provided by Elisabeth A. Oliver-Jones (Warwick University, Coventry, UK Coventry, England). After washing 5 times with PBT at room temperature, the embryos were incubated overnight at 4°C with Cy3-conjugated rat anti-mouse antibody diluted 1:1000 (Jackson ImmunoResearch, Pennsylvania, USA). Embryos were washed twice with PBT at room temperature, then analyzed using fluorescence microscopy (Leica, Köln).

6. Phenotype analysis

The phenotypic effects of overexpression were analyzed by comparing the injected and uninjected sides of stage 44-45 embryos. The areas through the widest part of the immunostained pronephros containing the pronephric tubules and through the anterior part of the pronephric duct were measured using the Kappa Metreo computer program (opto-electronics GmbH, Gleichen). No size difference between the injected and uninjected sides was set as 100. When it is over 100, the size of the pronephros in injected side is enlarged; while it is below 100, that is smaller. The Mann-Whitney test was used to compare the measurements obtained from each group of embryos with GFP control-injected embryos. Each group contained at least 30 embryos.

7. Western blotting

Embryonic lysates were prepared by homogenizing microinjected embryos cultured until stage 10 or later (60 embryos per 200 µl of lysis buffer (50 mM Tris (pH 7.9), 25% (v/v)

glycerol, 50 mM KCl, 2mM DTT, 0.1 mM EDTA, 0.1mM benzamidine, 0.1mM PMSF, 1µg/µl leupeptin and 1µg/µl pepstatin). Embryo lysates were pre-cleared by centrifugation at 15,000 RPM for 15 min at 4˚C (Beckmann TL-100 Ultracentrifuge). The total protein concentration was measured using the "BioRad Protein-Assay" system (BioRad). The lysates were combined with 1/3 volume SDS sample buffer (187.5 mM Tris-HCl (pH 6.8), 6% SDS, 3% glycerol, 0.03% phenol red and 125 mM DTT), boiled 3 min, resolved by 15% SDS-PAGE, and electroblotted at 1.5 mA/cm onto nitrocellulose membrane (Schleicher and Schuell, Dassel) using the Trans-Blot SD Electrophoretic Transfer Cell (BioRad) in transfer buffer (48 mM Tris, 39 mM glycine, 0.04% SDS, 20% methanol). The membrane was blocked with 0.5% blocking reagent (Liquid block, RPN 3601, Amersham, Braunschweig) in PBS at 4°C overnight, then incubated 90 min with the GE10 anti-myc monoclonal antibody (Evan et al., 1985) diluted 1:5 in PBS containing 0.1% Tween 20. After washing 3 x 10 min with 0.1% Tween 20 in PBS, the membrane was incubated for 1 h with peroxidase-conjugated rabbit anti-mouse antibody (Dianova) diluted 1:5000 in wash buffer. The membrane was washed 3 times, then the secondary antibody was detected using the Enhanced Chemoluminescence System (ECL-System, Amersham) according to manufacture's instructions. All steps were carried out at room temperature unless otherwise specified.

III. Results

1. The functionality of the GFP-HNF1β fusion protein

1.1 The GFP-HNF1β fusion protein has similar localization and transactivation properties as HNF1β in transfected HeLa cells.

Creation of a GFP-HNF1β fusion construct eliminated the necessity of coinjecting a marker into the injected side, and allowed the analysis of HNF1β function in cells and *Xenopus* embryos. The GFP-HNF1β construct was generated by inserting the coding sequence for GFP upstream to the HNF1β coding sequence. The pCS2 expression vector was more efficient for producing the protein in HeLa cells as well as *Xenopus* embryos than the Rc/CMV vector (data not shown). These expression vectors use different promoters and 3′ untranslated regions, which have an influence on mRNA stability and protein expression level. For this reason, the myc-tagged constructs were subcloned into pCS2 vector for injection into *Xenopus* embryos. The Rc/CMV vector was adequate for transfection assays in HeLa cells. The GFP-HNF1β fusion protein construct was transiently transfected into HeLa cells to show that the fusion protein is localized in the correct subcellular compartment to be functional. As has been shown previous for the native HNF1β protein, GFP-HNF1β was localized exclusively in the nuclei of transfected HeLa cells (Figure 3A) (Bohn et al., 2003). As a comparison, GFP was expressed both in the nucleus and cytoplasm of transfected HeLa cells (Figure 3A). These results indicate that the attached GFP domain does not influence the nuclear translocation of the HNF1β protein. To explore the transactivation potential of GFP-HNF1β, expression vectors encoding myc-HNF1β or GFP-HNF1β were cotransfected into HeLa cells lacking endogenous HNF1α and β expression together with a luciferase reporter plasmid containing an HNF1-inducible promoter. Figure 3B shows that equivalent amounts of either the GFP-HNF1β or myc-HNF1β constructs similarly transactivated the luciferase reporter in a dose-dependent manner. The results indicate that the GFP-HNF1β fusion protein functions similarly to the myc-HNF1β fusion protein in transfected cells. It has been shown previously that the myc-

HNF1β fusion protein behaves similarly to the native HNF1β protein (Wild et al., 2000), implying that the GFP-HNF1β fusion protein is functionally equivalent to native HNF1β.

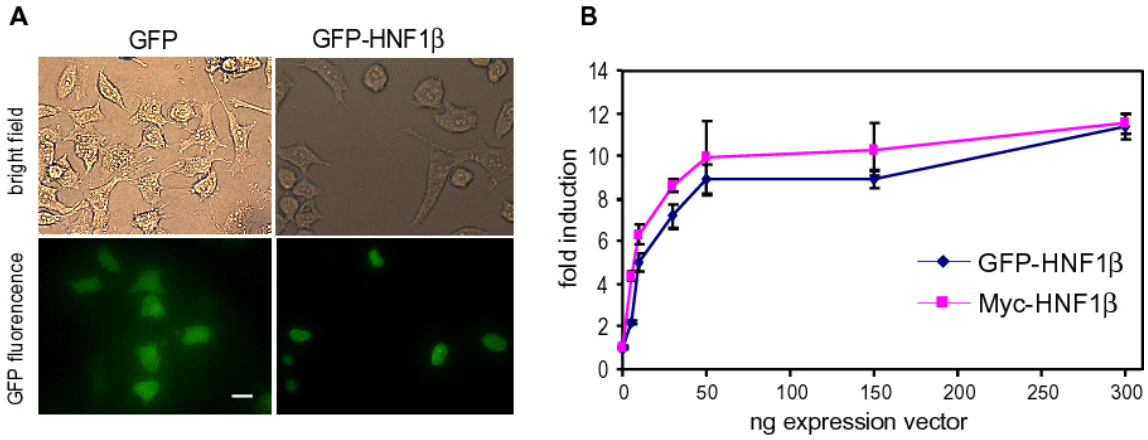

Figure 3. Subcellular localization and transactivation potential of GFP-HNF1β fusion construct. (A) HeLa cells expressing GFP or GFP-HNF1β fusion construct illuminated by bright field or green fluorescence. bar = 10µm. **(B)** Increasing amounts (5, 10, 30, 50, 150, 300ng) of GFP-HNF1β or myc-HNF1β were cotransfected into HeLa cells with an HNF1-dependent luciferase reporter plasmid. The fold-activation induced by each of the HNF1β expression constructs is shown. Error bars indicate standard deviation of the mean of at least six replicates.

1.2 Expression of the GFP-HNF1β fusion protein interferes with nephrogenesis in *Xenopus* embryos.

In order to analyze the function of HNF1β during nephrogenesis, the GFP-HNF1β fusion protein was overexpressed in the *Xenopus* embryos. The GFP-HNF1β mRNA was injected into one blastomere at the 2-cell stage of *Xenopus* embryos. As a comparison, GFP mRNA was coinjected with RNA encoding myc-tagged HNF1β. At the neurula stage, embryos were selected with strong GFP fluorescence restricted to only one side, and sorted into groups injected in either the right or left sides (Figure 4). The embryos injected with GFP mRNA and myc-tagged HNF1β showed very strong fluorescence at the initial tail bud stage (Figure 4A), and continued to fluoresce into the larval stage (Figure 4B). Embryos injected with GFP-HNF1β mRNA fluoresced more weakly at the initial tail bud stage (Figure 4C), and no fluorescence was observed at later stages. Strong GFP fluorescence was only observed in 10% (29/295) of embryos overexpressing GFP-HNF1β, as compared with 86% (259/300) of the embryos coinjected with myc-HNF1β and GFP mRNA (Figure 6). Some GFP-HNF1β-injected embryos exhibited strong enough fluorescence to see that

the fusion protein was expressed in a spotted pattern, representing a nuclear expression pattern that was never observed for injected GFP mRNA. Although expression indicated that the GFP-HNF1β fusion protein was functional, the reduced level of the fluorescence especially at later stages made it inadequate for phenotypic analysis in a whole embryo.

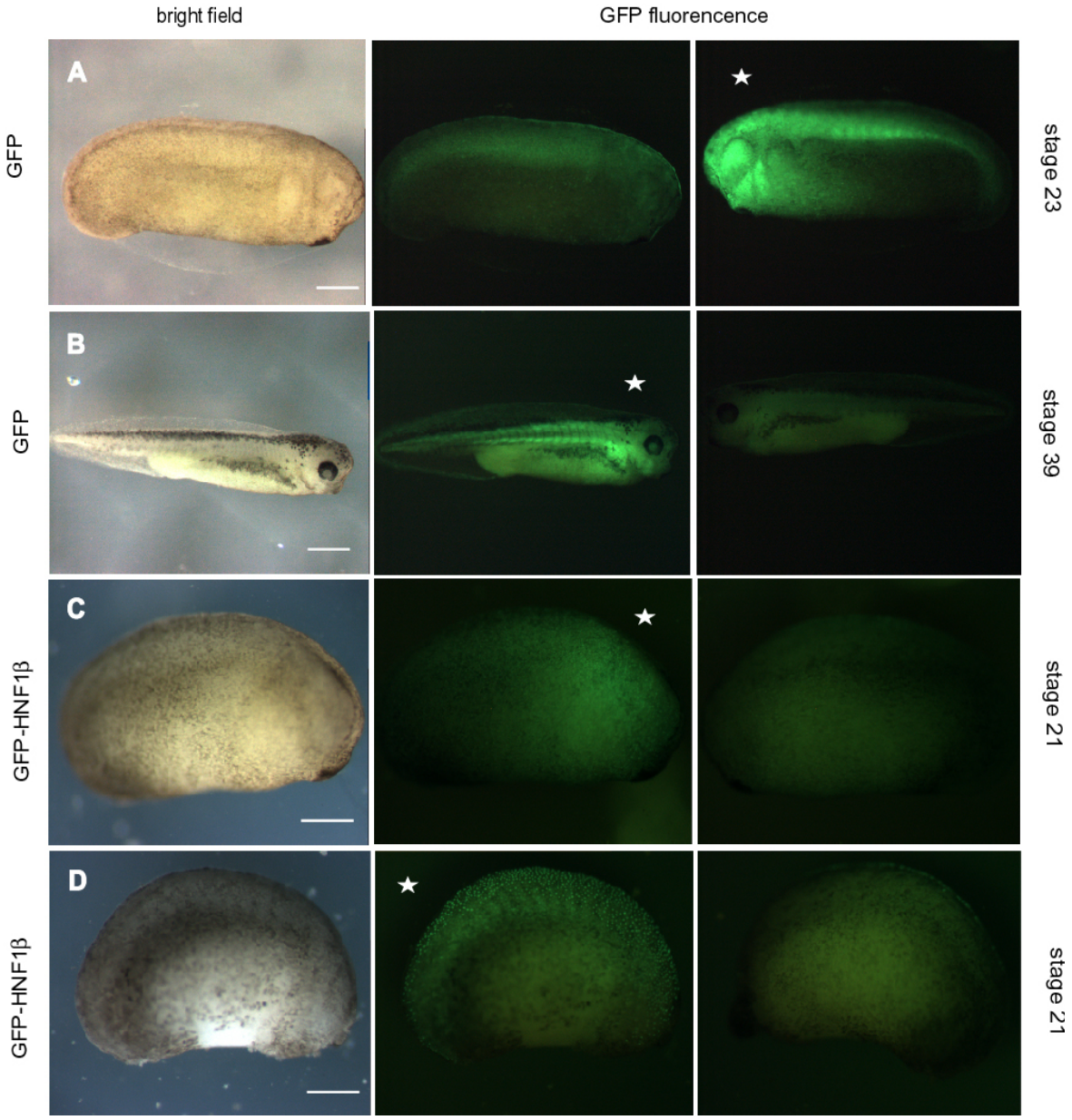

Figure 4. Expression of GFP alone or the GFP-HNF1β fusion protein in *Xenopus* embryos. (A) Lateral view of a typical larva (stage 23) expressing GFP on the left side. bar = 150m. **(B)** Lateral view of a typical larva (stage 39) expressing GFP on the right side. bar = 1mm. **(C)** Lateral view of a typical larva (stage 21) expressing GFP-HNF1β on the right side. bar = 300µm. **(D)** Lateral view of a typical larvae (stage 21) expressing GFP-HNF1β on the right side showing spotted GFP fluorescence pattern representing nuclear expression. bar = 300µm. Both uninjected and injected sides of each larva are shown, and the injected sides are marked by white stars.

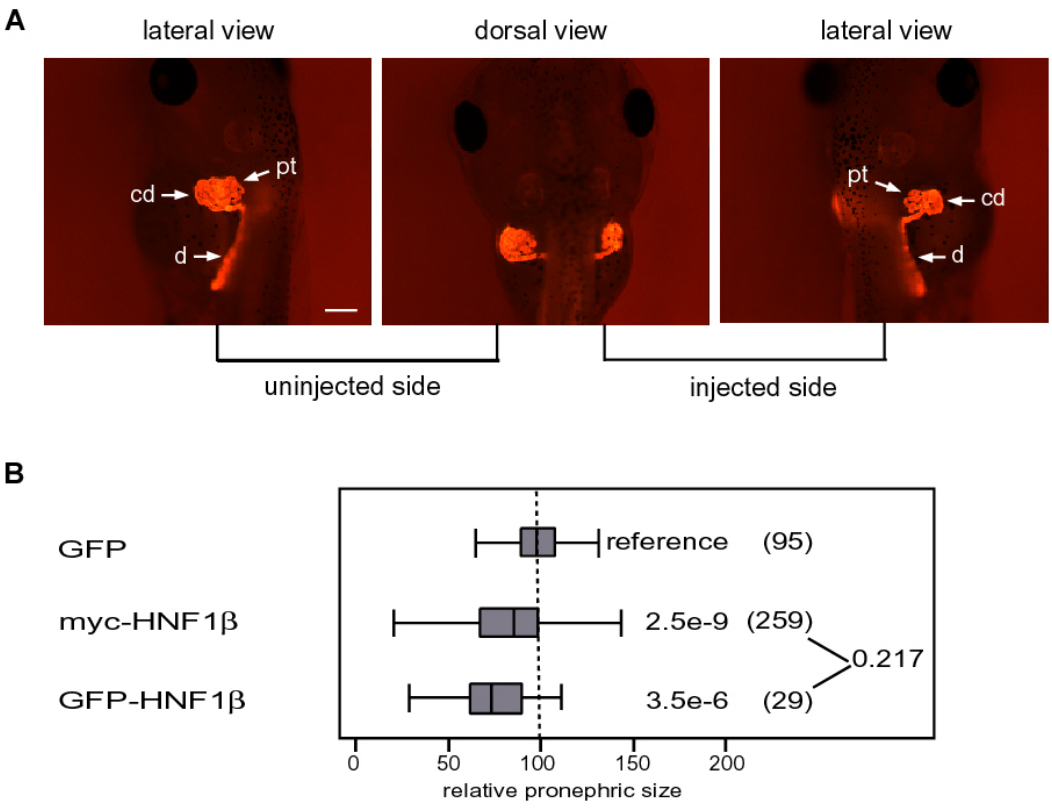

Figure 5. Expression of the HNF1β protein in *Xenopus* reduces kidney size. (A) Dorsal and lateral view of a larva (stage 44-45) expressing the HNF1β protein. Whole-mount immunostaining for the pronephric tubules and duct using a Cy3-conjugated secondary antibody is shown as red fluorescence. The injected side is indicated. Pronephric tubules: pt, pronephric duct: d, coiled duct: cd, bar = 300µm. **(B).** Box-and-whisker plots representing pronephric size in injected versus non-injected sides after expression of HNF1β proteins. Boxes include 75% of the values, the vertical line represents the group median and whiskers represent the outer quartile. The P-values comparing each group to the reference group are shown at the immediate right, and the animal number per group is shown in parenthesis. The P-value comparing Myc-HNF1β to GFP-HNF1β is shown at far right. The reference group was GFP-injected control animals.

To monitor the morphogenetic potential of the fusion construct, the injected embryos with strong GFP fluorescence were raised to free swimming tadpoles (stage 45), and immunostained to visualize the pronephros. Monoclonal antibodies for both the pronephric tubules and duct were used so that the entire pronephros was stained (Vize et al., 1995). Example of dorsal and lateral views of such larvae are shown in Figure 4. The size of the pronephros was measured from the lateral view (Figure 5A). The phenotypic effects of overexpression were analyzed by comparing the injected to the uninjected sides of the stage 44-45 embryo (Figure 5B). Pronephric development was analyzed only in otherwise phenotypically normal embryos. The expression of HNF1β protein was compared with GFP control-injected embryos. The Mann-Whitney test was used to compare the measurements obtained from each group of embryos, and differences were considered

significant when $P < 0.01$ (Figure 5B). As has been previously shown for myc-HNF1β (Wild et al., 2000; Bohn et al., 2003), the expression of the GFP-HNF1β fusion protein led to a significant reduction in the size of the pronephros (Figure 5B). There was no significant difference between the effect of GFP-HNF1β compared to myc-HNF1β ($P = 0.217$), indicating that GFP does not influence the function of HNF1β in GFP-HNF1β fusion protein. Taken together, these data show that the GFP-HNF1β fusion protein is functionally equivalent to the myc-HNF1β protein in the cellular as well as whole embryo context.

2. The specificity of HNFβ on pronephric development

Three human proteins were tested for their functional roles in kidney development in *Xenopus* embryos. One is the protein product of a gene associated with Oral Facial Digital Syndrome Type 1 (OFD1). OFD1 syndrome is an X-linked dominant condition which is lethal in the male, and is characterized by malformations of the face, oral cavity, and digits (Wettke-Schafer and Kantner, 1983; Donnai et al., 1987). The OFD1 gene, located on Xp22, has been shown to be mutated in a limited set of OFD1 patients (Feather et al., 1997). The OFD1 protein is expressed in the metanephric mesenchyme in human embryos, and plays a role in the differentiation of metanephric precursor cells (Romio et al., 2003). Consistent with this, polycystic kidney disease is commonly associated with OFD1 (Connacher et al., 1987; Donnai et al., 1987; Scolari et al., 1997). The OFD1312delG mutation, a single G deletion at nucleotide 312, was identified in an OFD1 syndrome family (Ferrante et al., 2001). The expression of this human mutation in *Xenopus* embryos had no significant effect on pronephric development (Figure 6). Osteopontin (OPN) is a multifunctional protein highly expressed in bone, and expressed to a lesser extent in various cell types including macrophages, endothelial cells, smooth muscle cells and epithelial cells (O'Brien et al., 1994; Malyankar et al., 1997). In rat, OPN is expressed both in the developing nephron and ureteric bud, and regulates kidney morphorgenesis *in vitro* (Rogers et al., 1997; Denda et al., 1998). The overexpression of OPN in *Xenopus* embryos did not have an effect on phenotypic size (Figure 6). Neither expression of OPN nor OFD1 interfered with kidney development in *Xenopus* embryos. These results indicate that the

function of the HNF1β protein is specific in the nephrogenesis and also conserved for all vertebrates.

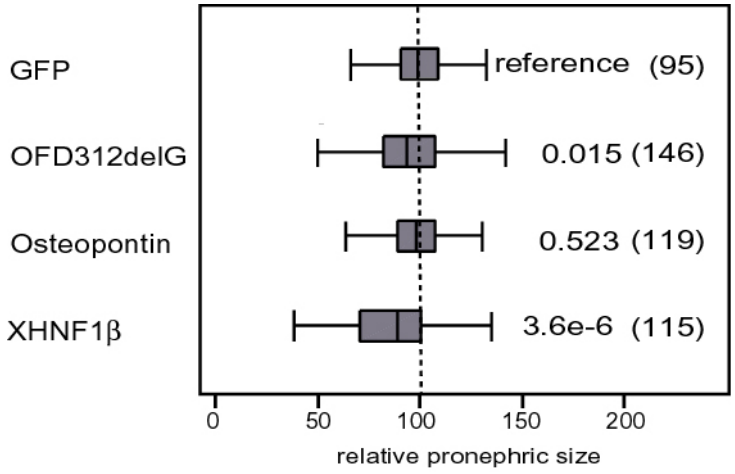

Figure 6. Expression of other human proteins known to hinder mammalian nephrogenesis in *Xenopus* does not reduce pronephric size. Box-and-whisker plots are shown comparing each group to the reference group. Boxes include 75% of the values, the vertical line represents the group median and whiskers represent the outer quartile. The P-values comparing each group to the reference group are shown at the right, and the animal number per group is shown in parenthesis. Note that only the XHNF1β-injected group was significantly different than the reference group. The reference group was GFP-injected control animals.

The expression of human HNF1β in *Xenopus* embryos led to agenesis of the pronephros (Wild et al., 2000; Bohn et al., 2003). In order to test whether human HNF1β is functionally equivalent to *Xenopus* HNF1β (XHNF1β) in this aspect, mRNA encoding the XHNF1β was coinjected with GFP mRNA (Vignali et al., 2000). Overexpression of XHNF1β in *Xenopus* embryos also led to a significant reduction of pronephric size (Figure 6). This result supports that the function of HNF1β in nephrogenesis is conserved from *Xenopus* to human.

3. Investigation of HNF1β domain functions using HeLa cells and *Xenopus* embryos

3.1 Construction of chimeric proteins for domain function experiments

It has been previously shown that the HNF1α protein plays no role in nephrogenesis (Wild et al., 2000). Based on this fact, I chose to use the HNF1α protein as an inactive backbone to construct chimeric proteins containing domains from HNF1β. In order to confirm that expression of the truncated HNF1α protein (HNF1aaa) has no effect on kidney development, HNF1aaa mRNA was coinjected with GFP mRNA into one blastomere at the 2-cell stage embryos. Injected embryos were raised to free swimming tadpoles (stage 44-45) and processed to visualize the pronephros using a mixture of monoclonal antibodies against proteins specific for the pronephric tubules and duct. Only embryos that were otherwise phenotypically normal were scored for effects on pronephric development. As expected, expression of the truncated HNF1α protein had no effect on kidney development (Figure 10). These data confirm that the truncated HNF1α protein could not interfere with kidney development as has been shown for the full-length HNF1α protein (Wild et al., 2000). To assure that the injected mRNAs were translated in the embryo, total protein was extracted from embryos exhibiting strong GFP fluorescence at the late gastrula stage. A western blot using the Myc antibody was performed to visualize the non-embryonic proteins. All constructs tested (HNF1aaa, HNF1bbb, HNF1aab and HNF1aabins26) were translated into proteins, as they could be detected using the Myc antibody (Figure 7). These constructs and their implication on HNF1β function will be discussed more fully below.

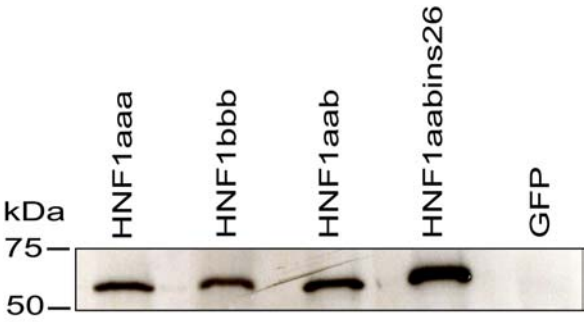

Figure 7. HNF1 chimeric protein expression in *Xenopus* embryos. Western blot analysis of total protein extracts form injected late gastrula stage embryos probed with the Myc antibody. The total protein from one embryo was separated in each lane. The 50 and 75 kDa molecular weight standards are indicated.

3.2 The conserved 26 aa segment of HNF1β plays a role in gene transactivation.

A 26 aa segment is present in the HNF1β-A splice variant of HNF1β, but deleted in the HNF1β-B splice variant. This segment exists in both the *Xenopus* and human HNF1β proteins, and is 88.5% identical at the protein level (Figure 1). The significance of this segment in kidney development was explored next. Two constructs lacking this 26 aa region were created (Figure 8A). The HNF1βD construct represents the HNF1β-B splice variant, as the 26 aa segment is deleted in the full-length HNF1β protein. The 26 aa segment was deleted from a truncated HNF1β protein (HNF1bbb) retaining the dimerization domain and the regions involved in DNA binding in the HNF1bbbD construct. The truncated protein (HNF1bbb) corresponds to the human Y352insA HNF1β mutation, and was shown in previous experiments to be sufficient to induce agenesis of the pronephros in *Xenopus* embryos (Bohn et al., 2003). A truncated HNF1α protein (HNF1aaa) lacking the transactivation domain was also generated. Additionally, the 26 aa segment was inserted between the POU_S and POU_H domains of truncated HNF1α (HNF1aaains26 construct, Figure 8A). The 26 aa segment is normally not present in the HNF1α protein.

The subcellular localization of these constructs was first assayed in transfected HeLa cells. Previous experiments have shown that HNF1α is localized primarily in the nucleus but to a lesser extent in the cytoplasm (Thomas et al., 2002). Localization of HNF1β is, however, exclusively nuclear (Bohn et al., 2003). To define the subcellular distribution of these various proteins, GFP-fusions of these constructs were expressed in HeLa cells. All HNF1β-derived constructs (HNF1β, HNF1βD, HNF1bbb and HNF1bbbD) were exclusively in the nucleus (Figure 8B). In contrast, the HNF1aaa and HNF1aaains26 constructs were present in both the nucleus and cytoplasm (Figure 8B). These expression patterns correlate well with previous published observations.

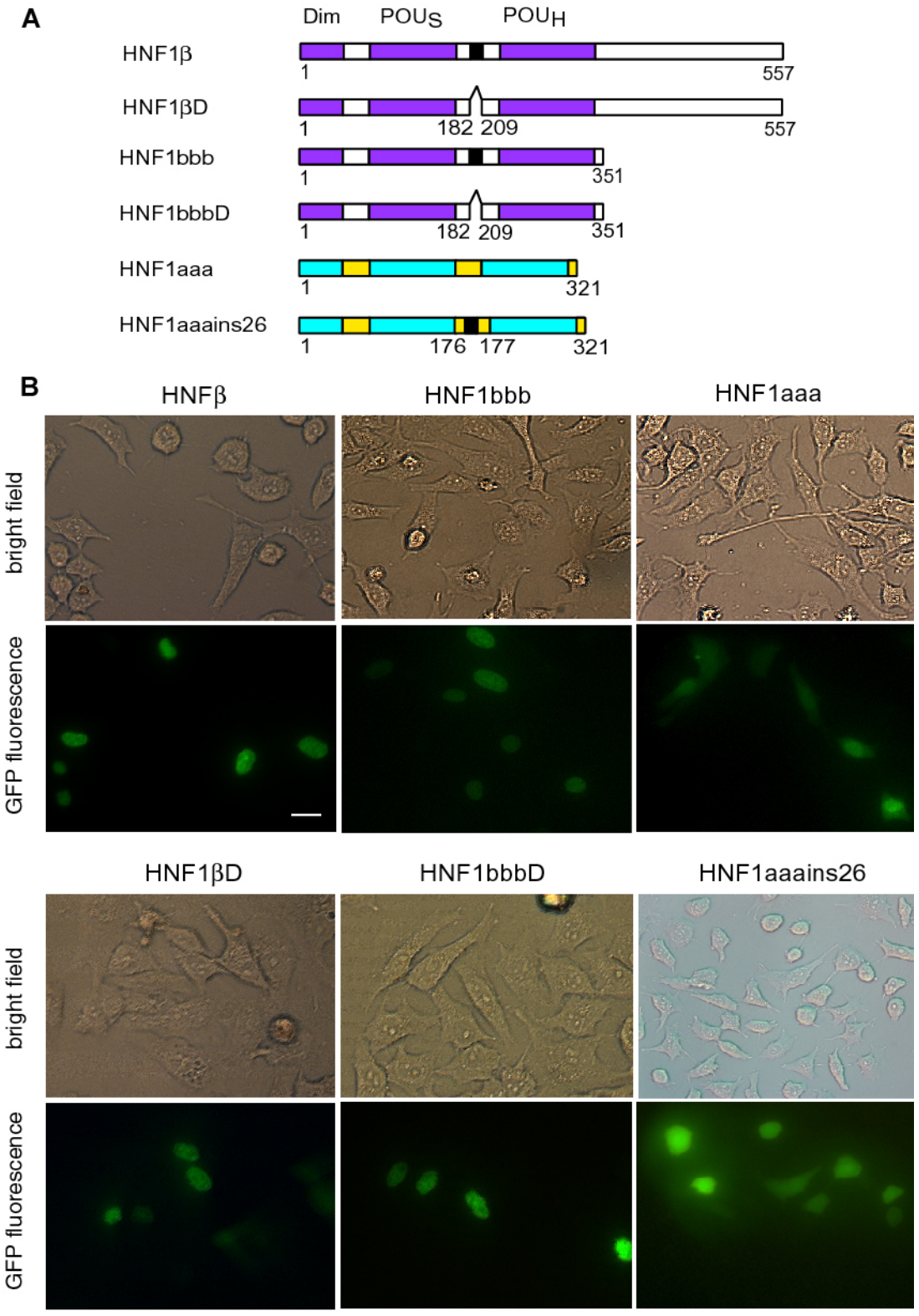

Figure 8. The 26 aa segment of the HNF1β protein is not responsible for nuclear localization. (A) The domains included in these HNF1 constructs are shown diagrammatically. HNF1β is shown in purple and HNF1α in blue. The black box indicates the 26 aa segment deleted from the HNF1β splice variant B.**(B)** Subcellular localization of the HNF1 constructs shown in A. Transiently transfected HeLa cells are shown under bright field and green fluorescence. bar = 10μm.

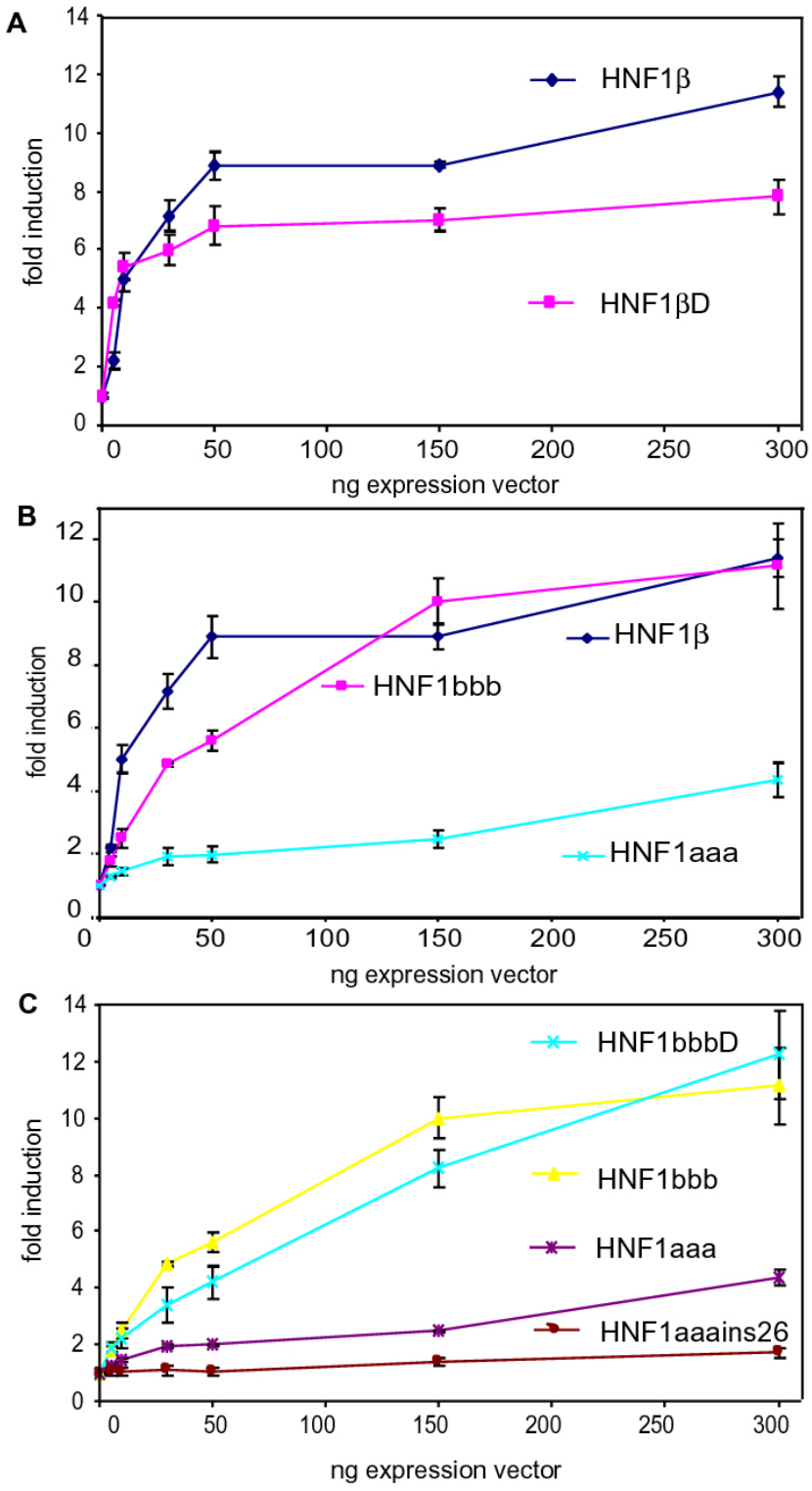

Figure 9. Transactivation potential of HNF1 constructs with deletion or insertion of the 26 aa segment. (A-C) Increasing amounts (5, 10, 30, 50, 150, 300ng) of the GFP-HNF1 expression construct indicated were cotransfected into HeLa cells together with an HNF1-dependent luciferase reporter plasmid. The fold-activation induced by each of the HNF1 expression constructs is shown. Error bars indicate standard deviation of the mean of at least six replicates.

The transactivation potential of the HNF1 domain function constructs were investigated. Expression vectors encoding these proteins were cotransfected into HeLa cells lacking endogenous HNF1 proteins together with a luciferase reporter plasmid containing an HNF1 inducible promoter. Deletion of the 26 aa segment reduced the transactivation potential approximately 30% compared to the full-length HNF1β transcription factor (Figure 9A). As previously observed (Bohn et al., 2003), the truncated HNF1β protein (HNF1bbb) lacking the transactivation domain retained substantial transactivation potential (11-fold, compare HNF1bbb with HNF1β in Figure 9B). Typically, HNF1bbb was less active at lower concentrations (10-50ng), but was as active as the full-length protein when 3-6 times the plasmid amount was transfected. The truncated HNF1β protein lacking the 26 aa segment (HNF1bbbD) transactivated the reporter gene to a similar level (12-fold) as the truncated HNF1β protein (HNF1bbb, Figure 9C). The truncated HNF1α protein (HNF1aaa) transactivated the reporter plasmid at a low level (4.5-fold) when high plasmid concentrations were transfected (300ng, Figure 9B). As the known transactivation domain is missing from the HNF1α truncated protein, this should be considered as residual activity. As expected, the full-length HNF1α protein transactivated the reporter gene at a high level (18-fold for 300ng, data not shown). These date are consistent with the initial description of the HNF1α transcription factor localizing the activation domain to the C-terminus (Nicosia et al., 1990; Sourdive et al., 1993). Insertion of the β-specific 26 aa segment into the truncated HNF1α protein abolished residual transactivation (1.7-fold) (Figure 9C). This indicates that the 26 aa segment plays distinct roles depending on the context of the rest of the protein.

3.3 The conserved 26 aa segment of HNF1β interferes with pronephric development in *Xenopus* embryos.

The morphogenetic potential of the various HNF1 constructs were examined in the developing *Xenopus* embryos by injecting mRNA encoding these proteins into one blastomere of the two-cell stage embryo. As initial experiments revealed that the GFP-HNF1β fusion protein fluorescenced too weakly for the identification of the injected side (Figure 4), GFP mRNA was coinjected with mRNA for the myc-tagged versions of the constructs (Figure 5). Injected embryos were raised to free swimming tadpoles (stage 44-

45) and processed to visualize the pronephros using a mixture of monoclonal antibodies against proteins specific for the pronephric tubules and duct. Only embryos that were otherwise phenotypically normal were scored for effects on pronephric development. Examples of dorsal views of such larvae are given in Figure 10A. The pronephric size was measured in the lateral view of the larvae, and the quantification of these phenotypic changes together with the statistical analysis for significance are summarized in Figure 10B.

As found previously (Wild et al., 2000; Bohn et al., 2003), expression of full-length HNF1β led to a significant reduction of pronephric size (Figure 10), and this effect was even more pronounced for the HNF1β truncated protein (HNF1bbb, Figure 10). Surprisingly, the full-length HNF1β protein lacking only the 26 aa segment (HNF1βD) had no effect on pronephric size (Figure 10). As expected, expression of neither the full-length nor the truncated HNF1α protein (HNF1aaa) had any effect on pronephric development (Figure 10, (Wild et al., 2000) The insertion of the 26 aa segment into the truncated HNF1α protein (HNF1aaains26) led to a reduction of size of the pronephros (Figure 10), implying a crucial role for this segment in nephrogenesis. Expression of the truncated HNF1β-protein (HNF1bbbD) lacking this 26 aa segment continued to produce pronephric agenesis (Figure 10). However, a dramatic gastrulation defect at the injected side was observed when this protein was expressed (Figure 11A and B), and more than 90% of the embryos in this group died during gastrulation. Even if the amount of HNF1bbbD mRNA injected was halved, 70% of the embryos died during gastrulation. The majority of the surviving tadpoles were distorted compared to control animals (Figure 11D-F, C). Therefore, a relatively small number (36) of healthy larvae were available for immunostaining and the examination of pronephros-specific effects. Nevertheless, this group size was sufficient for significance analysis. This abnormal development was not observed with any of the other constructs.

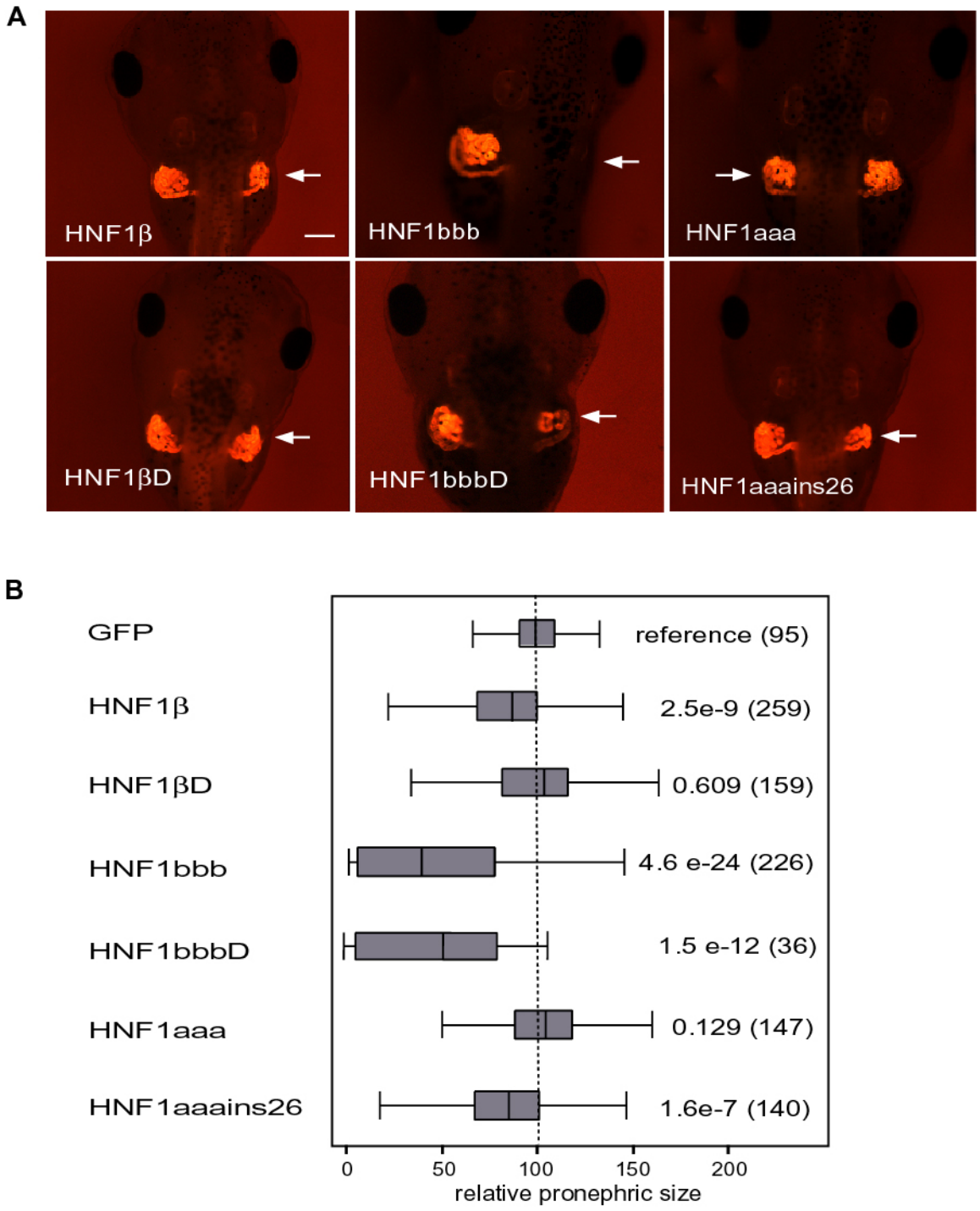

Figure 10. The HNF1β-specific 26 aa segment plays a role in nephrogenesis. (A) Dorsal view of larvae (stage 44-45) expressing the HNF1 chimeric proteins shown in Fig. 8. Whole-mount immunostaining for the pronephric tubules and duct using a Cy3-conjugated secondary antibody is shown as red fluorescence. The injected side is marked by an arrow. bar = 300µm. **(B)** Box-and-whisker plots representing pronephric size in injected versus non-injected sides after expression of HNF1 proteins. Boxes include 75% of the values, the vertical line represents the group median and whiskers represent the outer quartile. The P-values comparing each group to the reference group are shown at the right, and the animal number per group is shown in parenthesis. The reference group was GFP-injected control animals.

Figure 11. *Xenopus* embryos expressing truncated HNF1β lacking the 26 aa segment. Example of gastrulation defects observed in 70% of embryos under **(A)** bright field or **(B)** green fluorescence. Note cell death was only observed in the injected (GFP positive) region. bar = 300µm. **(C)** Stage 44-45 control embryo injected with GFP alone. bar = 1mm. **(D-F)** Developmental defects observed in tadpoles expressing the truncated HNF1β protein lacking the 26 aa segment (HNF1bbbD). Animals shown in panel D and E were not scored for pronephric morphology.

3.4 Function of the dimerization domain of HNF1β

As expression of the truncated HNF1β protein lacking the 26 aa segment (HNF1bbbD) also resulted in a smaller pronephros (Figure 10), protein areas other than the 26 aa segment appear to interfere with nephrogenesis. To explore the function of the dimerization domain of the HNF1β protein, chimeras of the HNF1α and HNF1β proteins were constructed by exchanging the dimerization domains between HNF1α and HNF1β as shown in Figure 12A. The molecular and cellular properties of these chimeric constructs were assayed in transfected cells as well as in developing *Xenopus* embryos.

Transfection of the chimeric HNF1 constructs together with an HNF1-dependent luciferase reporter plasmid was used to measure transactivation activity in HeLa cells. Only the construct encoding the POU_S and POU_H domains of the HNF1β protein (HNF1abb) resulted in transactivation of the reporter gene similar to that mediated by the truncated HNF1β protein (HNF1bbb, Figure 12C). The presence of the HNF1β dimerization domain in the chimeric protein (HNF1baa) was not sufficient for substantial transactivation of the reporter.

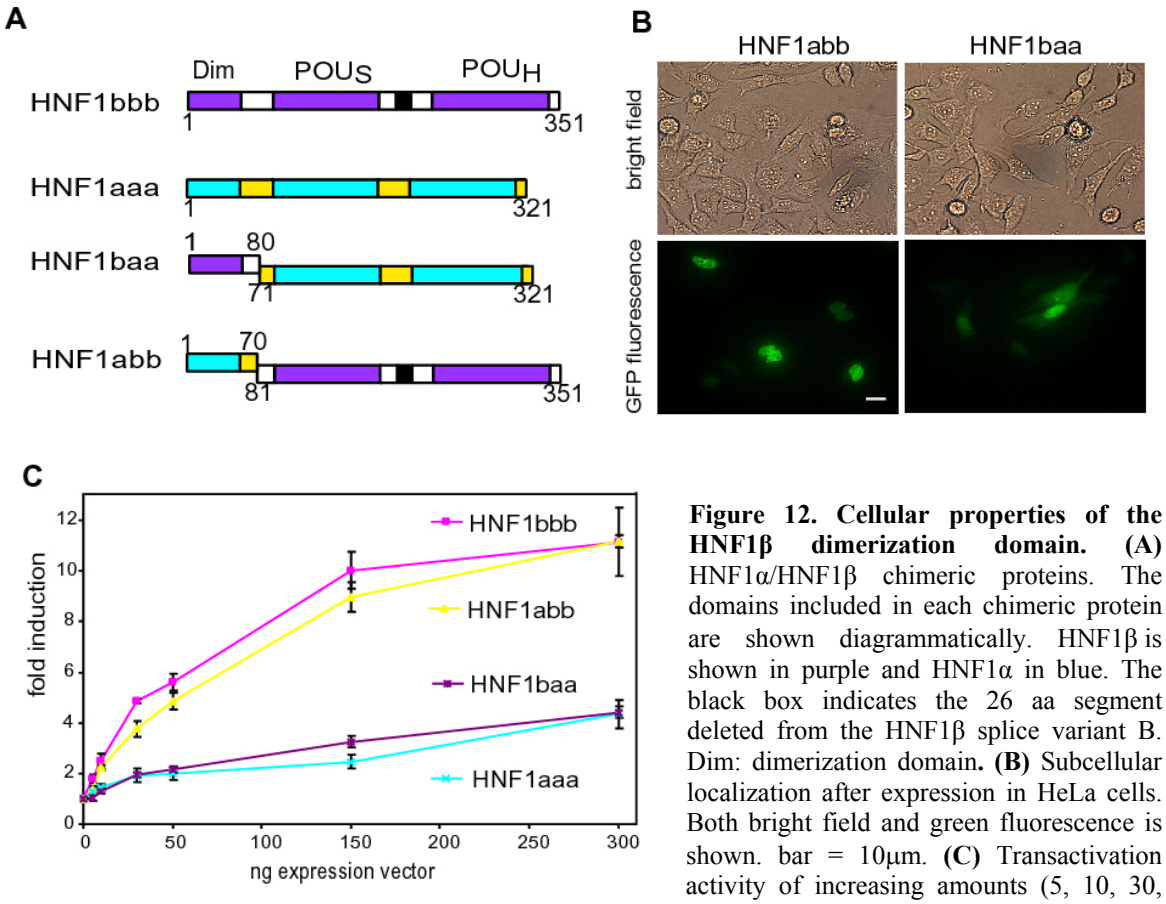

Figure 12. Cellular properties of the HNF1β dimerization domain. (A) HNF1α/HNF1β chimeric proteins. The domains included in each chimeric protein are shown diagrammatically. HNF1β is shown in purple and HNF1α in blue. The black box indicates the 26 aa segment deleted from the HNF1β splice variant B. Dim: dimerization domain**. (B)** Subcellular localization after expression in HeLa cells. Both bright field and green fluorescence is shown. bar = 10µm. **(C)** Transactivation activity of increasing amounts (5, 10, 30, 50, 150, 300ng) of HNF1 chimeric expression constructs that were cotransfected into HeLa cells with an HNF1-dependent luciferase reporter plasmid. The fold-activation induced by each of the HNF1 chimeric expression constructs is shown. Error bars indicate standard deviation of the mean of at least six replicates.

The influence of the chimeric HNF1 constructs on kidney development was tested by expression experiments in *Xenopus* embryos. Expression of either the HNF1β dimerization domain (HNF1baa) in the truncated HNF1α protein or the HNF1β DNA binding domains fused to the HNF1α dimerization domain (HNF1abb) led to a reduction in pronephric size (Figure 13). This indicates that the dimerization domain as well as the DNA binding domain of HNF1β interfere with pronephric development. However, expression of the fusion protein containing the HNF1β dimerization domain was less efficient at reducing pronephric size than expression of the protein containing the HNF1β POU_S and POU_H domains (Figure 13), implying that the HNF1β dimerization domain contributes to nephrogenesis, but not to the same extent as the DNA binding domains.

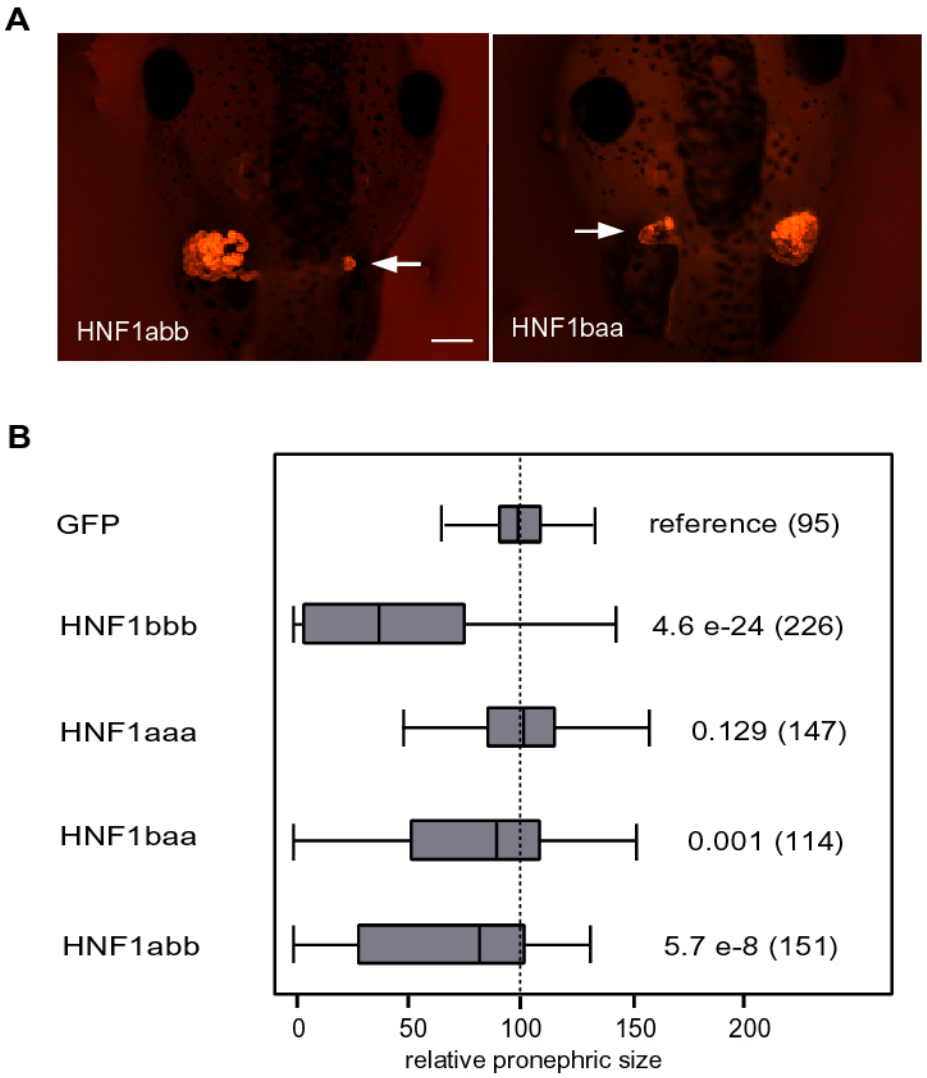

Figure 13. Functional analysis of the HNF1β dimerization domain in nephrogenesis. (A) Pronephric phenotype in *Xenopus* larvae expressing HNF1 chimeric proteins. Dorsal view of larvae (stage 44-45) expressing the HNF1 chimeric proteins shown in Fig 12. Whole-mount immunostaining for the pronephric tubules and duct using a Cy3-conjugated secondary antibody is shown as red fluorescence. The injected side is marked by an arrow. bar = 300µm. **(B)** Box-and-whisker plots representing pronephric size in injected versus non-injected sides. Boxes include 75% of the values, the vertical line represents the group median and whiskers represent the outer quartile. The P-values comparing each group to the reference group are shown at the right, and the animal number per group is shown in parenthesis. The reference group was GFP-injected control animals.

3.5 The homeodomain of HNF1β is essential for nuclear localization and interferes with pronephric development.

To explore the function of the HNF1β homeodomain (POU_H) in more detail, chimeric constructs were created containing various parts of the HNF1β homeodomain region in a

truncated HNF1α protein (HNF1aaa). The chimeric gene constructs generated are shown diagrammatically in Figure 14A. The molecular and cellular properties of these chimeric constructs were assayed in transfected HeLa cells as well as in developing *Xenopus* embryos.

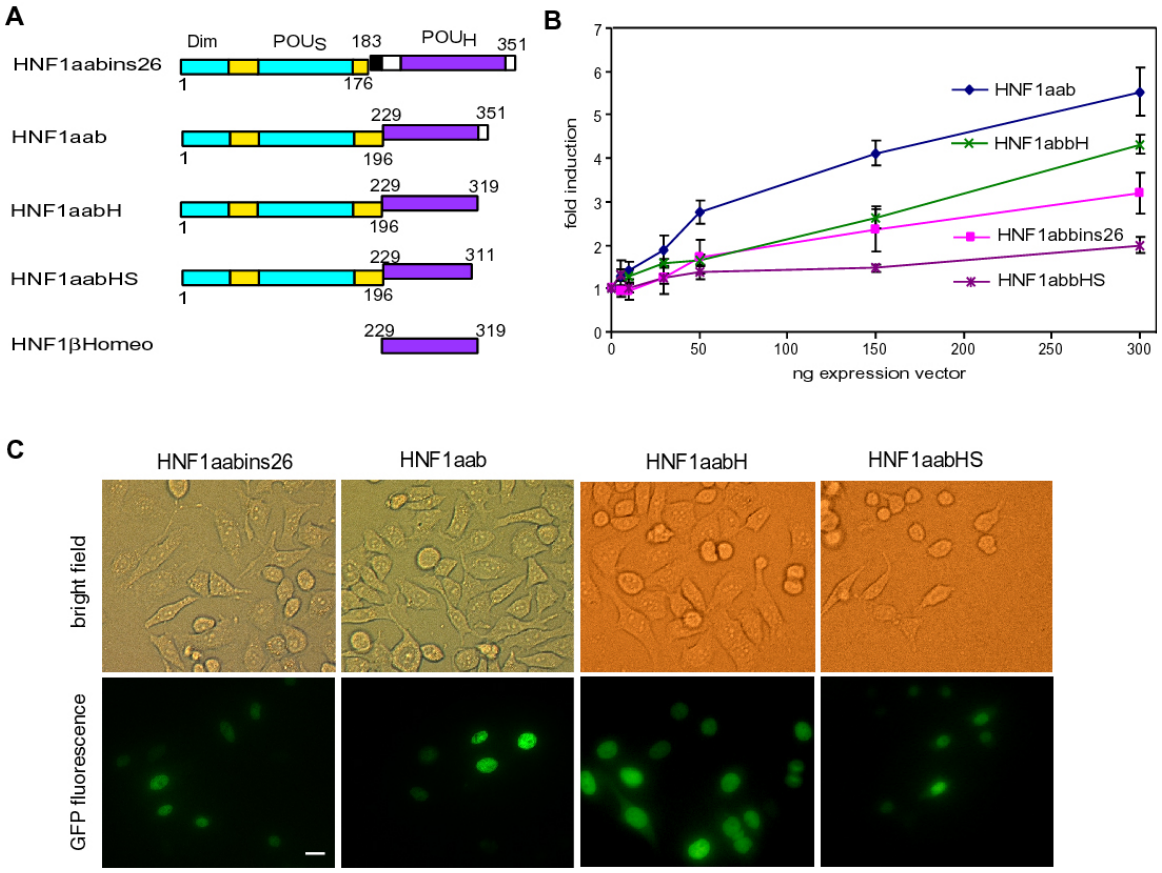

Figure 14. Cellular properties of HNF1β POU_H domain. (A) The domains included in the HNF1 chimeric constructs are shown diagrammatically. HNF1β is shown in purple and HNF1α in blue. The black box indicates the 26 aa segment deleted from the HNF1β splice variant B. **(B)** Transactivation activity of POU_H domain. Increasing amounts (5, 10, 30, 50, 150, 300ng) of these expression constructs were cotransfected into HeLa cells together with an HNF1-dependent luciferase reporter gene. The fold-activation induced by each expression construct is shown. Error bars indicate standard deviation of the mean of at least six replicates. **(C)** Subcellular localization of the chimeric proteins. Bright field and fluorescence microscopy of HeLa cells expressing GFP fusion proteins are shown. bar = 10µm

All chimeric constructs (HNF1aabins26, HNF1abb, HNF1aabH, HNF1aabHS) containing the HNF1β homeodomain were found exclusively in the nuclear compartment, implying that this domain contributes to the nuclear localization (Figure 14C). The truncated HNF1 protein (HNF1aaa) was localized both in the cytoplasm and the nucleus (Figure 8B). All chimeric constructs containing the HNF1β homeodomain were less active at transactivating the HNF1-dependent reporter gene than the truncated HNF1β protein in transfection assays

(Figure 14B). The construct containing the HNF1β POU_H domain (HNF1aab) was more active (5.5-fold) than the truncated HNF1α protein (HNF1aaa). The chimeric construct lacking the C-terminal transactivation region (aa 320-351, HNF1aabH) transactivated the reporter similar to the truncated HNF1α protein (apprx. 4-fold). The chimeric protein containing the β-specific 26 aa segment (HNF1aabins26) transactivated the reporter only 3-fold (Figure 14B). Crystalographic experiments have shown previously that the DNA binding domains of HNF1α lacking the C-terminal 8 aa of the POU_H domain could still form a complex with the high-affinity promoter DNA (Chi et al., 2002). The deletion of the corresponding amino acids in the HNF1β protein (aa 311-319, HNF1aabHS) reduced transactivation activity the most (Figure 14B), resulting in only a 2-fold activation. These data support a role for both the POU_H and POU_S domains in transactivation of HNF1β target genes.

To identify whether the homeodomain influences kidney development in *Xenopus* embryos, mRNA for chimeric constructs were injected into one cell at the 2-cell stage, and the pronephric size was measured at stage 44-45 (Figure 15). Expression of truncated HNF1α proteins containing the entire HNF1β POU_H domain (HNF1aab, HNF1aabins26 and HNF1aabH) led to a reduction of pronephric size (Figure 15). However, the HNF1β homeodomain alone (HNF1βHomeo) had no effect on pronephric size (Figure 15). Expression of the HNF1α chimera containing the HNF1β POU_H domain lacking the C-terminal 8 aa (HNF1aabHS) also had no effect on pronephric size. These results indicate that the entire HNF1β POU_H domain can interfere with kidney development, but only in the context of the HNF1 backbone. Expression of two of the truncated HNF1 chimeric constructs containing the entire HNF1β POU_H domain but lacking the 26 aa segment (HNF1aab and HNF1aabH) also resulted in gastrulation defects, as was observed for the truncated HNF1β construct lacking the 26 aa segment (HNF1bbbD, Figure 11D-F). In fact, approximately 60% of the injected animals (45/113 and 42/104, respectively) showed developmental defects not concerning the kidney, allowing only a minority to be analyzed at stage 44-45. However, this group was adequate for statistical analysis. Expression of the chimeric protein lacking the C-terminal 8 aa of POU_H domain (HNF1aabHS) resulted in no developmental abnormalities. Taken together, these data indicate that the entire homeodomain of HNF1β is required to interfere with renal development.

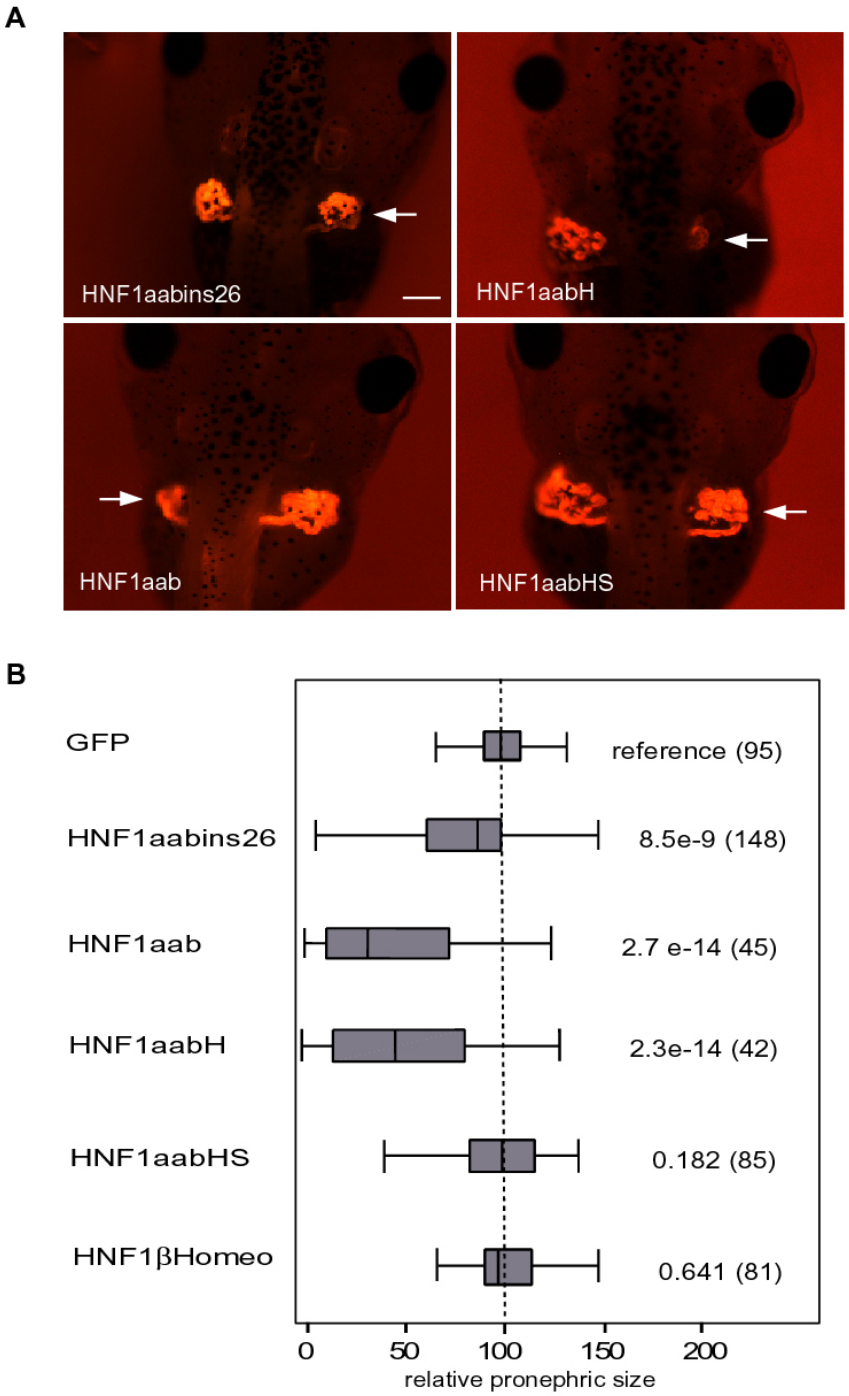

Figure 15. Functional analysis of the HNF1β POU_H domain in nephrogenesis. (A) Pronephric phenotype in *Xenopus*. Dorsal view of larvae (stage 44-45) expressing the HNF1 chimeric proteins shown diagrammatically in Fig. 14. Whole-mount immunostaining for the pronephric tubules and duct using a Cy3-conjugated secondary antibody is shown as red fluorescence. The injected side is marked by an arrow. bar = 300µm. **(B)** Box-and-whisker plots representing pronephric size in injected versus non-injected sides after expression of HNF1 proteins. Boxes include 75% of the values, the vertical line represents the group median and whiskers represent the outer quartile. The P-values comparing each group to the reference group are shown at the right, and the number of animals per group is shown in parenthesis. The reference group was GFP-injected control animals.

4. HNF1β partially rescues the Pax8/lim1-mediated nephrogenic phenotype.

It has been reported that overexpression of the transcription factors, Pax8 and lim1, in *Xenopus* embryos led to the development of an abnormally large pronephros as well as the formation of ectopic pronephric tubules (Carroll and Vize, 1999). Both of these transcription factors are expressed at the neurula stage together with HNF1β in the pronephric anlage. Since the overexpression of HNF1β led to agenesis of the pronephros, it is possible that a simple antagonistic relationship exists between HNF1β and Pax8/lim1. In this case, it would be expected that coexpressing HNF1β would rescue the Pax8/lim1-induced nephrogenic phenotype.

Overexpression of Pax8 or lim1 by themselves caused a mild nephrogenic phenotype that was difficult to quantify, but synergized to have a pronounced effect. At the 2-cell stage, mRNA for Pax8 and lim1 were coinjected into one blastomere together with GFP mRNA. Injected embryos were raised to the swimming tadpole stage (stage 44-45), and processed to visualize the pronephric tubules and duct. Overexpression of Pax8 together with lim1 led to an enlargement of the pronephros as compared to embryos injected with GFP alone (Figure 16). This size difference was shown to be significant using the Mann-Whitney test (Figure 16G). Ectopic pronephric tubules and cyst-like structures close to the main body of the pronephros were also observed in 16% of this group on the injected sides (Figure 16B, Table 2). Such structures were never observed in animals only expressing GFP or expressing any other HNF1 chimeric protein. Furthermore, the 24% of the larvae coexpressing Pax8 and lim1 displayed a thickening of the tubules on the injected side (Figure 16A). Such abnormalities were only observed in 4% of the larvae expressing truncated HNF1β (HNF1bbb, Table 2). These results are similar to those reported previously using a slightly different injection protocol (Carroll and Vize, 1999). The pronephri of embryos coexpressing Pax8, lim1 and HNF1β were similar to embryos expressing only Pax8 and lim1 (Figure 16C and D). The pronephros appeared smaller in some embryos also expressing HNF1β, but the size difference was not significant when compared with embryos injected with Pax8 and lim1 alone (Figure 16G). Furthermore, as in the group expressing only Pax8 and lim1, 17% of the group also expressing HNF1β were

found to have ectopic tubules and 27% exhibited cyst-like structures or thickened tubules (Table 2).

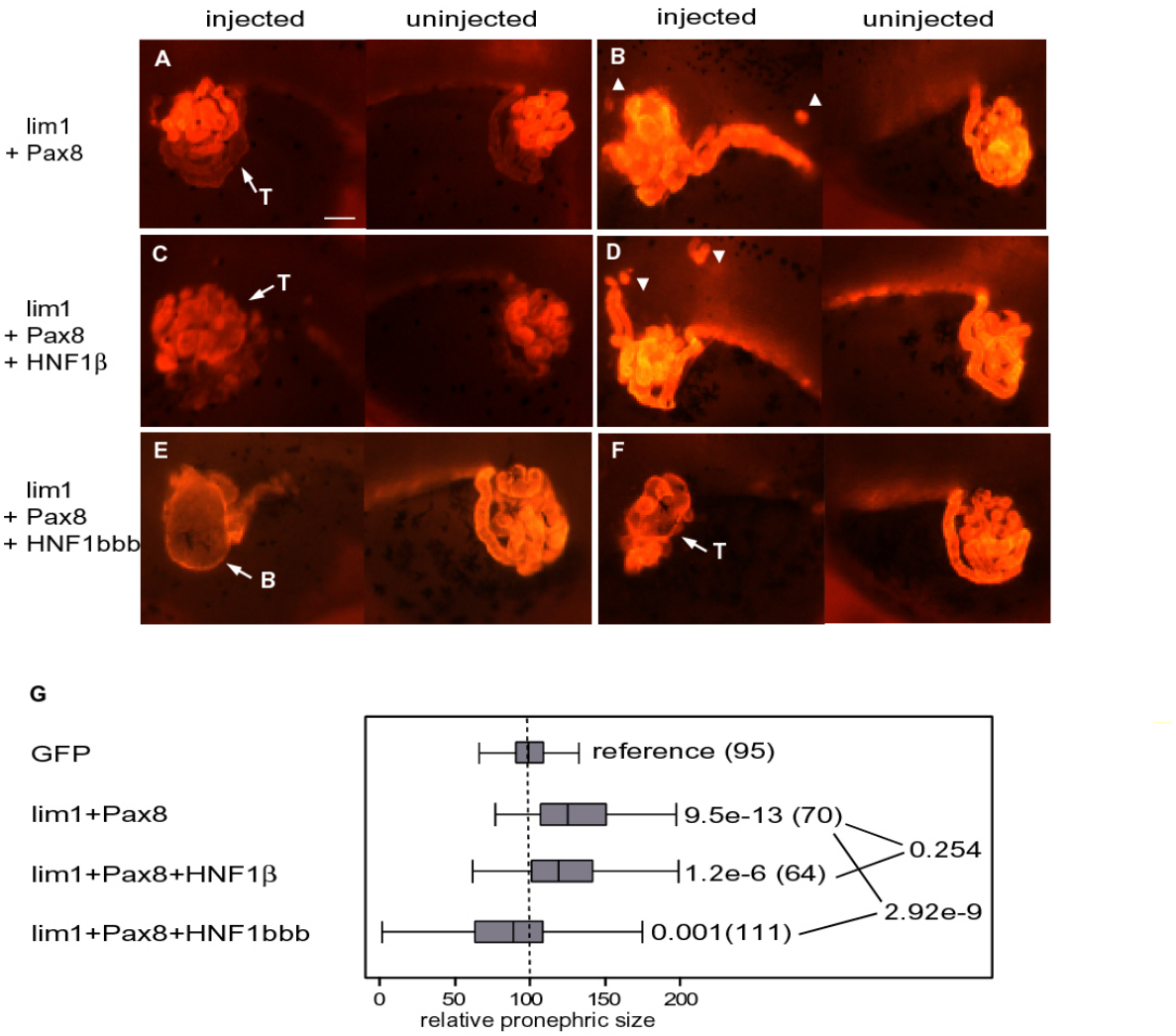

Figure 16: Partial rescue of Pax8/lim1-induced kidney malformation by HNF1β. (A-F) Lateral views of two representative larvae expressing the proteins listed at the left on one side. Larvae are immunostained to visualize the pronephric tubules and duct. **(A, B)** Enlarged pronephri in Pax8/lim (125 pg mRNA each/embryo) coinjected embryos. **(C, D)** Enlarged pronephri in embryos coinjected with Pax8 (125 pg mRNA/embryo), lim1 (125 pg mRNA/embryo), and HNF1β (250 pg mRNA/embryo). **(E, F)** Reduced pronephric size in embryos coinjected with Pax8 (125 pg mRNA/embryo), lim1 (125 pg mRNA/embryo) and truncated HNF1β (HNF1bbb, 250 pg mRNA/embryo). Anterior is to the left for the injected sides, to the right for the non-injected sides, and dorsal is up. Thickened tubules (T) and cyst-like structures or bubbles (B) are indicated by arrows. Ectopic pronephric tubules are indicated by arrow heads. bar = 200µm. **(G)** Statistical analysis of pronephric size in injected versus non-injected sides after expression of the proteins indicated at the left. Boxes include 75% of the values, the vertical line represents the group median and whiskers represent the outer quartile. The P-value comparing each group to the reference group is shown at the immediate right, and the animal number per group is shown in parenthesis. The P-values comparing the Pax8/lim1 expressing group to either the group coexpressing full-length or truncated HNF1β are shown at the far right. The reference group was GFP-injected control animals.

Table 2. Frequency of enlarged or ectopic pronephric tubules in *Xenopus* embryos

Proteins Expressed	Pronephric Tubules (%)				Cyst-like structures or thickened tubules (%)	N
	Enlarged[a]	Normal[b]	Smaller[c]	Ectopic		
Pax8 + lim1	49	34	1	16	24	83
Pax8 + lim1+ HNF1β	40	38	5	17	27	77
Pax8 + lim1 + HNF1bbb	20	38	42	0	28	111
HNF1bbb	5	17	78	0	4	226

[a] relative pronephric size of the injected side/uninjected side > 120%

[b] relative pronephric size of the injected side/uninjected side between 80%-120%

[c] relative pronephric size of the injected side/uninjected side < 80%

These data imply that the overexpression of Pax8 and lim1 is dominant to the effect of HNF1β. It was not possible to injected higher concentrations of HNF1β mRNA, otherwise the RNA injection may produce nonspecific defects in injected *Xenopus* embryos. Since truncated HNF1β (HNF1bbb) was more active in injected embryos for the reduction of the pronephric size (Figure 10), this construct was coinjected with Pax8/lim1. These embryos had slightly smaller pronephroi in the injected side (Figure 16F), suggesting that expression of the truncated HNF1β protein could overcome this effect mediated by Pax8 and lim1. More importantly, no larvae had ectopic tubules (Table 2). Surprisingly, 28% of the samples exhibited cyst-like structures or thickening of the tubules (Figure 16E and F, Table 2). These results suggest that HNF1β activity can overcome part of the nephrogenic potential of Pax8 and lim1. Most importantly, the data also reveal that Pax8/lim1 and HNF1β are not simple antagonists during nephrogenesis, but that Pax8/lim1 also have distinct morphogenetic properties.

IV. Discussion

1. Functional properties of the HNF1β domains

1.1 Homeodomain is responsible for nuclear localization of the HNF1β protein.

I have shown here that all chimeric proteins containing the HNF1β POU homeodomain (POU_H) were exclusively localized in the nucleus in transfected HeLa cells. This finding is consistent with previous published observations that all mutated forms of the HNF1β transcription factor lacking the POU_H domain were excluded from the nucleus (Bohn et al., 2003). A nuclear localization signal (NLS) is predicted in the N-terminal region of the POU_H domain of HNF1β (PredictNLS Online http://www.cubic.bioc.columbia.edu /predictNLS), but no potential NLS was found in the HNF1α protein. The amino acid sequence, KKMRRNR (amino acid 229 to 235, Figure 17), is the predicted NLS in the HNF1β protein. The corresponding sequence of the HNF1α protein (KKGRRNR) differs by only one amino acid (M to G). This change may hinder efficient nuclear translocation of HNF1α in transfected HeLa cells, and probably results in nuclear as well as cytoplasmic localization typical for HNF1α in transfected HeLa cells. Taken together, the KKMRRNR sequence in the homeodomain is most likely the NLS of the HNF1β protein.

1.2 The POU_S and POU_H domains are responsible for most of the transactivation activity of the HNF1β protein.

Deletion of the area of the HNF1β protein corresponding to the transactivation domain of the HNF1α protein did not abolish transactivition activity. Although the truncated HNF1β protein was slightly less effective at transactivating the reporter gene at low plasmid concentrations, the same maximal level of transactivation was reached at saturating plasmid concentrations. The N-terminal region includes the dimerization domain and two DNA binding domains, known as the POU_S and POU_H domains. I have shown here that the dimerization domain of the HNF1β protein has no transactivation activity. Replacement of

either the POU_S and POU_H domains or the POU_H domain alone in the HNF1α protein lacking the transactivation domain partially restored the transactivation potential. Even though the POU_S and POU_H domains are highly conserved between the HNF1α and HNF1β proteins, there appears to be functional differences. As a progressive increase was observed in the transactivation potential with the length of the β-protein derived segment, several features probably contribute to the transactivation potential of the POU_S and POU_H domains of HNF1β. The 26 aa segment located between the POU_S and POU_H domains of the HNF1β is evolutionarily highly conserved from *Xenopus* to human. This segment, deleted in splice variant HNF1β-B, is the most striking difference between the HNF1α and HNF1β proteins (Cereghini et al., 1992). I have shown here that the 26 aa segment contributes differently to transactivation potential based on the context of the rest of the protein. In the full-length HNF1β protein, it accentuated transactivation activity. This is consistent with previous results showing that the HNF1β splice variant B lacking this 26 aa segment was less effective at transactivating the reporter gene (Ringeisen et al., 1993; Haumaitre et al., 2003). Deletion of this segment from the truncated HNF1β protein made no difference on its transactivation potential. Finally, the insertion of this segment into the HNF1α protein lacking the transactivation domain abolished residual transactivation activity. The 26 aa segment may interact in a context-dependent manner with other transcription factors or components of the basal transcriptional machinery to cause these differences. Although the C-terminal region of the HNF1β protein is often referred to in the literature as the transactivation domain, I have shown here that primarily the POU_S and POU_H domains of the HNF1β protein carry out this function.

1.3 Domains of HNF1β involved in nephrogenesis

1.3.1 The effect of HNF1β on pronephros formation is conserved.

Although the analysis in this study was concentrated on HNF1 proteins of human origin, it is unlikely that protein functions are species specific. Both *Xenopus* and humans have homologs of the HNF1α and HNF1β proteins (Bartkowski et al., 1993; Demartis et al., 1994). As I have shown here, overexpression of either XHNF1β or hHNF1β in *Xenopus* embryos inhibited pronephric development, supporting the conserved function of HNF1β from *Xenopus* to humans. Only overexpression of HNF1β causes agenesis of the

pronephros in the *Xenopus* model system. Overexpression of HNF1α has no effect on development, even though they are closely related transcription factors (Wild et al., 2000).

Expression of two other human proteins known to be involved in mammalian kidney development had no effect on pronephric development in *Xenopus*. OFD1 is the gene responsible for OFD1 syndrome, and is commonly associated with polycystic kidney disease in humans (Connacher et al., 1987; Donnai et al., 1987; Scolari et al., 1997). The function of this protein remains unclear. In work shown here, expression of the OFD1312delG mutant in *Xenopus* had no significant effect on pronephric development. This mutant is a truncated protein only containing the predicted N-terminal lish motif (aa 68-101), but lacking the predicted coiled-coil domains and hepta repeats of this protein. (Emes and Ponting, 2001). Coiled-coil domains are involved in protein-protein interactions. The absence of critical functional areas of the protein that could be interacting with other transcription factors or proteins in transcriptional activating or repressing complexes may be the reason why this mutant did not interfere with pronephric development in *Xenopus*. Further experiments will be necessary to completely elucidate the function of OFD1 in nephrogenesis using full-length OFD1 protein and/or other mutants. Osteopontin (OPN), a multifunctional protein, is a prominent matricellular component of mineralized tissues of bones and teeth (O'Brien et al., 1994; Malyankar et al., 1997). In the rat, OPN is expressed both in the developing nephron and in the ureteric bud, and addition of neutralizing antibodies to metanephric organ cultures blocked normal metanephric tubulogenesis (Rogers et al., 1997). Importantly, OPN was induced by HNF1β in cell cultures, indicating it is a potential target gene for HNF1β (unpublished data). However, experiments presented in this thesis showed that overexpression of OPN in *Xenopus* had no effect on pronephric development. This implies that OPN may be not responsible for the effect of HNF1β on nephrogenesis in *Xenopus*. It is possible that OPN function in nephrogenesis is not conserved between mammalian and non-mammalian vertebrates. Alternatively, since this is a secreted protein, it is possible that secretion of the protein and its integration into the extracellular matrix was not efficiently carried out by the cells of the *Xenopus* embryo. Taken together, the *Xenopus* pronephros is good model for examining the nephrogenic function of human HNF1β, but not all proteins known to be involved in mammalian kidney development.

1.3.2 GFP-HNF1β fusion protein is inadequate for examining protein expression at later developmental stages.

Experiments presented here show that the GFP-HNF1β fusion protein is functionally equivalent to the myc-HNF1β fusion protein, and therefore the endogenous HNF1β protein, in a cellular as well as a whole embryo context. However, the fluorescence of the GFP-HNF1β fusion protein was inadequate for examining expression at later developmental stages in *Xenopus* embryos. Consequently, four- to five-fold more embryos had to be injected to analyze the pronephric phenotype as compared to embryos coinjected with mRNA for myc- HNF1β and GFP. The lower GFP fluorescence might be due to an altered GFP conformation caused by either the attached linker and HNF1β protein or by cofactors binding to HNF1β *in vivo*. It is reported that the number of linker amino acids is critical for the correct expression and/or fluorescence of the GFP (Tavoularis et al., 2001). In *Xenopus* embryos, the DCoH cofactor binds to HNF1β for proper *in vivo* function (Pogge v.Strandmann et al., 1997). The fluorescence of the GFP-HNF1β fusion protein was strong in transfected HeLa cells, which do not express DCoH. Alternatively, since mRNA is injected into the *Xenopus* embryo at the 2-cell stage, some of the mRNA will be degraded as development proceeds resulting in lower protein levels. Additionally, the mRNA is split up among the cells of the embryo during mitosis, also resulting in a lower concentration of mRNA per blasomere. It is likely that a combination of all of these possible explanations contribute to the low fluorescence of the GPF-HNF1β fusion protein in *Xenopus* larvae making it unsuitable for pronephric phenotype analysis.

1.3.3 Three domains in HNF1β are involved in nephrogenesis.

The *Xenopus* pronephros is a good system to define the function of proteins involved in vertebrate nephrogenesis. Overexpression of HNF1β, but not HNF1α, interfered with pronephros formation in *Xenopus* embryos. Using expression of HNF1α/HNF1β chimeric proteins, I have identified three domains of the HNF1β protein that contribute to this effect, including the dimerization domain, the 26 aa segment and the homeodomain. It is noteworthy repeat that the HNF1β dimerization domain had no transactivation activity in HeLa cells. This indicates that the function of HNF1β during nephrogenesis is more

complicated than the ability to transactivate a target gene. These results reinforce that analysis of protein function using only cell cultures is too simplistic to evaluate protein function in a developing organism. The analysis of the morphogenetic potential of chimeric HNF1 proteins during kidney development in *Xenopus* is more meaningful to this end.

The HNF1β dimerization region used in the experiments presented here includes the dimerization domain and part of the linker between the dimerization and POU_S domains. They may both contribute to reducing pronephric size. For the dimerization domain, nine of 32 residues differ between the HNF1α and HNF1β dimerization domains. One conservative substitution of Val21 in HNF1β for Leu21 in HNF1α is located in a buried position in the loop. The other eight residues occupy solvent exposed positions in the HNF1 structure (Rose et al., 2000). These surfaces may interact with another segment of HNF1 or with other proteins to create the functional differences of HNF1α and HNF1β during pronephric development.

The 26 aa segment located between the POU_S and POU_H domains of HNF1β plays an important role in nephrogenesis. This is most interesting, as this segment is the characteristic feature of the splice variant A. Whereas, the full-length splice variant A of HNF1β led to agenesis of the pronephros in *Xenopus* embryos, the splice variant B, lacking only the 26 aa segment, did not interfere with pronephric development (Figure 10). The ratio of splice variant A to B expression varies during the time of kidney development in the mouse (Cereghini et al., 1992). In this light, these results spawn the intriguing suggestion that expression changes of differentially spliced HNF1β proteins is an important level of control during vertebrate kidney development. The functional difference between the A and B splice variants in nephrogenesis contrasts to their role during early murine embryogenesis, where either variant can compensate for the loss of the endogenous HNF1β gene during the differentiation of visceral endoderm from embryonic stem cells (Haumaitre et al., 2003). Consistant with these results, the insertion of 26 aa segment into the nephrogenically inactive truncated HNF1α protein led to agenesis of the pronephros. The results presented in this thesis strongly support a role for the 26 aa segment of HNF1β in vertebrate nephrogenesis.

Although deletion of the 26 aa segment from the full-length HNF1β protein (splice variant B) blocked the effect on nephrogenesis, deletion of this segment from the truncated HNF1β protein did not affect its ability to interfere with pronephric formation. This indicates that complex interactions between different domains of the HNF1β protein itself or interactions with other proteins may be very important for the proper function of HNF1β in kidney development. In fact, chimeric proteins containing the HNF1β homeodomain, but lacking the 26 aa segment, also led to agenesis of the pronephros. These results demonstrate the importance of the HNF1β homeodomain in kidney development. The region of the homeodomain responsible for this effect was restricted to the POU_H domain between 229 and 319 aa. The homeodomain alone was unable to reduce pronephric size, emphasizing once again the importance of the remaining protein context for proper function. Deletion of the C-terminal 8 aa of homeodomain of the β protein (311-319 aa) abolished its potential to interfere with pronephric formation. Although the POU_H domain of HNF1α lacking the corresponding 8 aa still formed a complex with the high-affinity promoter as shown by X-ray crystalography (Chi et al., 2002). Since, two amino acids within this 8 aa region (Q311 and A317) are different in the HNF1α and HNF1β proteins, it is possible that one or both of these two amino acids play a functional role of the HNF1β POU_H domain during nephrogenesis. Alternatively, the entire POU_H domain may be necessary for proper function. Nine amino acids differ within the POU_H domain when the HNF1α and HNF1β proteins are compared (position α versus β: G199M, F215Y, E119D, T231A, I242L, Q250K, Q252H, H279Q, T285A). Three of these have chemically different side groups chains (F215Y: from nonpolar to uncharged polar, Q250K: from uncharged polar to basic, H279Q: from basic to uncharged polar), and these differences are strictly conserved in all vertebrate HNF1α and HNF1β proteins examined to date. These three amino acids in the POU_H domain may provide the functionality of the HNF1β protein in nephrogenesis. It is known that Q250 is involved in the interface between the POU_S and POU_H domains in the HNF1α protein, and this change to lysine (Q to K) in the HNF1β protein most probably influences the conformation and flexibility of the DNA binding domain. The substitution of methionine for glycine (G199M) may also be important for the conformation and function of the HNF1β protein, although this is a conservative change (remains nonpolar). This amino acid is located in the NLS, and could be at least partially responsible for the hindered nuclear transport of HNF1α protein in HeLa cells. Additionally, because methionine has a

more bulky side chain and is capable of disulfide bonding, other protein interactions may be possible. Further experiments will be necessary to determine the significance of these amino acids for HNF1β protein function.

In a recent report, the expression of human HNF1β mutants into *Xenopus* embryos was reported to lead to either a reduction or an enlargement of the pronephros (Bohn et al., 2003). These observed phenotypes could not be correlated directly to the structure of the mutated HNF1β protein (summarized in Figure 17). All truncated HNF1β proteins retaining the DNA binding domain (e.g. Y352insA) as well as an HNF1β mutant with an in-frame internal deletion in the POU_S domain (R137-K161) that destroys DNA binding resulted in a reduction in pronephric size. In contrast, all truncated HNF1β proteins with impaired DNA binding (e.g. A263insGG and E101X) resulted in an enlargement of the pronephros. In the present studies, three regions were identified with nephrogenic potential. It is plausible that all three regions must be present in an HNF1β mutant for it to cause a reduction in pronephric size, otherwise an enlargement occurs.

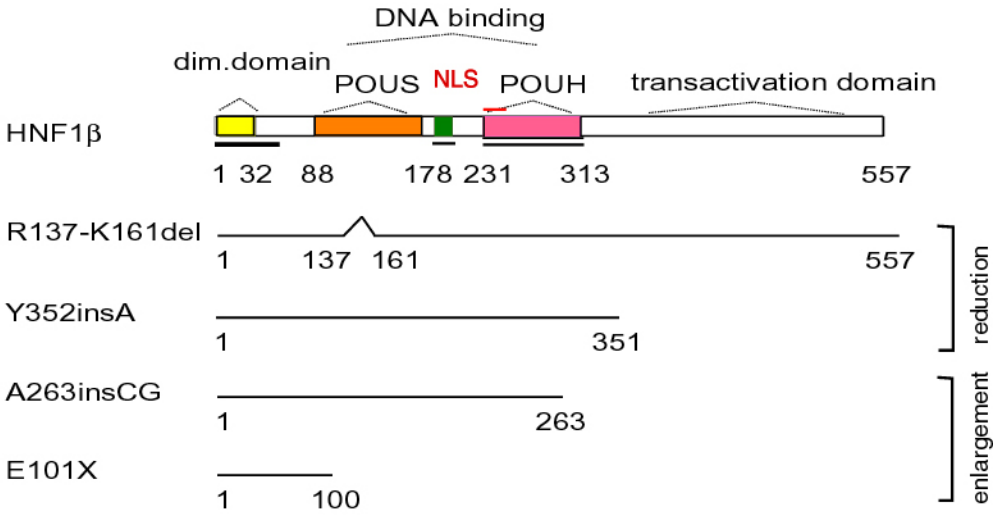

Figure 17. The nephrogenic effects of domains in the human HNF1β transcription factor and its mutants. Functional domains are indicated above the schematic representation of HNF1β, and numbers refer to the amino acid positions. The black box indicates the 26 aa segment deleted in the HNF1β splice variant B. The three regions involved in nephrogenesis are marked by black lines beneath the HNF1β diagram. The NLS is marked by a red line above the HNF1β diagram. Naturally occurring HNF1β mutations are shown below as line diagrams to indicate what regions of the protein are missing. Whether these HNF1β mutants cause an enlargement or a reduction of pronephric size is indicated at the far right (Bohn et al., 2003)

Expression of truncated HNF1 proteins lacking the 26 aa segment but containing the HNF1β homeodomain resulted in 70-80% of the embryos dying during gastrulation and 15-

20% embryos having general developmental malformations not related to nephrogenesis. It is not clear why the expression of these HNF1 proteins caused these early developmental problems. One explanation is, that HNF1β has several functions distinct from nephrogenesis in early embryogenesis. Knock-out experiments in the mouse established that HNF1β is required for yolk sac differentiation (Coffinier et al., 1999b; Barbacci et al., 1999), and overexpression in *Xenopus* of a dominant negative form of HNF1β interferes with mesoderm induction (Vignali et al., 2000). Furthermore, HNF1β mRNA injection into zebra fish showed it to be involved in the specification of the rhombomeres identity in the hindbrain (Wiellette and Sive, 2003). It is possible that some of our constructs may have disturbed similar early developmental processes outside of the pronephric anlage in the frog. Alternatively, these results may be explained by nonspecific gene regulation by these proteins. The deletion of the 26 aa segment and/or absence of C-terminal areas of the protein may result in a more open 3-dimensional protein conformation which allows regulatory proteins increased access to the homeodomain. Expression of these constructs may activate a maternal apoptotic program as a "fail-safe" mechanism of early embryogenesis (Kai et al., 2003). Hensey and Gautier have proposed that *Xenopus* embryonic cells perform a check shortly after MBT to see if they are physiologically fit (Hensey and Gautier, 1997). If a cell is physiologically aberrant at the G1 phase, it executes an apoptotic program and dies so that the embryo as a whole can continue normal development. The checkpoints may be normal RNA transcription and protein translation. However, if a large number of cells are abnormal, movements during gastrulation may be disturbed resulting in a surviving embryo with non-lethal malformations. If a critical number of abnormal cells is reached, development of the embryo is not able to continue, and it dies during gastrulation. It is most likely that the embryo death at gastrulation and general malformations observed after expression of these constructs was a result of nonspecific gene regulation as a result of binding of many other regulatory proteins to the homeodomain.

2. HNF1β partially antagonizes the Pax8/lim1-induced nephrogenic phenotype

There are at least two other transcription factors involved in early kidney development in vertebrates. In the *Xenopus* embryo, both Pax8 and lim1 are initially expressed in the pronephric anlage at the time when HNF1β is expressed (Ryffel, 2003). Both these transcription factors are functionally important, since overexpression of either protein led to an enlarged pronephros with ectopic pronephric tubules (Carroll and Vize, 1999). This effect was additive when both transcription factors were coexpressed, and the effect of Pax8 could be mimicked by Pax2 (Carroll and Vize, 1999), whose expression starts shortly after Pax8 in the pronephric anlage (Heller and Brändli, 1999). The importance of lim1 (Shawlot and Behringer, 1995) and Pax2 (Torres et al., 1995) in mammalian development was shown in knockout mice that had severe defects in organogenesis including agenesis of the kidney. The nephrogenic role of Pax8 has only been identified in a Pax2-deficient background. Mice lacking Pax8 and Pax2 are unable to form any nephric structure due to a block in the mesenchymal-epithelial transition (Bouchard et al., 2002).

I have shown here that overexpression of Pax8 and lim1 resulted in an enlargement of the pronephros and the development of ectopic pronephric tubules. This is consistent with previous studies using the different injection protocol (Carroll and Vize, 1999). In this paper, Pax8 was injected together with lim1 into different regions of the marginal zone of 16- to 32-cell stage *Xenopus* embryos in order to restrict expression only to certain areas of organ development (Carroll and Vize, 1999). Cells injected included the C-2, C-3, and C-4 blastomeres of 32-cell-stage embryos (nomenclature of (Dale and Slack, 1987). The C-2 cells will form the anterior somites and heart, whereas the C-3 cells form more posterior somites, pronephroi and part of the lateral plate mesoderm. The C-4 cells form lateral plate and ventral mesoderm as well as the posterior somites. Since HNF1β overexpression inhibits kidney formation and Pax8/lim1 overexpression is nephrogenic, it is possible that a simple antagonism exists between these factors during kidney development. Overexpression of the full-length HNF1β protein did not rescue the Pax8/lim1-induced nephrogenic phenotype. However, overexpression of the truncated HNF1β protein rescued lim1/Pax8-induced enlargement and ectopic tubule formation (Figure 16G). Pax8/lim1-

induced thickening of tubules and cyst-like structure formation, however, remained essentially unchanged (Figure 16E and F). The truncated HNF1β protein was a more potent inhibitor of nephrogenesis in the expression experiments presented here. It is likely that the ability of the truncated protein, but not the full-length HNF1β protein, to rescue the Pax8/lim1 nephrogenic phenotype is a function of the increased activity of the truncated protein. These results suggest that Pax8/lim1 and HNF1β are not simple antagonists during nephrogenesis, but that Pax8/lim1 also have distinct morphogenetic properties.

3. HNF1α and HNF1β have acquired different functions during evolution

The tissue-specific transcription factors, HNF1α and HNF1β, are two closely related homeodomain factors, and both are expressed in defined embryonic regions including the developing kidney during vertebrate development (Mendel et al., 1991; Tronche and Yaniv, 1992; Pogge v.Strandmann et al., 1997). They display extensive structural similarities with indistinguishable DNA sequence binding specificity (Cereghini, 1996). However, they also display distinct properties with different expression patterns and distinct phenotypes in knock-out mice (Cereghini et al., 1992; Ott et al., 1991; Lazzaro et al., 1992; Barbacci et al., 1999; Coffinier et al., 1999a; Reber and Cereghini, 2001; Barbacci et al., 1999; Coffinier et al., 1999b; Pontoglio et al., 1996; Pontoglio et al., 1998). Different human disease states have been linked to heterzygous mutations of the HNF1α and HNF1β genes (see review, (Ryffel, 2001). It has been reported previously that the different expression patterns are responsible for the different functions (Haumaitre et al., 2003). However, I have shown here that the homologous protein domains have also acquired distinct functional properties. The C-terminal transactivation domain is the most divergent region between the HNF1 proteins. In most transactivation assays HNF1α is approximately two-fold more potent than HNF1β (Cereghini, 1996; Wild et al., 2000). The C-terminal transactivation domain in HNF1α is responsible for this high transactivation activity. In contrast, the POU_S and POU_H DNA binding domains in HNF1β are required for the transactivation activity of HNF1β. The N-terminal three domains are highly conserved between the HNF1β and HNF1α proteins. The HNF1β dimerization domain, the 26 aa

segment only present in the splice variant HNF1β-A, and the POU_H domain contribute to nephrogenensis. However, the HNF1α protein has no function in nephrogenesis.

Divergence of protein function across evolution also occurs in other protein families. The transcription factors, Pax2 and Pax8, are structurally closely related proteins, but have acquired distinct functions during the evolution. Pax proteins are defined by the presence of a DNA-binding domain called the paired domain (PD), which makes sequence-specific contacts with DNA. In mammals, nine Pax genes have been identified to date. Homologs exist in worms, flies, fish, frogs and birds. Pax genes have been divided into four subgroups based on genomic structure, sequence similarity and conserved function. Pax2 and Pax8 belong to the same subgroup containing a PD domain, an octapeptide motif, the first helix of the homeodomain, and a carboxy-terminal transactivation domain. Both Pax2 and Pax8 are expressed in the central nervous system and kidney, but Pax8 is also expressed in the thyroid. Some functions of Pax2 and Pax8 appear to be similar in kidney development. Whereas mice deficient in Pax2 show defective kidney development, mice lacking Pax8 in a Pax2 deficient background are unable to form any nephric structure (Bouchard et al., 2002). Additionally, Pax8-deficient mice had normal kidneys, but lacked the thyroid gland (Mansouri et al., 1998). In humans, haploinsufficiency of Pax2 has been linked to the renal coloboma syndrome, an autosomal dominant human disease characterized by renal and ocular defects (Eccles, 1998). Conversely, Pax2 overexpression has been associated with fetal and infantile multicystic kidneys, autosomal dominant polycystic kidney disease, renal cell carcinoma and Wilm's tumor (Winyard et al., 1996). Humans heterozygous for Pax8 mutations exhibit hypothyroidism, but no kidney defects (Macchia et al., 1998). Although Pax2 and Pax8 are structurally very similar proteins, they have different functions during development.

4. Complex transcriptional regulation in nephrogenesis

Evidence is accumulating that specific gene transcription is regulated by large complexes of proteins including transcriptional activators, repressors, coactivators and corepressors that interact with sequence-specific regulatory elements on the DNA and/or various components of the general/basal transcription machinery as well as proteins that have a recruiting

function for other regulatory proteins and proteins that function as scaffolding elements for the binding of many proteins in the active or inactive complex (Bernstein et al., 2000; Knoepfler and Kamps, 1995; Wheeler et al., 2000). In light of experiments showing that

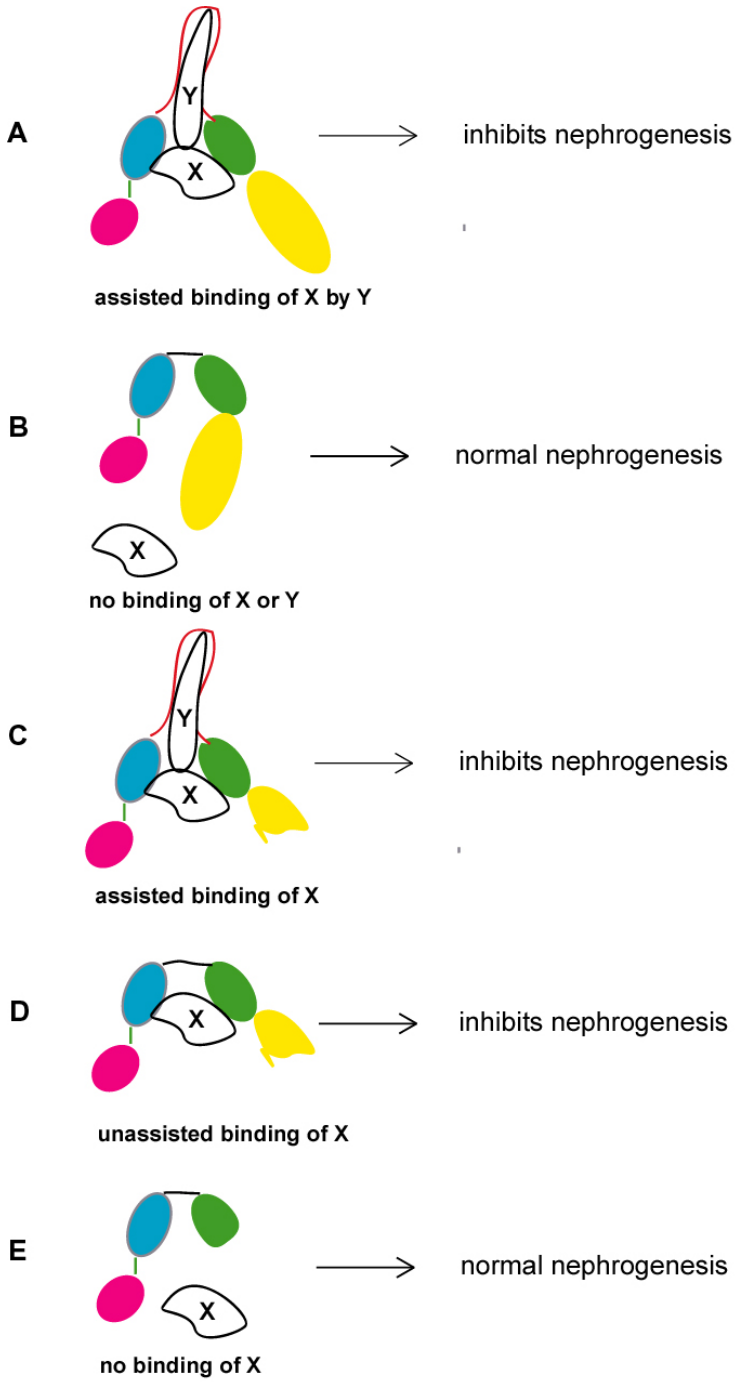

Figure 18. Model for the inhibition of nephrogenesis by HNF1β
A cartoon of the functional domains of HNF1β is shown. Unknown proteins, X and Y, are coregulators of gene transcription during nephrogenesis. red: dimerization domain, blue: POU_S domain, green: POU_H domain, yellow: C-terminal region, red line: 26 aa segment.

those transcription regulatory complexes examined contain hundreds of proteins (Holmes and Tjian, 2000; Hassel et al., 2004), it is necessary to examine functional domains of proteins involved in transcriptional regulation in the context of other possible necessary binding proteins.

I have shown that three domains of the HNF1β protein are involved in nephrogenesis: the dimerization domain, the 26 aa segment, and the POU_H domain. Using the expression of chimeric HNF1α/HNF1β proteins in *Xenopus*, several functional aspects of the 26 aa segment and the POU_H domain have come to light. The deletion of the 26 aa segment in full-length HNF1β abolished its inhibition of nephrogenesis. However, the chimeric protein containing the POU_H domain in the context of the truncated HNF1α protein which does not have the 26aa segment inhibited nephrogenesis, as does the full-length or truncated HNF1β protein. By insertion of the 26 aa segment into the truncated HNF1α protein, a partial gain of nephrogenic inhibition could be accomplished. A possible model for how the nephrogenic function of HNF1β can be achieved in presented in Figure 18. In light of the effects of these proteins during the expression experiments, at least two other proteins must be involved to achieve this functionality. These proteins are referred to as protein X and protein Y in the figure. I propose that protein Y binds to the 26 aa segment, and helps to recruit protein X to bind the POU_H domain (Figure 18A). If the 26 aa segment is absent, Y cannot bind and protein X cannot be recruited to the full-length protein (Figure 18B). It can be imagined that without the 26 aa segment, the 3-dimensional conformation of the HNF1β protein assumes a more closed structure, masking the binding site of protein X in the POU_H domain. If the C-terminal domain is removed, the binding site of the protein X is no longer masked due to a more open 3-dimensional conformation of the protein (Figure 18C). In this more open conformation, the 26 aa segment and the recruiting function of protein Y are no longer necessary for the binding of protein X (Figure 18D). The binding site for protein X is likely to be located in the C-terminal 8 aa of the POU_H domain, as deletion of these 8 aa from the truncated protein abolished the nephrogenic effect (Figure 18E). The large proportion of embryos dying after the expression of the truncated protein also lacking the 26 aa segment can also be explained by this model. Since a more open 3-dimensional conformation could also facilitate the binding of many other nonspecific regulatory proteins. This could result in several genes being improperly regulated and initiate the cell

death program. It is interesting to note that the presence of the 26 aa segment in such a truncated protein eliminated the lethal effects of protein expression. This segment may have restored more specific binding to the POU_H domain in this construct. It will be necessary to examine proteins interacting with the different domains of the HNF1β protein to understand the molecular function of HNF1β in nephrogenesis.

In this thesis, three domains of HNF1β were identified to be involved in nephrogenesis. These functionally unique features of HNF1β are not only important for understanding the distinct roles of HNF1α and HNF1β during development, but may also assist in the interpretation of data from human patients with heterozygous mutations in HNF1β, leading to a better understanding of the mechanisms of kidney disease.

V. References

Amores,A., Force,A., Yan,Y.L., Joly,L., Amemiya,C., Fritz,A., Ho,R.K., Langeland,J., Prince,V., Wang,Y.L., Westerfield,M., Ekker,M., and Postlethwait,J.H. (1998). Zebrafish hox clusters and vertebrate genome evolution. Science *282*, 1711-1714.

Arbeitman,M.N., Furlong,E.E., Imam,F., Johnson,E., Null,B.H., Baker,B.S., Krasnow,M.A., Scott,M.P., Davis,R.W., and White,K.P. (2002). Gene expression during the life cycle of Drosophila melanogaster. Science *297*, 2270-2275.

Bach,I., Mattei,M.G., Cereghini,S., and Yaniv,M. (1991). Two members of an HNF1 homeoprotein family are expressed in human liver. Nucleic Acids Res. *19*, 3553-3559.

Bach,I. and Yaniv,M. (1993). More potent transcriptional activators or a transdominant inhibitor of the HNF1 homeoprotein family are generated by alternative RNA processing. EMBO J. *12*, 4229-4242.

Barbacci,E., Reber,M., Ott,M.O., Breillat,C., Huetz,F., and Cereghini,S. (1999). Variant hepatocyte nuclear factor 1 is required for visceral endoderm specification. Development *126*, 4795-4805.

Bartkowski,S., Zapp,D., Weber,H., Eberle,G., Zoidl,C., Senkel,S., Klein-Hitpass,L., and Ryffel,G.U. (1993). Developmental regulation and tissue distribution of the liver transcription factor LFB1 (HNF1) in Xenopus laevis. Mol. Cell Biol. *13*, 421-431.

Beddington,R.S. and Robertson,E.J. (1998). Anterior patterning in mouse. Trends Genet. *14*, 277-284.

Belaoussoff,M., Farrington,S.M., and Baron,M.H. (1998). Hematopoietic induction and respecification of A-P identity by visceral endoderm signaling in the mouse embryo. Development *125*, 5009-5018.

Bergmann,C., Senderek,J., Schneider,F., Dornia,C., Kupper,F., Eggermann,T., Rudnik-Schoneborn,S., Kirfel,J., Moser,M., Buttner,R., and Zerres,K. (2004). PKHD1 mutations in families requesting prenatal diagnosis for autosomal recessive polycystic kidney disease (ARPKD). Hum. Mutat. *23*, 487-495.

Bernardini,G., Prati,M., Bonetti,E., and Scari,G. (1999). Atlas of *Xenopus* Development. Springer-Verlag Italia).

Bernstein,B.E., Tong,J.K., and Schreiber,S.L. (2000). Genomewide studies of histone deacetylase function in yeast. Proc. Natl. Acad. Sci. U. S. A *97*, 13708-13713.

Bingham,C., Ellard,S., Allen,L., Bulman,M., Shepherd,M., Frayling,T., Berry,P.J., Clark,P.M., Lindner,T., Bell,G.I., Ryffel,G.U., Nicholls,A.J., and Hattersley,A.T. (2000). Abnormal nephron development associated with a frameshift mutation in the transcription factor hepatocyte nuclear factor-1 beta. Kidney Int. *57*, 898-907.

Bohn,S., Thomas,H., Turan,G., Ellard,S., Bingham,C., Hattersley,A.T., and Ryffel,G.U. (2003). Distinct molecular and morphogenetic properties of mutations in the human HNF1beta gene that lead to defective kidney development. J. Am. Soc. Nephrol. *14*, 2033-2041.

Bouchard,M., Souabni,A., Mandler,M., Neubuser,A., and Busslinger,M. (2002). Nephric lineage specification by Pax2 and Pax8. Genes Dev. *16*, 2958-2970.

Brändli,A.W. (1999). Towards a molecular anatomy of the Xenopus pronephric kidney. Int. J. Dev. Biol. *43*, 381-395.

Brennan,H.C., Nijjar,S., and Jones,E.A. (1999). The specification and growth factor inducibility of the pronephric glomus in Xenopus laevis. Development *126*, 5847-5856.

Briancon,N., Bailly,A., Clotman,F., Jacquemin,P., Lemaigre,F.P., and Weiss,M.C. (2004). Expression of the alpha 7 isoform of HNF4 is activated by HNF6/OC-2 and HNF1, and repressed by HNF4alpha 1 in the liver. J. Biol. Chem.

Carroll,T., Wallingford,J., Seufert,D., and Vize,P.D. (1999). Molecular regulation of pronephric development. Curr. Top. Dev. Biol. *44*, 67-100.

Carroll,T.J. and Vize,P.D. (1999). Synergism between Pax-8 and lim-1 in embryonic kidney development. Dev. Biol. *214*, 46-59.

Cereghini,S. (1996). Liver-enriched transcription factors and hepatocyte differentiation. FASEB J. *10*, 267-282.

Cereghini,S., Ott,M.O., Power,S., and Maury,M. (1992). Expression patterns of vHNF1 and HNF1 homeoproteins in early postimplantation embryos suggest distinct and sequential developmental roles. Development *116*, 783-797.

Chan,T.C., Takahashi,S., and Asashima,M. (2000). A role for Xlim-1 in pronephros development in Xenopus laevis. Dev. Biol. *228*, 256-269.

Chi,Y.I., Frantz,J.D., Oh,B.C., Hansen,L., Dhe-Paganon,S., and Shoelson,S.E. (2002). Diabetes mutations delineate an atypical POU domain in HNF-1alpha. Mol. Cell *10*, 1129-1137.

Chouard,T., Blumenfeld,M., Bach,I., Vandekerckhove,J., Cereghini,S., and Yaniv,M. (1990). A distal dimerization domain is essential for DNA-binding by the atypical HNF1 homeodomain. Nucleic Acids Res. *18*, 5853-5863.

Coffinier,C., Barra,J., Babinet,C., and Yaniv,M. (1999a). Expression of the vHNF1/HNF1beta homeoprotein gene during mouse organogenesis. Mech. Dev. *89*, 211-213.

Coffinier,C., Gresh,L., Fiette,L., Tronche,F., Schutz,G., Babinet,C., Pontoglio,M., Yaniv,M., and Barra,J. (2002). Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. Development *129*, 1829-1838.

Coffinier,C., Thepot,D., Babinet,C., Yaniv,M., and Barra,J. (1999b). Essential role for the homeoprotein vHNF1/HNF1beta in visceral endoderm differentiation. Development *126*, 4785-4794.

Connacher,A.A., Forsyth,C.C., and Stewart,W.K. (1987). Orofaciodigital syndrome type I associated with polycystic kidneys and agenesis of the corpus callosum. J. Med. Genet. *24*, 116-118.

Coucouvanis,E. and Martin,G.R. (1999). BMP signaling plays a role in visceral endoderm differentiation and cavitation in the early mouse embryo. Development *126*, 535-546.

Courtois,G., Baumhueter,S., and Crabtree,G.R. (1988). Purified hepatocyte nuclear factor 1 interacts with a family of hepatocyte-specific promoters. Proc. Natl. Acad. Sci. U. S. A *85*, 7937-7941.

Dale,L. and Slack,J.M. (1987). Fate map for the 32-cell stage of Xenopus laevis. Development *99*, 527-551.

De Simone,V., De Magistris,L., Lazzaro,D., Gerstner,J., Monaci,P., Nicosia,A., and Cortese,R. (1991). LFB3, a heterodimer-forming homeoprotein of the LFB1 family, is expressed in specialized epithelia. EMBO J. *10*, 1435-1443.

Demartis,A., Maffei,M., Vignali,R., Barsacchi,G., and De,S., V (1994). Cloning and developmental expression of LFB3/HNF1 beta transcription factor in Xenopus laevis. Mech. Dev. *47*, 19-28.

Denda,S., Reichardt,L.F., and Muller,U. (1998). Identification of osteopontin as a novel ligand for the integrin alpha8 beta1 and potential roles for this integrin-ligand interaction in kidney morphogenesis. Mol. Biol. Cell *9*, 1425-1435.

Deryckere,F., Byrnes,L., Wagner,A., McMorrow,T., and Gannon,F. (1995). Salmon HNF1: cDNA sequence, evolution, tissue specificity and binding to the salmon serum albumin promoter. J. Mol. Biol. *247*, 1-10.

Donnai,D., Kerzin-Storrar,L., and Harris,R. (1987). Familial orofaciodigital syndrome type I presenting as adult polycystic kidney disease. J. Med. Genet. *24*, 84-87.

Drewes,T., Senkel,S., Holewa,B., and Ryffel,G.U. (1996). Human hepatocyte nuclear factor 4 isoforms are encoded by distinct and differentially expressed genes. Mol. Cell Biol. *16*, 925-931.

Dukes,I.D., Sreenan,S., Roe,M.W., Levisetti,M., Zhou,Y.P., Ostrega,D., Bell,G.I., Pontoglio,M., Yaniv,M., Philipson,L., and Polonsky,K.S. (1998). Defective pancreatic beta-cell glycolytic signaling in hepatocyte nuclear factor-1alpha-deficient mice. J. Biol. Chem. *273*, 24457-24464.

Eccles,M.R. (1998). The role of PAX2 in normal and abnormal development of the urinary tract. Pediatr. Nephrol. *12*, 712-720.

Eeckhoute,J., Moerman,E., Bouckenooghe,T., Lukoviak,B., Pattou,F., Formstecher,P., Kerr-Conte,J., Vandewalle,B., and Laine,B. (2003). Hepatocyte nuclear factor 4 alpha isoforms originated from the P1 promoter are expressed in human pancreatic beta-cells and exhibit stronger transcriptional potentials than P2 promoter-driven isoforms. Endocrinology *144*, 1686-1694.

Emes,R.D. and Ponting,C.P. (2001). A new sequence motif linking lissencephaly, Treacher Collins and oral-facial-digital type 1 syndromes, microtubule dynamics and cell migration. Hum. Mol. Genet. *10*, 2813-2820.

Evan,G.I., Lewis,G.K., Ramsay,G., and Bishop,J.M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol. Cell Biol. *5*, 3610-3616.

Feather,S.A., Woolf,A.S., Donnai,D., Malcolm,S., and Winter,R.M. (1997). The oral-facial-digital syndrome type 1 (OFD1), a cause of polycystic kidney disease and associated malformations, maps to Xp22.2-Xp22.3. Hum. Mol. Genet. *6*, 1163-1167.

Ferrante,M.I., Giorgio,G., Feather,S.A., Bulfone,A., Wright,V., Ghiani,M., Selicorni,A., Gammaro,L., Scolari,F., Woolf,A.S., Sylvie,O., Bernard,L., Malcolm,S., Winter,R., Ballabio,A., and Franco,B. (2001). Identification of the gene for oral-facial-digital type I syndrome. Am. J. Hum. Genet. *68*, 569-576.

Frain,M., Swart,G., Monaci,P., Nicosia,A., Stampfli,S., Frank,R., and Cortese,R. (1989). The liver-specific transcription factor LF-B1 contains a highly diverged homeobox DNA binding domain. Cell *59*, 145-157.

Froguel,P. and Velho,G. (1999). Molecular Genetics of Maturity-onset Diabetes of the Young. Trends Endocrinol. Metab *10*, 142-146.

Gehring,W.J., Affolter,M., and Burglin,T. (1994). Homeodomain proteins. Annu. Rev. Biochem. *63*, 487-526.

Gresh,L., Fischer,E., Reimann,A., Tanguy,M., Garbay,S., Shao,X., Hiesberger,T., Fiette,L., Igarashi,P., Yaniv,M., and Pontoglio,M. (2004). A transcriptional network in polycystic kidney disease. EMBO J.

Habener,J.F. and Stoffers,D.A. (1998). A newly discovered role of transcription factors involved in pancreas development and the pathogenesis of diabetes mellitus. Proc. Assoc. Am. Physicians *110*, 12-21.

Hassel,S., Eichner,A., Yakymovych,M., Hellman,U., Knaus,P., and Souchelnytskyi,S. (2004). Proteins associated with type II bone morphogenetic protein receptor (BMPR-II) and identified by two-dimensional gel electrophoresis and mass spectrometry. Proteomics. *4*, 1346-1358.

Hattersley,A.T. (1998). Maturity-onset diabetes of the young: clinical heterogeneity explained by genetic heterogeneity. Diabet. Med. *15*, 15-24.

Haumaitre,C., Reber,M., and Cereghini,S. (2003). Functions of HNF1 family members in differentiation of the visceral endoderm cell lineage. J. Biol. Chem. *278*, 40933-40942.

Hausen,P. and Ribesel,M. (1991). The early development of *Xenopus laevis*: An atlas of the histology. (Berlin: Springer-Verlag).

Heller,N. and Brändli,A.W. (1999). Xenopus Pax-2/5/8 orthologues: novel insights into Pax gene evolution and identification of Pax-8 as the earliest marker for otic and pronephric cell lineages. Dev. Genet. *24*, 208-219.

Hensey,C. and Gautier,J. (1997). A developmental timer that regulates apoptosis at the onset of gastrulation. Mech. Dev. *69*, 183-195.

Hiesberger,T., Bai,Y., Shao,X., McNally,B.T., Sinclair,A.M., Tian,X., Somlo,S., and Igarashi,P. (2004). Mutation of hepatocyte nuclear factor-1beta inhibits Pkhd1 gene expression and produces renal cysts in mice. J. Clin. Invest *113*, 814-825.

Holmes,M.C. and Tjian,R. (2000). Promoter-selective properties of the TBP-related factor TRF1. Science *288*, 867-870.

Huang,H.P. and Tsai,M.J. (2000). Transcription factors involved in pancreatic islet development. J. Biomed. Sci. *7*, 27-34.

Iyer,V.R., Horak,C.E., Scafe,C.S., Botstein,D., Snyder,M., and Brown,P.O. (2001). Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF. Nature *409*, 533-538.

Jones,E.A. (2003). Molecular control of pronephric development: an overview. In The kidney, Peter D.Vize, Adrian S.Woolf, and Johnathan B.L.Bard, eds. (London, UK: Elsevier Science).

Kai,M., Kaito,C., Fukamachi,H., Higo,T., Takayama,E., Hara,H., Ohya,Y., Igarashi,K., and Shiokawa,K. (2003). Overexpression of S-adenosylmethionine decarboxylase (SAMDC) in Xenopus embryos activates maternal program of apoptosis as a "fail-safe" mechanism of early embryogenesis. Cell Res. *13*, 147-158.

Kawazoe,Y., Sekimoto,T., Araki,M., Takagi,K., Araki,K., and Yamamura,K. (2002). Region-specific gastrointestinal Hox code during murine embryonal gut development. Dev. Growth Differ. *44*, 77-84.

Kayo,T., Allison,D.B., Weindruch,R., and Prolla,T.A. (2001). Influences of aging and caloric restriction on the transcriptional profile of skeletal muscle from rhesus monkeys. Proc. Natl. Acad. Sci. U. S. A *98*, 5093-5098.

Knoepfler,P.S. and Kamps,M.P. (1995). The pentapeptide motif of Hox proteins is required for cooperative DNA binding with Pbx1, physically contacts Pbx1, and enhances DNA binding by Pbx1. Mol. Cell Biol. *15*, 5811-5819.

Kudo,E., Kamatani,N., Tezuka,O., Taniguchi,A., Yamanaka,H., Yabe,S., Osabe,D., Shinohara,S., Nomura,K., Segawa,M., Miyamoto,T., Moritani,M., Kunika,K., and Itakura,M. (2004). Familial juvenile hyperuricemic nephropathy: detection of mutations in the uromodulin gene in five Japanese families. Kidney Int. *65*, 1589-1597.

Lazzaro,D., De,S., V, De Magistris,L., Lehtonen,E., and Cortese,R. (1992). LFB1 and LFB3 homeoproteins are sequentially expressed during kidney development. Development *114*, 469-479.

Lee,T.I. and Young,R.A. (2000). Transcription of eukaryotic protein-coding genes. Annu. Rev. Genet. *34*, 77-137.

Lee,Y.H., Sauer,B., and Gonzalez,F.J. (1998). Laron dwarfism and non-insulin-dependent diabetes mellitus in the Hnf-1alpha knockout mouse. Mol. Cell Biol. *18*, 3059-3068.

Lemon,B. and Tjian,R. (2000). Orchestrated response: a symphony of transcription factors for gene control. Genes Dev. *14*, 2551-2569.

Macchia,P.E., Lapi,P., Krude,H., Pirro,M.T., Missero,C., Chiovato,L., Souabni,A., Baserga,M., Tassi,V., Pinchera,A., Fenzi,G., Gruters,A., Busslinger,M., and Di Lauro,R. (1998). PAX8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis. Nat. Genet. *19*, 83-86.

Malyankar,U.M., Almeida,M., Johnson,R.J., Pichler,R.H., and Giachelli,C.M. (1997). Osteopontin regulation in cultured rat renal epithelial cells. Kidney Int. *51*, 1766-1773.

Mansouri,A., Chowdhury,K., and Gruss,P. (1998). Follicular cells of the thyroid gland require Pax8 gene function. Nat. Genet. *19*, 87-90.

Mendel,D.B., Hansen,L.P., Graves,M.K., Conley,P.B., and Crabtree,G.R. (1991). HNF-1 alpha and HNF-1 beta (vHNF-1) share dimerization and homeo domains, but not activation domains, and form heterodimers in vitro. Genes Dev. *5*, 1042-1056.

Mody,M., Cao,Y., Cui,Z., Tay,K.Y., Shyong,A., Shimizu,E., Pham,K., Schultz,P., Welsh,D., and Tsien,J.Z. (2001). Genome-wide gene expression profiles of the developing mouse hippocampus. Proc. Natl. Acad. Sci. U. S. A *98*, 8862-8867.

Nammo,T., Yamagata,K., Hamaoka,R., Zhu,Q., Akiyama,T.E., Gonzalez,F.J., Miyagawa,J., and Matsuzawa,Y. (2002). Expression profile of MODY3/HNF-1alpha protein in the developing mouse pancreas. Diabetologia *45*, 1142-1153.

Narayana,N., Hua,Q., and Weiss,M.A. (2001). The dimerization domain of HNF-1alpha: structure and plasticity of an intertwined four-helix bundle with application to diabetes mellitus. J. Mol. Biol. *310*, 635-658.

Nicosia,A., Monaci,P., Tomei,L., De Francesco,R., Nuzzo,M., Stunnenberg,H., and Cortese,R. (1990). A myosin-like dimerization helix and an extra-large homeodomain are essential elements of the tripartite DNA binding structure of LFB1. Cell *61*, 1225-1236.

Nielsen,D.A. and Shapiro,D.J. (1986). Preparation of capped RNA transcripts using T7 RNA polymerase. Nucleic Acids Res. *14*, 5936.

Nieuwkoop,P.D. and Faber,J. (1975). Normal table of Xenopus laevis (Daudin). (Amsterdam: The netherlands: Elsevier/North-Holland Publishing Co.).

O'Brien,E.R., Garvin,M.R., Stewart,D.K., Hinohara,T., Simpson,J.B., Schwartz,S.M., and Giachelli,C.M. (1994). Osteopontin is synthesized by macrophage, smooth muscle, and endothelial cells in primary and restenotic human coronary atherosclerotic plaques. Arterioscler. Thromb. *14*, 1648-1656.

Olive,M., Thiebaud,P., Landry,M., Duvert,M., Verna,A., Barillot,W., and Theze,N. (2003). Using Xenopus as a model system for an undergraduate laboratory course in vertebrate development at the University of Bordeaux, France. Int. J. Dev. Biol. *47*, 153-160.

Orphanides,G., Lagrange,T., and Reinberg,D. (1996). The general transcription factors of RNA polymerase II. Genes Dev. *10*, 2657-2683.

Orphanides,G. and Reinberg,D. (2002). A unified theory of gene expression. Cell *108*, 439-451.

Ott,M.O., Rey-Campos,J., Cereghini,S., and Yaniv,M. (1991). vHNF1 is expressed in epithelial cells of distinct embryonic origin during development and precedes HNF1 expression. Mech. Dev. *36*, 47-58.

Pogge v.Strandmann,E.P., Nastos,A., Holewa,B., Senkel,S., Weber,H., and Ryffel,G.U. (1997). Patterning the expression of a tissue-specific transcription factor in embryogenesis: HNF1 alpha gene activation during Xenopus development. Mech. Dev. *64*, 7-17.

Pontoglio,M. (2000). Hepatocyte nuclear factor 1, a transcription factor at the crossroads of glucose homeostasis. J. Am. Soc. Nephrol. *11 Suppl 16*, S140-S143.

Pontoglio,M., Barra,J., Hadchouel,M., Doyen,A., Kress,C., Bach,J.P., Babinet,C., and Yaniv,M. (1996). Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome. Cell *84*, 575-585.

Pontoglio,M., Sreenan,S., Roe,M., Pugh,W., Ostrega,D., Doyen,A., Pick,A.J., Baldwin,A., Velho,G., Froguel,P., Levisetti,M., Bonner-Weir,S., Bell,G.I., Yaniv,M., and Polonsky,K.S. (1998). Defective insulin secretion in hepatocyte nuclear factor 1alpha-deficient mice. J. Clin. Invest *101*, 2215-2222.

Reber,M. and Cereghini,S. (2001). Variant hepatocyte nuclear factor 1 expression in the mouse genital tract. Mech. Dev. *100*, 75-78.

Reinberg,D., Orphanides,G., Ebright,R., Akoulitchev,S., Carcamo,J., Cho,H., Cortes,P., Drapkin,R., Flores,O., Ha,I., Inostroza,J.A., Kim,S., Kim,T.K., Kumar,P., Lagrange,T., LeRoy,G., Lu,H., Ma,D.M., Maldonado,E., Merino,A., Mermelstein,F., Olave,I., Sheldon,M., Shiekhattar,R., Zawel,L., and . (1998). The RNA polymerase II general

transcription factors: past, present, and future. Cold Spring Harb. Symp. Quant. Biol. *63*, 83-103.

Rey-Campos,J., Chouard,T., Yaniv,M., and Cereghini,S. (1991). vHNF1 is a homeoprotein that activates transcription and forms heterodimers with HNF1. EMBO J. *10*, 1445-1457.

Ringeisen,F., Rey-Campos,J., and Yaniv,M. (1993). The transactivation potential of variant hepatocyte nuclear factor 1 is modified by alternative splicing. J. Biol. Chem. *268*, 25706-25711.

Roeder,R.G. (1998). Role of general and gene-specific cofactors in the regulation of eukaryotic transcription. Cold Spring Harb. Symp. Quant. Biol. *63*, 201-218.

Rogers,S.A., Padanilam,B.J., Hruska,K.A., Giachelli,C.M., and Hammerman,M.R. (1997). Metanephric osteopontin regulates nephrogenesis in vitro. Am. J. Physiol *272*, F469-F476.

Romio,L., Wright,V., Price,K., Winyard,P.J., Donnai,D., Porteous,M.E., Franco,B., Giorgio,G., Malcolm,S., Woolf,A.S., and Feather,S.A. (2003). OFD1, the gene mutated in oral-facial-digital syndrome type 1, is expressed in the metanephros and in human embryonic renal mesenchymal cells. J. Am. Soc. Nephrol. *14*, 680-689.

Rose,R.B., Endrizzi,J.A., Cronk,J.D., Holton,J., and Alber,T. (2000). High-resolution structure of the HNF-1alpha dimerization domain. Biochemistry *39*, 15062-15070.

Rudnicki,M.A. and Jaenisch,R. (1995). The MyoD family of transcription factors and skeletal myogenesis. Bioessays *17*, 203-209.

Rupp,R.A., Snider,L., and Weintraub,H. (1994). Xenopus embryos regulate the nuclear localization of XMyoD. Genes Dev. *8*, 1311-1323.

Ryffel,G.U. (2001). Mutations in the human genes encoding the transcription factors of the hepatocyte nuclear factor (HNF)1 and HNF4 families: functional and pathological consequences. J. Mol. Endocrinol. *27*, 11-29.

Ryffel,G.U. (2003). What can a frog tell us about human kidney development. Nephron Exp. Nephrol. *94*, e35-e43.

Sambrook,M., Fritsch,E.F., and Maniatis,T. (1989). Molecular cloning - a laboratory manual. (New York: Cold Spring Harbour Laboratory, Cold Spring Harbour).

Scolari,F., Valzorio,B., Carli,O., Vizzardi,V., Costantino,E., Grazioli,L., Bondioni,M.P., Savoldi,S., and Maiorca,R. (1997). Oral-facial-digital syndrome type I: an unusual cause of hereditary cystic kidney disease. Nephrol. Dial. Transplant. *12*, 1247-1250.

Shawlot,W. and Behringer,R.R. (1995). Requirement for Lim1 in head-organizer function. Nature *374*, 425-430.

Sive,H., Grainger,R., and Harland,R. (2000). Early Development of *Xenopus laevis*, A Laboratory manual. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Sourdive,D.J., Chouard,T., and Yaniv,M. (1993). The HNF1 C-terminal domain contributes to transcriptional activity and modulates nuclear localisation. C. R. Acad. Sci. III *316*, 385-394.

Sun,Z. and Hopkins,N. (2001). vhnf1, the MODY5 and familial GCKD-associated gene, regulates regional specification of the zebrafish gut, pronephros, and hindbrain. Genes Dev. *15*, 3217-3229.

Taira,M., Otani,H., Jamrich,M., and Dawid,I.B. (1994). Expression of the LIM class homeobox gene Xlim-1 in pronephros and CNS cell lineages of Xenopus embryos is affected by retinoic acid and exogastrulation. Development *120*, 1525-1536.

Tavoularis,S., Scazzocchio,C., and Sophianopoulou,V. (2001). Functional expression and cellular localization of a green fluorescent protein-tagged proline transporter in Aspergillus nidulans. Fungal. Genet. Biol. *33*, 115-125.

Thomas,H., Badenberg,B., Bulman,M., Lemm,I., Lausen,J., Kind,L., Roosen,S., Ellard,S., Hattersley,A.T., and Ryffel,G.U. (2002). Evidence for haploinsufficiency of the human HNF1alpha gene revealed by functional characterization of MODY3-associated mutations. Biol. Chem. *383*, 1691-1700.

Tomura,H., Nishigori,H., Sho,K., Yamagata,K., Inoue,I., and Takeda,J. (1999). Loss-of-function and dominant-negative mechanisms associated with hepatocyte nuclear factor-1beta mutations in familial type 2 diabetes mellitus. J. Biol. Chem. *274*, 12975-12978.

Torres,M., Gomez-Pardo,E., Dressler,G.R., and Gruss,P. (1995). Pax-2 controls multiple steps of urogenital development. Development *121*, 4057-4065.

Tronche,F. and Yaniv,M. (1992). HNF1, a homeoprotein member of the hepatic transcription regulatory network. Bioessays *14*, 579-587.

Uchiyama,H., Komazaki,S., Asashima,M., and Kikuyama,S. (1996). Occurrence of immunoreactive activin/inhibin beta(B) in gonadotrophs, thyrotrophs, and somatotrophs of the Xenopus pituitary. Gen. Comp Endocrinol. *102*, 1-10.

Vignali,R., Poggi,L., Madeddu,F., and Barsacchi,G. (2000). HNF1(beta) is required for mesoderm induction in the Xenopus embryo. Development *127*, 1455-1465.

Vize,P.D., Jones,E.A., and Pfister,R. (1995). Development of the Xenopus pronephric system. Dev. Biol. *171*, 531-540.

Vize,P.D., Seufert,D.W., Carroll,T.J., and Wallingford,J.B. (1997). Model systems for the study of kidney development: use of the pronephros in the analysis of organ induction and patterning. Dev. Biol. *188*, 189-204.

Vize,P.D., Woolf,A.S., and Bard,J.B.L. (2003). The Kidney (From Normal Development to Congenital Disease). (London, UK: Elsevier Science).

Weber,H., Pogge v.Strandmann,E., Holewa,B., Bartkowski,S., Zapp,D., Zoidl,C., and Ryffel,G.U. (1996). Regulation and function of the tissue-specific transcription factor HNF1 alpha (LFB1) during Xenopus development. Int. J. Dev. Biol. *40*, 297-304.

Wettke-Schafer,R. and Kantner,G. (1983). X-linked dominant inherited diseases with lethality in hemizygous males. Hum. Genet. *64*, 1-23.

Wheeler,J.C., Shigesada,K., Gergen,J.P., and Ito,Y. (2000). Mechanisms of transcriptional regulation by Runt domain proteins. Semin. Cell Dev. Biol. *11*, 369-375.

White,K.P., Rifkin,S.A., Hurban,P., and Hogness,D.S. (1999). Microarray analysis of Drosophila development during metamorphosis. Science *286*, 2179-2184.

Wiellette,E.L. and Sive,H. (2003). vhnf1 and Fgf signals synergize to specify rhombomere identity in the zebrafish hindbrain. Development *130*, 3821-3829.

Wild,W., Pogge,v.S., Nastos,A., Senkel,S., Lingott-Frieg,A., Bulman,M., Bingham,C., Ellard,S., Hattersley,A.T., and Ryffel,G.U. (2000). The mutated human gene encoding hepatocyte nuclear factor 1beta inhibits kidney formation in developing Xenopus embryos. Proc. Natl. Acad. Sci. U. S. A *97*, 4695-4700.

Winter,W.E., Nakamura,M., and House,D.V. (1999). Monogenic diabetes mellitus in youth. The MODY syndromes. Endocrinol. Metab Clin. North Am. *28*, 765-785.

Winter,W.E. and Silverstein,J.H. (2000). Molecular and genetic bases for maturity onset diabetes of youth. Curr. Opin. Pediatr. *12*, 388-393.

Winyard,P.J., Risdon,R.A., Sams,V.R., Dressler,G.R., and Woolf,A.S. (1996). The PAX2 tanscription factor is expressed in cystic and hyperproliferative dysplastic epithelia in human kidney malformations. J. Clin. Invest *98*, 451-459.

Wu,G., Tian,X., Nishimura,S., Markowitz,G.S., D'Agati,V., Park,J.H., Yao,L., Li,L., Geng,L., Zhao,H., Edelmann,W., and Somlo,S. (2002). Trans-heterozygous Pkd1 and Pkd2 mutations modify expression of polycystic kidney disease. Hum. Mol. Genet. *11*, 1845-1854.

Zapp,D., Bartkowski,S., Zoidl,C., Klein-Hitpass,L., and Ryffel,G.U. (1993). Genomic structure of the Xenopus laevis liver transcription factor LFB1. Gene *134*, 251-256.

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CURRICULUM VITAE

Name: Guizhi Wu

Date of Birth: 27.10.1971

Place of Birth: Shandong, China

Married States: Married

Nationality: China

MAJOR EDUCATION:

2001.2– 2004.7 Ph D work in the field of Cell & Developmental Biology
Institut für Zellbiologie (Tumorforschung), Universitätsklinikum Essen, Essen, Germany

1994.8 – 1997.7 Master of Science in Pharmacochemistry
Institute of Medical Science, Academy of Military Medical Science, Beijing, P.R. China

1990.8 - 1994.7 Bachelor of Science in Chemistry
Department of Chemistry, Shandong Normal University.
Jinan, Shandong Province, P.R. China

WORKING EXPERIENCE:

1997.8 – 1999.3 Pharmacist in Department of Pharmacy
309 Hospital of PLA, Beijing, P.R. China

1999.4 – 2001.1 Pharmacologist in Department of Pharmacology
309 Hospital of PLA, Beijing, P.R. China

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., daß ich das Arbeitsgebiet, dem das Thema „The transcription factor HNF1β has several domains involved in nephrogenesis andpartially rescues Pax8/lim1 induced kidney malformations“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Frau Guizhi Wu befürworte.

Essen, 30.04.2004

(Prof. Dr. Gerhart U. Ryffel)

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., daß ich die vorliegende Dissertation selbständig verfaßt und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, 30.04.2004

(Guizhi Wu)

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., daß ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe, und daß diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

Essen, 30.04.2004

(Guizhi Wu)