Impact of transcriptional and posttranscriptional regulation of *HNF4A* and its target genes on diabetes and cancer

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Andrea Wirsing

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1. Gutachter: Professor Dr. G. U. Ryffel
2. Gutachter: Professor B. Opalka
3. Gutachter: Professor Dr. B. Horsthemke

Vorsitzende des Prüfungsausschusses: Professor Dr. A. Vortkamp

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Abbreviations

A	Adenine
AF	Activation function
agRNA	Antigene RNA
Ago	Argonaute
ARE	AU-rich element
AREBP	ARE binding protein
BAC	Bacterial artificial chromosome
BSA	Bovine serum albumin
bp	Base pair
C	Cytosine
CaCo2	Human intestinal cell line
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CIDEB	Cell death-inducing DFFA-like effector b (human gene, mRNA, cDNA)
CIDEB	Cell death-inducing DFFA-like effector b (human protein)
Cideb	Cell death-inducing DFFA-like effector b (mouse and rat gene, mRNA, cDNA)
Cideb	Cell death-inducing DFFA-like effector b (mouse and rat protein)
CMV	Cyctomegalovirus
cRNA	Complementary RNA
C-terminus	Carboxyterminus
DMEM	Dulbecco`s Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuleic acid
dNTP	Deoxyribonucleotidetriphosphate
Dox	Doxycycline
ds	Double-stranded
ECL	Enhanced chemiluminescence
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
esiRNA	Endoribonuclease-prepared short interfering RNA
et al.	And others (*et alii*)
FCS	Fetal calf serum
FL	Firefly
FRT	Flp recombination target
Fig	Figure
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GOI	Gene of interest
HCC	Hepatocellular carcinoma
HEK293	Human embryonic kidney cells
HepG2	Human liver hepatocellular carcinoma cell line
HK120	Kidney cell line
HNF	Hepatocyte nuclear factor (human gene, mRNA, cDNA)
HNF	Hepatocyte nuclear factor (human protein)

Hnf	Hepatocyte nuclear factor (mouse and rat gene, mRNA, cDNA)
Hnf	Hepatocyte nuclear factor (mouse and rat protein)
INS-1	Rat insulinoma cell line
kb	Kilo base
lac-Z	Gene encoding β-galactosidase
MIN6	Mouse insulinoma cell line
miRNA	MicroRNA
MODY	Maturity-onset diabetes of the young
mRNA	Messenger ribonucleic acid
Myc	V-myc myelocytomatosis viral oncogene homolog
n.d.	Not determined
nt	Nucleotide
N-terminal	Amino-terminal
N-terminus	Amino-terminus
OD	Optical density
ORF	Open reading frame
PAS	Polyadenylation signal
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potentia hydrogenii
qRT-PCR	Quantitative real-time PCR
RCC	Renal cell carcinoma
RISC	RNA-induced silencing complex
RL	*Renilla*
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulfate
SINE	Short interspersed repetitive element
siRNA	Short interfering RNA
SNP	Single nucleotide polymorphism
SV40	Simian Virus 40
SYBR	Asymmetrical cyanine dye
T	Thymine
T2DM	Type 2 diabetes mellitus
Tab	Table
Tet	Tetracycline
TGF	Transforming growth factor
VHL	Von Hippel-Lindau tumor suppressor gene

The international system of units (SI units) was used in this thesis.

A Introduction

1 The cell-specific transcription factor HNF4A

The cell-specific transcription factor hepatocyte nuclear factor 4 alpha (HNF4A, NR2A1) is a highly conserved member of the nuclear receptor superfamily (Sladek et al., 1990). Other members of subfamily 2, group A (Nuclear Receptors Nomenclature Committee 1999), include HNF4B (Holewa et al., 1997), so far exclusively identified in *Xenopus*, and HNF4G present in humans (Drewes et al., 1996) and mice (Taraviras et al., 2000).
HNF4A consists of six structural domains A-F responsible for specific functions (Fig. 1A). The A/B domain is positioned at the N-terminus and includes the transactivation domain AF-1 comprised of the N-terminal 24 amino acids (Hadzopoulou-Cladaras et al., 1997; Green et al., 1998). The DNA binding domain (DBD, C domain), highly conserved among nuclear receptors, consists of two zinc fingers and is linked by the flexible D domain to the large hydrophobic ligand binding domain (LBD, E domain). This second highly conserved region functions as a ligand binding, homodimerisation and second activation domain (AF-2; Jiang and Sladek, 1997; Hadzopoulou-Cladaras et al., 1997). Hence, it is involved in transcriptional activation and interactions with other transcription factors and coregulators (Ktistaki and Talianidis, 1997). In contrast to the majority of nuclear receptors, the C-terminal F domain of HNF4A is unusually long and includes a repressor function that inhibits access of coactivators to AF-2, and possibly to other regions (Suaud et al., 1999; Sladek et al., 1999).

HNF4A was long considered an orphan receptor as no activity modulating ligand could be identified. The search for ligands caused much controversy. Long-chain fatty acids were shown to bind as acyl-CoA thioesters to the LBD of HNF4A and function as transactivational agonists or antagonists, depending on their chain length and degree of saturation (Hertz et al., 1998). Crystal structures of the LBD of bacterially expressed HNF4A confirmed that the ligand-binding pocket is occupied by fatty acids, but excluded acyl-CoAs (Dhe-Paganon et al., 2002; Wisely et al., 2002; Duda et al., 2004). Fatty acids were bound firmly and could not be exchanged, suggesting that HNF4A is constitutively bound and activated by fatty acids. Hence, fatty acids seemed to act more as structural cofactors rather than classical regulatory ligands (Benoit et al., 2004). However, in a recent study, mammalian expressed HNF4A was shown to be bound to the essential fatty acid linoleic acid (LA; C18:2). Although binding is reversible, no effect was observed on the transactivation function of HNF4A (Yuan et al., 2009).

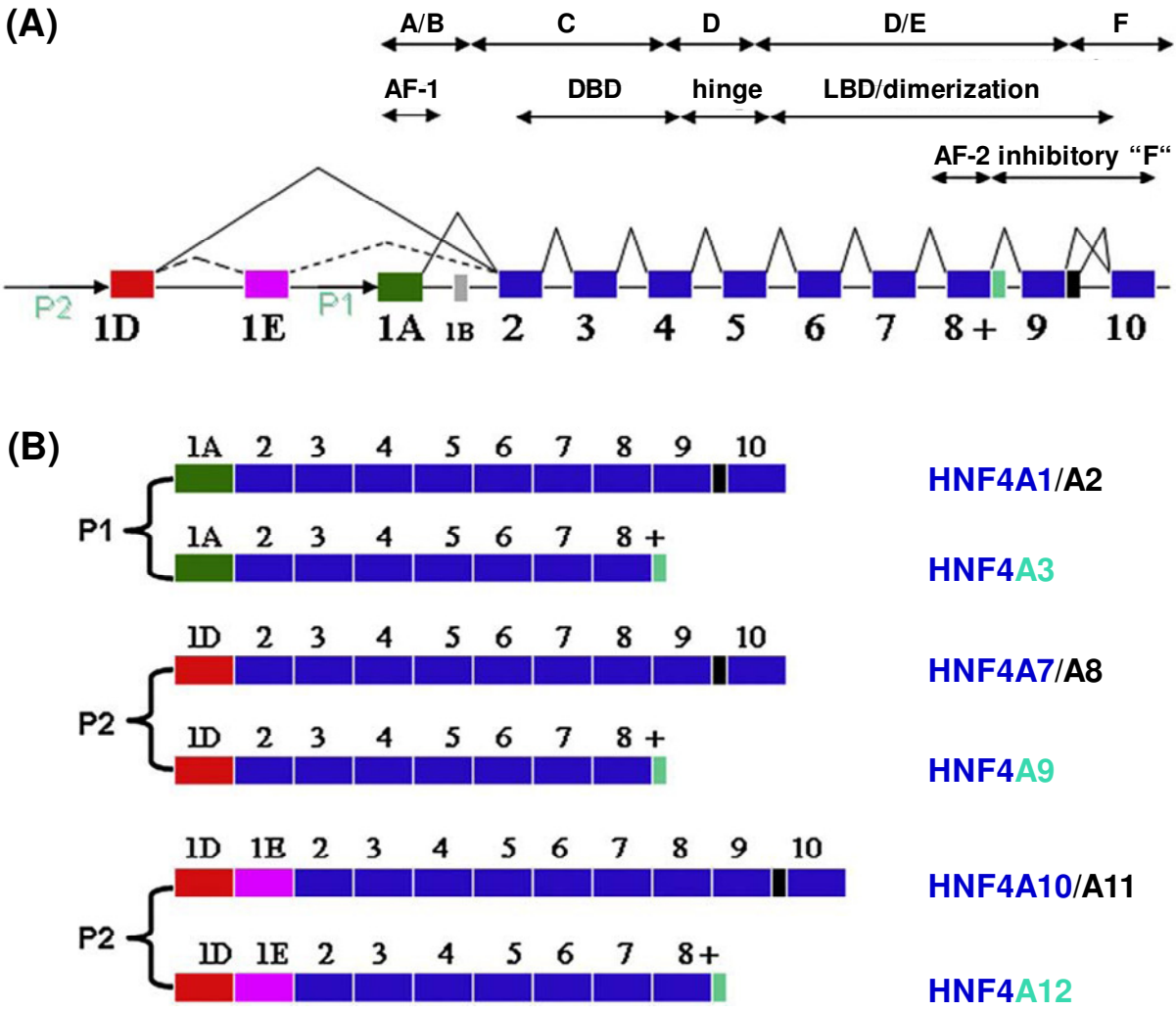

Figure 1: *HNF4A* gene structure (A) and isoforms (B).
(A) The six structural (A-F) and functional domains are illustrated above the exon structure (not drawn to scale). The P2 and P1 specific exon 1D and 1A are indicated by red and green, respectively. Novel exon 1E (P2) is given in pink. Transcriptional termination at a polyadenylation signal (PAS) in intron 8 (8+) results in an alternative C-terminus (light blue box). Presence of 10 amino acids due to alternative splicing at exon 9 is indicated by a black box. The presence and significance of exon 1B is not clearly established. **(B)** The P1 and P2 specific isoforms and their exon structure are listed, excluding disputable isoforms HNF4A4/5/6. Abbreviations: Activation function 1 (AF-1); DNA binding domain (DBD); non-conserved "hinge" region; multi-functional domain for ligand-binding domain (LBD), receptor dimerization and activation function 2 (AF-2); inhibitory "F" domain. (Figure adapted from Huang et al., 2009a).

Several other endogenous or external circumstances are known to influence the transcriptional activity or expression of *HNF4A*. These include bile acids (Zhang and Chiang, 2001), cytokines (Li et al., 2006b), hypoxia (Mazure et al., 2001), diet (Viollet et al., 1997), exposure to drugs (Hertz et al., 2001) and nitric oxide (NO; Vossen and Erard, 2002). Furthermore, HNF4A function is modulated by phosphorylation (Jiang et al., 1997; Sun et al., 2007; Gonzalez, 2008) as well as methylation (Barrero and Malik, 2006) and acetylation (Soutoglou et al., 2000). Its expression is regulated by a variety of different transcription factors that target both promoter and enhancer sequences (Hatzis and Talianidis, 2001; Bailly et al., 2009) and is also autoregulated by HNF4A itself (Hatzis and Talianidis, 2001;

Magenheim et al., 2005; Bailly et al., 2009). Interestingly, HNF4A, HNF1A and HNF1B, which have key roles in embryonic development and in mature homeostasis, are part of an autoregulatory network in mammalian pancreas, kidney, liver and gut (Ferrer, 2002; Harries et al., 2009). However, expression of *HNF4A* is not dependent on HNF1A in hepatocytes (Boj et al., 2001; Ferrer, 2002). Taken together, the versatile interactions of HNF4A with a variety of different transcription factors, coregulators and modifying enzymes can cause up- and downregulation of *HNF4A* as well as increased and decreased transcriptional activity (Sladek and Seidel, 2001; Kyrmizi et al., 2006; Gonzalez, 2008; Tomaru et al., 2009).

In humans, the *HNF4A* gene spans about 74 kb on chromosome 20 and comprises at least 12 exons (Avraham et al., 1992; Drewes et al., 1996; Sladek and Seidel, 2001; Huang et al., 2009a). Two promoters, P1 and P2, have been identified that drive the expression of at least six different splice variants (*HNF4A1-A3* and *HNF4A7-A9*; Fig. 1B). Expression of predicted variants *HNF4A4-A6* is controversial (Drewes et al., 1996; Huang et al., 2008; Harries et al., 2008) and therefore not included in Figure 1B. Recently, three new isoforms (*HNF4A10-A12*) were described (Huang et al., 2009a) including exon 1E that was previously not detected. The proximal P1 and distal P2 promoter are separated by about 45.5 kb in the human *HNF4A* gene (Thomas et al., 2001). Despite the conserved structure in rodents, the two promoters are located approximately 36.6 kb and 40.2 kb apart in rat and mouse, respectively (Huang et al., 2009a). AF-1 is exclusively contained in proteins derived from the P1 promoter due to the P1 and P2 promoter specific first exon 1A and 1D, respectively. The isoform specific differences in the F domain are splice dependent. Importantly, the variant-specific domain makeup causes functional variations (Sladek et al., 1999; Torres-Padilla et al., 2001; Eeckhoute et al., 2003; Briancon and Weiss, 2006). HNF4A2 (P1), used in this work contains a 10 amino acid insert in the middle of the F domain in comparison to the initially identified HNF4A1 (Sladek et al., 1999). The corresponding isoform HNF4A8 is expressed from the P2 promoter and thus lacks AF-1. The use of the two promoters including distinct regulatory elements, in a temporal and spatial-specific fashion results in a complex regulation of the isoforms and their different physiological roles (Torres-Padilla et al., 2001; Kyrmizi et al., 2006; Huang et al., 2008; Harries et al., 2008). The major tissues in which the P1 promoter is active includes the adult kidney, liver, stomach and colon as well as fetal liver and pancreas. The P2 promoter is predominantly expressed in adult colon, pancreas, stomach and small intestine as well as in fetal pancreas and liver (Bolotin et al., 2010). In other tissues expressing *HNF4A* the promoter usage has not been established.

HNF4A usually binds as a homodimer (Jiang et al., 1995) to a direct repeat element (AGGTCA) with either a one or two nucleotide spacer, designated DR1 or DR2, respectively,

in the regulatory sequences of its target genes (Jiang and Sladek, 1997; Ellrott et al., 2002). However, HNF4A is predicted to bind to thousands of different variations of the response element (Badis et al., 2009). To regulate gene expression, transcriptional coactivators and other accessory proteins are recruited by HNF4A. Many sites targeted by HNF4A are also bound by other nuclear receptors including COUP transcription factors, RXR and PPARs, resulting in the expression of many of the same genes.

The impact of HNF4A on gene regulation has been elucidated by identifying numerous target genes in several tissues involved in various processes such as homeostasis, metabolism, immune and stress response, cell structure, apoptosis and cancer. A list of target genes can be found at http://www.sladeklab.ucr.edu/hnf43.pdf. In the liver many target genes of HNF4A were initially identified by classical techniques such as promoter deletions, gel shifts and luciferase assays. Recently genome-wide techniques have been applied to identify more than a thousand potential target genes in the liver, but also in other tissues such as kidney and pancreas. Expression profiles of HNF4A regulated genes have been determined in different human cell lines including HEK293 (embryonic kidney; Lucas et al., 2005; Grigo et al., 2008), HuH-7 (hepatocyte; Naiki et al., 2002), HepG2 (hepatocyte; Bolotin et al., 2009), HCT116 (colon; Yuan et al., 2009) and in human liver (Boj et al., 2009), as well as in different mouse and rat tissues (Garrison et al., 2006; Battle et al., 2006; Waxman and O'Connor, 2006; Erdmann et al., 2007; Gupta et al., 2007; Ishikawa et al., 2008; Boj et al., 2009; Darsigny et al., 2009). ChIP-chip analyses were performed in hepatocytes purified from human (Odom et al., 2004; Odom et al., 2006; Odom et al., 2007) and mouse liver (Odom et al., 2007), HepG2 cells (Rada-Iglesias et al., 2005; Wallerman et al., 2009), pancreatic islets (Odom et al., 2004), the human intestinal cell line CaCo2 (Boyd et al., 2009) and in liver of human, mouse, dog, opossum and chicken by ChIP-seq (Schmidt et al., 2010). However, which of these genes are directly dependent on HNF4A *in vivo* and the functional significance of this binding, remains to be analyzed.

HNF4A plays an important role in early embryogenesis. This transcription factor is present as a maternal component in the *Xenopus* egg (Holewa et al., 1996) and is detected in the primary endoderm of mouse embryos at day 4.5 (Duncan et al., 1994). Its essential function in vertebrate development is evident in homozygous knockout mice that die during early gastrulation due to dysfunction of the visceral endoderm (Chen et al., 1994; Duncan et al., 1997). Furthermore, HNF4A is essential in the adult as shown by severe defects in mice lacking hepatic *Hnf4a* expression resulting in death within six weeks (Hayhurst et al., 2001). HNF4A is crucial to establish and maintain the heptatocyte phenotype by regulating genes involved in the control of lipid homeostasis (Li et al., 2000; Hayhurst et al., 2001; Naiki et al., 2005) and the liver architecture (Parviz et al., 2003; Battle et al., 2006). In the embryonic

liver, *HNF4A* expression is driven by the P1 and P2 promoter, while in adults, the P1 promoter is mainly active. Hence, in the adult liver *Hnf4a2* is the main isoform besides *Hnf4a1*, while *Hnf4a7* and *Hnf4a8* are absent (Nakhei et al., 1998; Torres-Padilla et al., 2001).

In addition to the liver, where HNF4A (A1) was initially identified (Costa et al., 1989), HNF4A function in the pancreas has been quite thoroughly investigated. It is expressed in the endocrine and exocrine cells of the pancreas, although at a lower level than in liver (Miquerol et al., 1994; Tanaka et al., 2006; Nammo et al., 2008). Hnf4a was described to regulate the expression of genes associated with β-cell glucose metabolism and insulin secretion in rat insulinoma cells (INS-1; Wang et al., 2000). Location analysis, which combined ChIP with a custom DNA microarray containing parts of the promoter regions of 13,000 human genes, resulted in the presumption that HNF4A regulates >40% of the active promoters in the islets (Odom et al., 2004). The vast majority of genes were not verified in β-cell-specific *Hnf4a* knockout mice. Although there is substantial discrepancy in the different studies concerning HNF4A targets genes and HNF4A dependent phenotype, disruption of *Hnf4a* in β-cells of mice causes impaired glucose tolerance due to attenuated glucose-stimulated insulin secretion (Gupta et al., 2005; Miura et al., 2006; Gupta et al., 2007). Furthermore, *Hnf4a* seems to be essential for adult β-cell mass expansion upon enhanced metabolic demand (Gupta et al., 2007). In humans, transcripts derived from the P1 promoter comprise up to 23% of total *HNF4A* expression in fetal pancreas from nine weeks until at least 19-26 weeks post-conception (Harries et al., 2008). *Hnf4a* mRNAs transcribed from both promoters are also detected in mouse pancreas during embryonic periods (Kanazawa et al., 2009). Several reports constrained *HNF4A* expression in the adult to the P2 promoter in human and rat pancreases (Thomas et al., 2001; Boj et al., 2001; Hansen et al., 2002; Ihara et al., 2005; Tanaka et al., 2006). This is in contrast to one study reporting the expression of P1 specific isoforms in human adult β-cells (Eeckhoute et al., 2003).

Despite high expression of *HNF4A* in selected parts of the kidney, little is known about its functions in this organ. In the metanephros of the mouse, *Hnf4a* is initially detected in the epithelial cells of the comma-shaped body, then distributed widely throughout the developing nephron and is finally restricted to the proximal tubules (Taraviras et al., 1994; Kanazawa et al., 2009). *Hnf4a* expression in those embryonic periods is driven by both promoters, but predominantly by P1 (Kanazawa et al., 2009). In the adult kidney, *HNF4A* expression is observed in the proximal tubules as determined in human kidney tissue specimens (Chabardes-Garonne et al., 2003; Tanaka et al., 2006) and verified on protein level (Jiang et al., 2003). *HNF4A* is not detected in the glomerulus, distal and collecting tubular epithelial cells of the kidney nor in HEK293 cells (Jiang et al., 2003; Lucas et al., 2005; Tanaka et al., 2006). In the adult kidney, *HNF4A* expression is restricted to the P1 promoter in humans,

mouse and rat as established on RNA and protein level (Nakhei et al., 1998; Jiang et al., 2003; Tanaka et al., 2006; Kanazawa et al., 2009).

2 *HNF4A* and human diseases

In humans, no homozygous mutations have been identified in *HNF4A*, consistent with the embryonic lethality in mice (Chen et al., 1994; Ellard and Colclough, 2006). However, monoallelic mutations in the *HNF4A* gene have been directly linked to Maturity Onset Diabetes of the Young 1 (MODY1; Yamagata et al., 1996). In addition, a mutation in the HNF4A binding site within the *HNF1A* promoter has been associated with MODY3 (Gragnoli et al., 1997). Indirectly, HNF4A is linked to many human diseases via the target genes it regulates. Due to the impact on the majority of apolipoproteins in the liver, a role in atherosclerosis is suggested (Sladek and Seidel, 2001). Increasing evidence links *HNF4A* misregulation to the pathogenesis of various human cancers (Tanaka et al., 2006). The tumor repressive effect is supported by findings that HNF4A inhibits cell proliferation in various cell types, including murine hepatocellular carcinoma cells (Lazarevich et al., 2004; Yin et al., 2008), endothelial lung and embryonal carcinoma cells (Chiba et al., 2005), insulinoma cells (Erdmann et al., 2007) as well as embryonic kidney cells (Lucas et al., 2005; Grigo et al., 2008).

2.1 Diabetes

The pancreas is comprised of exocrine and endocrine (<5%) parts. The latter consists of the islets of Langerhans which includes five cell types: glucagon-producing α-cells, insulin-producing β-cells, somatostatin-producing δ-cells, ghrelin-producing ε-cells and pancreatic polypeptide-producing cells. Heterozygous mutations in the coding sequence of the human *HNF4A* gene or in the P2 promoter lead to MODY1 (Bell et al., 1991; Yamagata et al., 1996; Harries et al., 2008), while mice heterozygous for *Hnf4a* show no signs of diabetes (Stoffel and Duncan, 1997). This form of type 2 diabetes mellitus (T2DM) is characterized by an autosomal dominant mode of inheritance, early onset around 20 to 40 years of age and impaired glucose-stimulated insulin secretion due to pancreatic β-cell dysfunction (Yamagata et al., 1996; Ryffel, 2001; Owen and Hattersley, 2001). Infants heterozygous for *HNF4A* may exhibit macrosomia and hypoglycemia at birth, reflecting increased insulin secretion *in utero* and during the neonatal period, respectively (Pearson et al., 2007). However, pancreatic β-cells usually produce adequate insulin at first and insulin deficiency is slowly progressive

resulting in overt hyperglycemia typically in early adulthood (Hattersley, 1998). The age-related penetrance varies considerably, but by the age of 55, about 95% of mutation carriers have developed diabetes (Frayling et al., 2001). Since many studies exclude a dominant-negative effect of the mutated *HNF4A*, but rather imply a loss-of-function mechanism, a haploinsufficiency mechanism is discussed (Stoffel and Duncan, 1997; Sladek et al., 1998; Navas et al., 1999; Lausen et al., 2000). In accordance with that assumption, are the identified mutations in the P2 promoter of *HNF4A*. The complex regulation of *HNF4A* via both promoters, resulting in various isoforms at different time points in the pancreas has been described above. The distinct isoforms give rise to proteins with different properties functioning in a unique network. Hence, depending on the location of the mutation, different isoforms and subsequent interaction partners are affected, which might at least in part explain the differential diabetic phenotype of *HNF4A* mutation carriers (Harries et al., 2008; Harries et al., 2009). Up to date 45 different *HNF4A* mutations in 190 patients from 58 families have been identified (Harries et al., 2008). The R154X MODY mutation, which results in a truncated protein lacking most of the ligand binding domain (Lindner et al., 1997; Laine et al., 2000) is used in this work. Given the large number of genes regulated by HNF4A, a pleiotropic phenotype is expected. However, MODY1 patients show only few symptoms in other organs (Froguel and Velho, 1999). In some *HNF4A* mutation carriers low levels of triglycerides, lipoprotein(a) and apolipoproteins (AII and CIII) have been noticed, indicating a primary hepatic defect (Lehto et al., 1999; Shih et al., 2000).

The common late-onset T2DM is characterized by relative insulin deficiency due to defective insulin secretion and/or insulin sensitivity (Martin et al., 1992; DeFronzo et al., 1992; Weyer et al., 1999). Although this complex heterogenous disease is considered a polygenic disorder, little is known about the responsible genes. Several groups have observed linkage of T2DM to chromosome 20q12-q13.1, the region *HNF4A* is localized in (Zouali et al., 1997; Bowden et al., 1997; Ghosh et al., 1999; Klupa et al., 2000; Permutt et al., 2001). Hence, an important role for *HNF4A* in T2DM is suggested (Gupta and Kaestner, 2004), which is significantly downregulated in pancreatic islets of patients with T2DM (Gunton et al., 2005). One study reported that a deletion of seven base pairs in the proximal promoter, deleting a single putative Sp1 binding site, can confer a severe form of T2DM causing renal target organ damage (Price et al., 2000). Single nucleotide polymorphisms (SNPs) in the promoter area as well as in exon 1-3 of *HNF4A* were associated with T2DM (Silander et al., 2004; Love-Gregory et al., 2004; Damcott et al., 2004). Furthermore, *Hnf4a* was shown to be essential for adult β-cell mass expansion upon increased metabolic demand, the failure of which is a hallmark of T2DM (Dickson and Rhodes, 2004; Gupta et al., 2007). In addition

there is evidence that loss of *Hnf4a* in other organs such as liver contribute to the progression to T2DM. (Zhu et al., 2003; Gupta et al., 2005).

Recently, microRNAs (miRNAs) were shown to be required during pancreas development by conditional *Dicer* knockout early in pancreas development in mice (Lynn et al., 2007). Although severe defects were observed in all pancreatic lineages, the β-cells were reduced the most. miRNAs are reported to play significant roles in insulin production, action and secretion as well as in diverse parts of glucose and lipid metabolism, indicating a critical role in the pathogenesis and progression of diabetes (Tang et al., 2008; Pandey et al., 2009). An example is miR-375, which is the most abundant intra-islet miRNA (Bravo-Egana et al., 2008) and was shown to directly target myotrophin (*Mtpn*), which inhibits insulin secretion and 3'-phosphoinositide-dependent protein kinase-1 (*PDK1*). miR-375 suppresses glucose-stimulated insulin secretion in a calcium independent manner, while inhibition of miR-375 enhanced insulin release (Poy et al., 2004; El Ouaamari A. et al., 2008). Furthermore, a role in pancreatic β-cell development is suggested due to decreased total β-cell mass and insulin levels in mice with homozygous deletion of miR-375 (Poy et al., 2007). Several other miRNAs have been experimentally linked to diabetes (Tang et al., 2008; Pandey et al., 2009). A few miRNA microarrays have been performed, comparing miRNA expression in mouse embryonic pancreas at two different developmental stages (Baroukh et al., 2007), in the mouse insulinoma cell line MIN6B1 exposed to fatty acid (palmitate; Lovis et al., 2008) and in MIN6 cells in response to changes in glucose concentrations (Tang et al., 2009). Although those analyses shed some light on the potential role of miRNAs in diabetes, it is not known, whether miRNAs are dysregulated in T2DM or MODY and whether they influence *HNF4A* expression.

2.2 Cancer

Hepatocellular carcinoma (HCC) is one of the world´s most common cancers. Even though epidermal growth factor (*EGF*) and transforming growth factor α (*TGF- α*) seem to play an important role (Tonjes et al., 1995), the molecular mechanism underlying HCC progression remains obscure. HNF4A is known to be a central regulator of the differentiated hepatocyte phenotype (Li et al., 2000; Hayhurst et al., 2001; Parviz et al., 2003). Initially, differences in the biologic properties of experimental systems and tumor samples gave rise to conflicting reports concerning the role of *HNF4A* in HCC progression (Stumpf et al., 1995; Flodby et al., 1995; Kalkuhl et al., 1996; Xu et al., 2001; Choi et al., 2004). However, dysfunction of *HNF4A* due to structural aberrations or modification of upstream regulatory signaling cascades, has been associated with the progression of rodent and human HCC and

contributes to accelerated cell proliferation, loss of epithelial morphology, dedifferentiation and the ability for invasion and metastasis (Lazarevich and Fleishman, 2008). Furthermore, re-expression of *HNF4A* in dedifferentiated hepatoma cells results in partial reversion of the malignant phenotype both *in vitro* and *in vivo* (Lazarevich et al., 2004; Yin et al., 2008). Usually the *HNF4A* P1 promoter is active in the adult liver and decreased P1 promoter expression has been reported in HCC (Tanaka et al., 2006). Recently a switch from P1 to P2 expression was detected in transgenic livers and HCCs of *EGF* overexpressing mice and human HCCs. The switch to fetal liver programs in HCC is presumed to predispose liver cells to malignant transformation prior to loss of *HNF4A* expression (Niehof and Borlak, 2008).

Renal cell carcinoma (RCC) is a type of kidney cancer that accounts for 3% of all malignancies and is classified into different subtypes including clear cell (cc), papillary (p), chromophobe (ch) and colleting duct (c) RCC (Kovacs et al., 1997). Those carcinomas are associated with distinct molecular alterations and different clinical outcomes. ccRCC is the most common and aggressive form in adults, accounting for 70-80% of kidney cancers (Jones and Libermann, 2007). The genetics of ccRCC are distinctive, but in most cases somatic or germline inactivating mutations in the von Hippel-Lindau (*VHL)* gene have been reported (Kaelin and Maher, 1998; Dalgliesh et al., 2010). Under normal oxygen pressure VHL causes the degradation of hypoxia-inducible factors (HIFs). *VHL* inactivation results in accumulation of HIFs which triggers transcription of genes such as *VEGF*, *PDGF-β*, *TGF-α* and *EPO* involved in angiogenesis, cell growth, migration and proliferation (Gnarra et al., 1993; Calzada and del, 2007; Rathmell and Chen, 2008). However, other molecular factors associated with RCC initiation and progression are largely unknown. To gain insight into the mechanism of RCC, several microarray analyses have been performed over the years. However, there is very little agreement as to which genes are differentially regulated among these studies (Lenburg et al., 2003). Those genes repeatedly identified as differentially expressed genes in RCC are involved in a broad range of processes such as glycolysis, cell adhesion, signal transduction, or nucleotide metabolism (Greenman et al., 2007). However, due to the various discrepancies among the studies, genes that failed to be identified multiple times might still be essential for RCC progression. Gene specific analyses are needed to clarify which factors are indeed associated with RCC. Expression of *HNF4A* is 4.7 fold downregulated in RCC compared to normal tissue (Lenburg et al., 2003). Furthermore, the amount and DNA binding activity of HNF4A is reduced in RCC compared to normal tissue (Sel et al., 1996). Overexpression of *HNF4A* in the HEK293 cell line results in a decrease in cell proliferation and is accompanied by a failure of cells to grow in an epithelium-like monolayer (Lucas et al., 2005). HNF4A dependent microarray analyses in those cells revealed several target genes that have been shown to be deregulated in RCC (*ACY1*, *WT1*,

SELENBP1, *COBL*, *EFHD1*, *AGXT2L1*, *ALDH5A1*, *THEM2*, *ABCB1*, *FLJ14146*, *CSPG2*, *TRIM9* and *HEY1*; Lucas et al., 2005). HNF4A has been described to function in a network of transcription factors including HNF1A and HNF1B that control gene expression in embryonic and adult tissues, particularly in liver, pancreas and kidney (Ferrer, 2002; Harries et al., 2009). Misregulation of *HNF1A* and *HNF1B* has been suggested as predisposing factors contributing to renal tumors (Sel et al., 1996; Rebouissou et al., 2005). *HNF1B* is expressed along the length of the nephron, whereas *HNF1A* expression is restricted to the proximal tubules comparable to *HNF4A*. The two most common forms of RCC, ccRCC and pRCC, originate from the proximal tubules as well. mRNA expression of *HNF1A* and *HNF4A* seems to be co-regulated in tumor and non-tumor renal tissue (Rebouissou et al., 2005) and disruption of the HNF4A/HNF1A pathway is assumed to be a molecular event contributing to renal cell carcinogenesis (Sel et al., 1996). Taken together, loss of HNF4A function might contribute to the progression of RCC (Lucas et al., 2005). However, so far no mutation in the *HNF4A* gene has been identified (Lausen et al., 2000; Dalgliesh et al., 2010) that may explain the downregulation of *HNF4A* in RCC.

Various mice with conditional *Dicer* knockout in different parts of the kidney have been generated, revealing a critical role for miRNAs in kidney development and maintenance of function (Saal and Harvey, 2009). In addition, several miRNA expression profiles from mouse, rat and human kidney, identified an overlap of 73 miRNAs with conserved expression in the kidney (Saal and Harvey, 2009). For a few miRNAs a specific target and function have been described and some of them have been linked to kidney diseases such as diabetic nephropathy and polycystic kidney disease (Saal and Harvey, 2009; Kato et al., 2009). miRNA expression profilings in RCC have revealed a large number of miRNAs that are either up- or downregulated in the tumors compared to normal tissue (Gottardo et al., 2007; Dutta et al., 2007; Kort et al., 2008; Nakada et al., 2008; Jung et al., 2009; Petillo et al., 2009; Huang et al., 2009b; Chow et al., 2010; Juan et al., 2010). A recent study set out to identify direct mRNA targets of miRNAs dysregulated in RCC (Liu et al., 2010). The method is mainly based on the anti-correlation of miRNA/mRNA levels strongly dysregulated in tumor versus normal cells of the same patient. Several miRNA/mRNA pairs were identified and the reduction of *SEMA6A* upon pre-miR-141 expression was confirmed by semi-quantitative RT-PCR. Another study reported the downregulation of Kallikrein-related peptidase 1 (KLK1) protein by miR-224 and a decrease in luciferase activity of a *KLK1* reporter upon let-7f transfection (White et al., 2010). Even in those two examples where functional assays were applied, the specific interaction of the miRNA with the target site in the mRNA was not proven. *Ago1* is expressed at a low to medium level in most tissues, but particularly high in embryonic kidney. In Wilms` tumor, the most frequent renal tumor in children, that lack the

Wilms` tumor suppressor gene *WT1*, *Ago1* expression is increased (Carmell et al., 2002). Despite good indication for miRNA misregulation in RCC, direct linkage of those miRNAs to the corresponding mRNAs with regards to RCC by functional assays is still missing.

3 Transcriptional and posttranscriptional regulation

3.1 Promoter regulation by transcription factors

Transcription is the first step of a process that converts the encoded information from the DNA into RNA which is then translated into protein. Gene expression is regulated at several steps, but regulation at transcription initiation is most commonly studied (Maston et al., 2006). A promoter is composed of a core promoter and proximal regulatory elements which together usually span less than 1 kb. Distal regulatory elements can include enhancers, silencers, insulators, and locus control regions (LCR) which can be spread up to 1 Mb away from the promoter. All *cis*-acting transcriptional regulatory elements are targeted by *trans*-acting transcription factors that can either enhance or repress transcription. In case of protein coding genes, general transcription factors, required for transcription of almost all genes, assemble on the core promoter, direct RNA polymerase II to the transcription start site and can cause basal transcription. About 1850 promoter specific transcription factors have been discovered that bind to upstream regulatory elements (6-12 bp DNA binding site) and greatly enhance transcriptional activity in a spatial and temporal fashion. The numerous transcription factors are distinguished from each other by different DNA-binding domains such as zinc finger (Laity et al., 2001), helix-turn-helix (Wintjens and Rooman, 1996), basic leucine zipper domain (Vinson et al., 2002) and many more (Pabo and Sauer, 1992). Interaction of different regulatory elements is achieved by looping out intervening DNA and coactivators often provide a link between different proteins without binding to DNA themselves (Maston et al., 2006). However, transcriptional regulation is even more complex and is influenced by chromatin structure and by histone modifications such as methylation and acetylation (Li et al., 2007a). Transcriptional elongation, in which the RNA transcript is synthesized, is followed by the termination process, when dissociation of the polymerase, DNA template and RNA transcript takes place.

As sequence specific binding of transcription factors to promoters is a critical component of transcriptional control, sequence variations in the target site may alter or abolish the binding capacity (Kadonaga, 2004). Disruption of the normal process of gene expression, subsequently increases or decreases the amount of mRNA and thus protein (Cooper, 2002).

Mutations in transcription factor binding sites likely underlie a substantial component of the phenotypic variability within and across species (Wray, 2007). Furthermore, several mutations in the different transcriptional regulatory elements have been linked to human diseases (Maston et al., 2006) such as β-thalassemia (Hardison et al., 2002), Bernard-Soulier syndrome (Ludlow et al., 1996) and pyruvate kinase deficiency (Manco et al., 2000; van et al., 2003). In hemophila B (Crossley and Brownlee, 1990; Reijnen et al., 1992; Carew et al., 2000) and MODY3 (Gragnoli et al., 1997), the mutations are in part located within HNF4A binding sites. Despite the known impact of promoter mutations on gene expression, promoter analysis is not a regular part of DNA diagnostics and of a total of 85,558 registered mutations in the Human Gene Mutation Database (HGMD) only 1.6% are regulatory (Stenson et al., 2009). The majority of those regulatory mutations are located between nucleotides +50 and -500 from the transcription start site (de Vooght et al., 2009). In addition, 59% of functional SNPs were identified in the first 500 nucleotides upstream of the transcription start site in human promoters (Rockman and Wray, 2002). Sequence variations identified in the *HNF4A* P2 promoter that are linked to MODY1 have so far been located in close vicinity to the transcription start site as well. The first identified *HNF4A* promoter mutation was a heterozygous -146T>C substitution that impairs binding and attenuates the transactivation potential of the β-cell-specific transcription factor insulin promoter factor-1 (IPF-1; Thomas et al., 2001; Hansen et al., 2002). The second mutation causing reduced *HNF4A* activation is located within the HNF1 binding sites at position -181G>A and impairs binding of the transcription factor HNF1A (Hansen et al., 2002). Further artificial mutations in various transcription factor target sites in the regulatory elements of *HNF4A* have been shown to interfere with gene expression, but have not yet been identified in any diseases (Bailly et al., 2009). In other cases, rare variants in the P2 promoter of patients have either not correlated with diabetes and/or failed to cause an impaired function *in vitro* (Mitchell et al., 2002; Vaxillaire et al., 2005). It is known that effects of promoter mutations are often subtle and difficult to detect (de Vooght et al., 2009). Interestingly, a -192C>G mutation in the *HNF4A* P2 promoter is linked to diabetes in several families as revealed by two independent studies and was even shown to disrupt binding of an unidentified protein *in vitro* (Ek et al., 2006; Raeder et al., 2006b). However, reporter gene assays did not confirm an effect of this mutation *in vitro*.

3.2 3'UTR regulation by RNA-binding proteins

Transcription is intimately linked to processing of pre-mRNA (Proudfoot et al., 2002; Rosonina et al., 2006; Moore and Proudfoot, 2009). In human cells, the polyadenylation machinery that recognizes and processes poly(A) sites has been shown to involve about 90

protein factors (Shi et al., 2009). Both upstream (e.g., PAS) and downstream (e.g., U-rich and GU-rich) elements surrounding a poly(A) site are critical for mRNA polyadenylation (Hu et al., 2005; Nunes et al., 2010). The length of the poly(A) is species specific and in mammals about 150-250 nucleotides long (Brown and Sachs, 1998). About half of the genes in mammals contain multiple poly(A) sites that produce transcript variants with different 3'UTRs or coding regions if the poly(A) site is located within an alternative intron (Tian et al., 2005). The length of 3'UTRs varies a lot within a species, ranging from several nucleotides to a few thousand and is on average about one thousand nucleotides long (Mignone et al., 2002). Alternative 3'UTRs are usually about two fold longer than constitutive regions and contain more *cis*-elements (Ji et al., 2009). Hence, variant 3'UTRs have been shown to alter mRNA metabolism depending on the different *cis*-elements located in the corresponding 3'UTR (Majoros and Ohler, 2007; Ji et al., 2009; Mayr and Bartel, 2009). Although posttranscriptional regulation was long neglected in research in contrast to transcriptional control, it has become evident that the former process is equally as important for normal cell function and that its dysfunction is linked to the pathogenesis of many diseases (Danckwardt et al., 2008; Chatterjee and Pal, 2009). Eukaryotic 3'UTRs contain several types of repeats including short interspersed repetitive elements (SINEs), long interspersed repetitive elements (LINEs), minisatellites and microsatellites (Mignone et al., 2002). In general there are two main classes, regulatory proteins (Moore, 2005) and miRNAs (Bartel, 2009; Inui et al., 2010) that target *cis*-elements and mediate the 3'UTR dependent control of mRNA localization, stability, translation and even transcriptional initiation (Pesole et al., 2000; Chatterjee and Pal, 2009; Thomas et al., 2010).

Localization of mRNAs to different subcellular regions allows for a spatial and temporal specific regulation of protein expression due to local stimuli (Martin and Ephrussi, 2009; Meignin and Davis, 2010). Furthermore, translation of localized mRNAs is more efficient than transporting each protein one by one to a specific region. Several localized mRNAs have been reported in various species and processes such as *bicoid*, *oskar* and *nanos* mRNAs in *Drosophila* (Johnstone and Lasko, 2001) and *VegT* in *Xenopus* (King et al., 2005). In addition, localization of several mRNAs has been identified during brain development (Lin and Holt, 2007), but also in the mature brain (Martin and Zukin, 2006). Localization is determined by the sequence of *cis*-acting elements located mainly in the 3'UTR of mRNAs (Oleynikov and Singer, 1998; Martin and Ephrussi, 2009). Those elements ranging in length from five to several hundred nucleotides are often repeated and a combination of unique elements mediates different functions in mRNA localization (Macdonald et al., 1993; Lewis et al., 2004; Martin and Ephrussi, 2009). Although, no clear consensus motif has been established yet, bioinformatic analysis suggests that repeats of CAC motifs may be important

(Betley et al., 2002). RNA-binding proteins targeting those elements seem to be loaded on the mRNA already during transcription and nuclear mRNA processing and often function both in localization and translational regulation (Martin and Ephrussi, 2009). mRNAs are transported together with RNA-binding proteins within large RNA transport particles by motor proteins along cytoskeletal elements (Oleynikov and Singer, 1998; Meignin and Davis, 2010). During delivery, mRNAs are often translationally silenced in part through the association with eukaryotic translation initiation factor-4G and get re-activated appropriately (Besse and Ephrussi, 2008).

The control of mRNA stability is crucial, since changes in mRNA turnover alters the abundance of the corresponding protein. Hence, mRNAs of early-response-genes have half-lives of 5 to 30 minutes, whereas other mRNAs (e.g., *β-globin*) are stable for several hours or even days (Laroia et al., 1999). The most common *cis*-acting elements are the highly conserved AU-rich elements (AREs) that have been identified in mRNAs of functionally diverse proteins (Chen and Shyu, 1995; Khabar, 2005; Halees et al., 2008). AREs differ in their sequence feature, but are divided broadly into three classes: (1) 1-3 copies of AUUUA motifs including nearby U-rich region or U stretch; (2) minimum of two overlapping copies of the nonamer UUAUUUA(U/A)(U/A) in a U-rich region; (3) lacks a core AUUUA sequence but has a U-rich region (Chen and Shyu, 1995; Chen et al., 2006). Several ARE-binding proteins (AREBPs) have been identified (e.g., AUF1 and HuR) that can either promote or inhibit the degradation of mRNAs containing AREs in their 3'UTR in response to various stimuli such as development, stress and proliferation (Khabar, 2005; Barreau et al., 2006; Glisovic et al., 2008). Various other elements have been described to impact the stability of its mRNAs such as cytosine- or pyrimidine-rich elements, AG-rich elements and iron-responsive elements (IREs; Chen et al., 2006).

Translational regulation provides a rapid mechanism to control gene expression and is often linked to mRNA localization. Such a dual role is described for the zipcode binding protein 1 (ZBP1) on *β-actin* mRNA. The block of translational initiation is assumed to be abolished upon phosphorylation of ZBP1 which leads to a decreased binding affinity to *β-actin* mRNA (Hüttelmaier et al 2005). AREs and AREBPs also seem to play a minor role in the translational control of some genes. T-cell intracellular antigen 1 (TIA-1) is a translational repressor known to target AREs located in the tumor necrosis factor-α (*TNFA*) and cyclooxygenase 2 (*COX2*) 3'UTRs (Piecyk et al., 2000; Dixon et al., 2003). Immunoprecipitation experiments identified a 30-37 nucleotide long motif in TIA-1 targeted mRNAs that is highly U-rich in the 5' segment and AU-rich in the 3' stem (Lopez, et al., 2005).

3.3 Regulation by miRNAs

The first miRNA gene (*lin-4*) was described in *Caenorhabditis elegans* 17 years ago (Lee et al., 1993; Wightman et al., 1993). Since then, miRNA research has been extensive, resulting in 15,632 mature miRNAs entries across 133 virus, plant and animal species in the records of miRBase (http://www.microrna.sanger.ac.uk, V 15.0 April 2010). The 940 discovered human miRNAs seem to play a crucial role in posttranscriptional gene regulation of almost every process investigated including development, cell proliferation, differentiation, apoptosis, signaling pathways, metabolism and life span (Kloosterman and Plasterk, 2006).

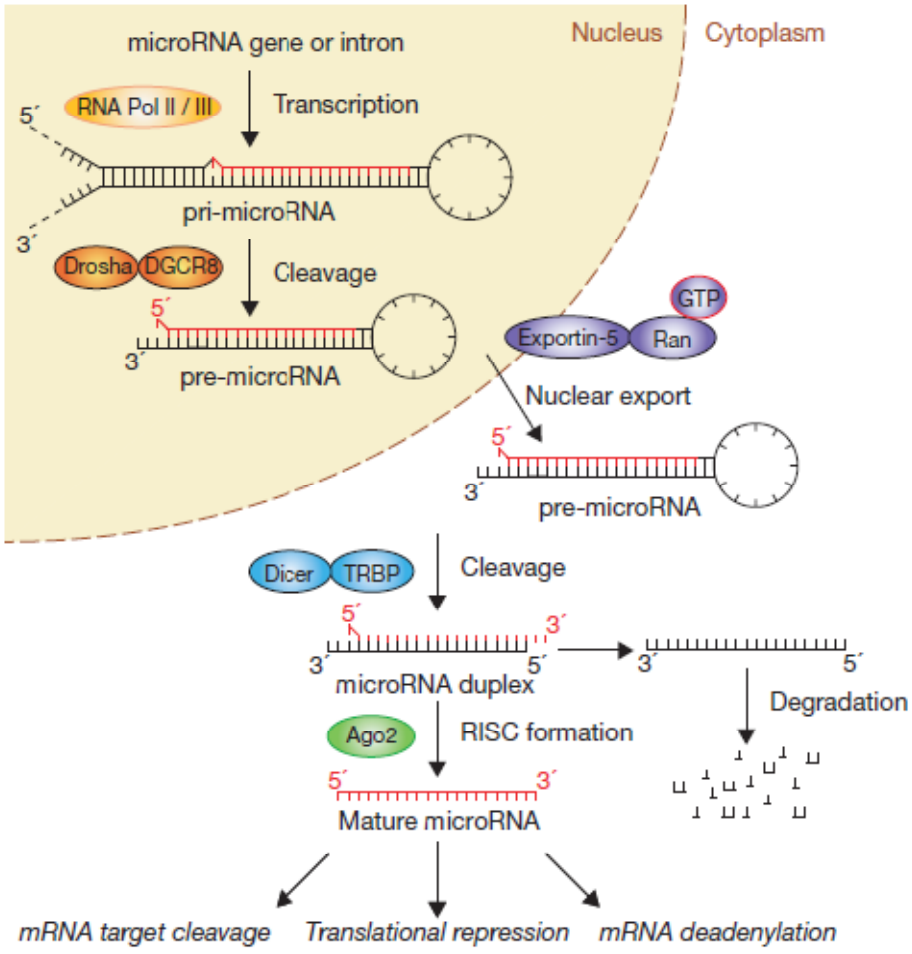

Figure 2: Biogenesis and mechanism of action of miRNAs.
The primary miRNA transcript (pri-miRNA) is generated mainly by RNA polymerase II (RNA Pol II). After cleavage, the precursor miRNA (pre-miRNA) is exported into the cytoplasm and processed by Dicer. The mature miRNA is loaded into the RNA-induced silencing complex (RISC) together with Argonaute (Ago) proteins and functions mostly by inhibiting gene expression. For a detailed description of miRNA biogenesis and function refer to text. (Figure from Winter et al., 2009).

miRNAs are 20-25 nucleotide-long noncoding RNAs (ncRNAs) that are mainly described to modulate gene expression by base-pairing to partially complementary sequences in the 3'UTR of its target mRNAs. Their genes can be monocistronic or expressed as clusters of

miRNAs from within one locus (polycistronic; Lagos-Quintana et al., 2001; Zeng, 2006). miRNA genes have been found within introns of protein coding genes and in introns and exons of longer ncRNAs, located in sense and anti-sense orientation (Rodriguez et al., 2004; Zeng, 2006). Furthermore they can be transcribed from their own promoter, promoters from nearby genes or be regulated together with their host genes. Usually miRNAs are transcribed by RNA polymerase II as part of longer primary constructs (pri-miRNA) including a 5' cap structure and 3' poly(A) tail (Bartel, 2004; Fig. 2). Those pri-miRNAs fold into a hairpin structures and are processed in the nucleus by a microprocessor complex, which consists of the RNase III type endonuclease Drosha and its partner DGCR8 (Denli et al., 2004). The resulting 65-85 nucleotide stem loop termed precursor miRNA (pre-miRNA) is actively transported into the cytoplasm by Exportin-5 and its cofactor RAN-GTP (Lund et al., 2004). Final processing is carried out by the RNase II type endonuclease Dicer in association with TAR RNA-binding protein (TRBP or TARBP2; Hammond, 2005). Usually one strand of the resulting 20-25 nucleotide mature miRNA duplex is degraded, while the mature miRNA is loaded into the RNA-inducing silencing complex (RISC) containing Argonaute (Ago) proteins (Gregory et al., 2005). Within the RISC complex, the miRNA guides target selection, resulting in mainly negative regulation of target mRNAs by different mechanism (Carthew and Sontheimer, 2009). Aside from this linear miRNA processing pathway, various miRNA specific differences have been described such as transcription by RNA polymerase III or RNA editing, allowing for multiple regulatory options to express and process individual miRNAs differentially (Winter et al., 2009). The importance of certain factors involved in miRNA biogenesis and function have been demonstrated by mouse models of *Dicer* (Bernstein et al., 2003), *Dgcr8* (Wang et al., 2007) or *Ago2* (Liu et al., 2004) knockout that displayed embryonic lethality.

The main mechanisms causing gene silencing influence mRNA cleavage, stability and translational repression of the target gene. The choice of mechanism depends in part upon the degree of complementarity between a miRNA and its target (Hutvagner and Zamore, 2002; Zeng et al., 2003). Perfect or near perfect base pairing promotes cleavage of the mRNA, a rare event in animals that depends on the slicer activity of Ago2 (Yekta et al., 2004; Liu et al., 2004; Meister et al., 2004). In vertebrates, miRNA base pairing is usually imperfect and is thought to cause inhibition of translation of the target mRNA, followed by a variable degree of mRNA degradation (Pillai et al., 2005). Several mechanisms of translational repression by miRNAs have been suggested, including blocking of both initiation and post-initiation steps and sequestrations of the mRNA targets together with miRNAs and Ago proteins into P-bodies, specialized cytoplasm compartments where translational repression and mRNA turnover takes place (Pillai et al., 2007). This miRNA mediated repression and

P-body localization of mRNAs seems to be reversible in response to certain environmental or developmental cues (Bhattacharyya et al., 2006; Pillai et al., 2007). Destabilization has been shown to occur by mRNA deadenylation, followed by decapping and subsequent 5'-3' exonucleolytic degradation (Wu et al., 2006; Behm-Ansmant et al., 2006; Chekulaeva and Filipowicz, 2009). A recent study combined ribosome and mRNA profiling analyses and it is claimed that >84% of the miRNA dependent decrease in protein production is caused by destabilization of target mRNAs while the influence on translational efficiency is modest (Guo et al., 2010).

In a few cases activation of gene expression was reported involving different circumstances. One group reported that miRNAs act as translational activators only in cells arrested in G0/G1 involving Ago2 and FXR1 proteins (Vasudevan and Steitz, 2007; Vasudevan et al., 2007). In another case, miR-10a enhanced translation by targeting the 5'UTR of ribosomal protein mRNAs (Orom et al., 2008). miR-122 stimulated translation of hepatitis C virus if bound to the 5'UTR, while binding to the 3'UTR of a reporter resulted in downregulation of its activity (Jopling et al., 2005; Jopling et al., 2008; Henke et al., 2008).

The RNA interference (RNAi) process triggered by small RNAs was thought to silence gene expression at the posttranscriptional level. Over the past few years, components of the RNAi machinery have also been identified to function in the nucleus. Transcriptional gene silencing in mammals was discovered by the use of promoter-directed, synthetic, small interfering RNAs (siRNAs; Morris et al., 2004; Ting et al., 2005) and shown to be accompanied by dimethylation of lysine 9 (H3K9) and trimethylation of lysine 27 (H3K27) in histone H3 (Ting et al., 2005; Kim et al., 2006; Weinberg et al., 2006; Han et al., 2007). Ago1 and Ago2 proteins are recruited to the promoter and involved in the formation of silent chromatin domains (Janowski et al., 2006; Kim et al., 2006). More recently, such synthetic, promoter-directed antigene RNAs (agRNAs) were also described to activate gene expression in human cancer cell lines (Li et al., 2006a; Janowski et al., 2007). Transcriptional activation is associated with demethylation of H3K9 (Li et al., 2006a) and increased di- and trimethylation of H3K4 (Janowski et al., 2007). Both, gene activation and inactivation by agRNAs is sequence specific, but no position dependent rules could be identified that cause gene silencing versus activation (Li et al., 2006a; Janowski et al., 2007). Recently, it has been reported that for both inhibition and activation, agRNAs recruit Ago proteins to antisense transcripts of the promoter and mediate formation of complexes with proteins and chromosomal DNA (Schwartz et al., 2008).

Currently, there are only a few reports in mammals providing evidence of miRNAs modulating gene expression by promoter recognition. In one study an epigenetic mechanism of miRNA directed transcriptional gene silencing has been suggested (Kim et al., 2008). They found that miR-320 targets the promoter location of the *POLR3D* gene from which it is

transcribed in antisense direction and showed that expression of this miRNA and protein coding gene, are anti-correlated. Another study reported the activating effect of miR-373 and pre-miR-373 in the presence of Dicer on *E-cadherin* and cold-shock domain-containing protein C2 (*CSDC2*), which was associated with an enrichment of RNA polymerase II at both promoters (Place et al., 2008). Activation was specific to the miR-373 sequence and dependent on the predicted target site within the promoter sequences.

Not all protein coding genes are regulated by miRNAs. Some genes that are involved in basic cellular processes seem to even avoid miRNA regulation due to short 3'UTRs that are specifically depleted of miRNA binding sites (Stark et al., 2005). However, about 30% of protein coding genes are estimated to be regulated by miRNAs (Lewis et al., 2005) with a high probability for transcription factors (John et al., 2004). Several target prediction programs have been developed to predict miRNA binding sites (John et al., 2004; Krek et al., 2005; Grimson et al., 2007). However, accurate prediction is made difficult since target sequences are very short and base pairing is mostly imperfect between the miRNA and regulated mRNAs. Perfect complementarily of the seven nucleotides between positions two to eight (seed sequence) of the miRNA and its target mRNA have been shown to be crucial in various cases, resulting in perfect seed sequences as a prerequisite in the majority of search algorithms (Lewis et al., 2005). Other features include thermodynamically stability of the duplex miRNA-mRNA, phylogenetic conservation, position within the 3'UTR, multiple target sites in a single mRNA by the same or different miRNAs and absence of stable secondary structures (Grimson et al., 2007). In contrast to the majority of programs available, RNA22 can upload and analyze a specific target sequence and miRNAs of interest and does not rely upon cross-species conservation (Miranda et al., 2006). Although many miRNA genes have been identified and potential targets have been bioinformatically predicted, the challenge is to provide experimental evidence of miRNA-mRNA interactions and identify their biological relevance (Kuhn et al., 2008).

Because miRNAs are important regulators of gene expression, misregulation or mutations of miRNAs seem to play key roles in several diseases including many types of cancer, genetic disorders and viral infections (Croce and Calin, 2005; Garofalo et al., 2008). They can function as oncogenes (e.g., miR-21 regulating the tumor-suppressor tropomyosin 1 (*TPM1*; Zhu et al., 2008)) or tumor suppressors (e.g., let-7 regulating *RAS* oncongene mRNAs; Johnson et al., 2005) and affect various steps of tumor formation from initiation to metastasis (Hanahan and Weinberg, 2000; Kent and Mendell, 2006; Shenouda and Alahari, 2009; Ventura and Jacks, 2009; Croce, 2009). Interestingly, dual oncogenic and tumor-suppressive roles are reported for several miRNAs, depending on the cell type and pattern of gene expression (Fabbri et al., 2007).

4 Objective of this study

HNF4A2 overexpression results in a significant decrease in cell proliferation. The aim of the first part of this project is to narrow down the number of potential HNF4A target genes to those crucial for proliferation control. For this purpose, a HNF4A isoform with no impact on cell proliferation decrease is sought. A cell line conditionally expressing this isoform will be established to subsequently determine its target genes by microarray analyses. Any identified gene is likely irrelevant for proliferation control and can thus be eliminated from the numerous potential HNF4A2 target genes identified previously. Detailed analyses, by means of qRT-PCR, RNAi and generation of inducible cell lines, of promising HNF4A target genes will be conducted to corroborate the impact on cell proliferation control.

The second part of this project addresses the transcriptional regulation of *HNF4A* itself. The impact of mutations identified within the P2 promoter, active in the pancreas, of patients with clinical signs of MODY1, will be analyzed in reporter gene studies. The aim is to link novel mutations in the regulatory sequences of *HNF4A* to MODY1.
In addition, evidence for the potential regulation of the *HNF4A* P1 and P2 promoter by miRNAs will be investigated. Considering an activating effect of miRNAs on gene promoters, downregulation of miRNAs in RCC or diabetes might contribute to the downregulation of *HNF4A* in those diseases due to dysfunction of the P1 and P2 promoter, respectively. To approach this hypothesis, the impact of miRNA depletion on both *HNF4A* promoters as well as truncated constructs will be investigated with reporter analyses in a cell system allowing for the conditional knock-down of Dicer.

The third part of this project will elucidate the previously unrecognized posttranscriptional regulation of *HNF4A*. Initially, the predicted as well as possible alternative *HNF4A* 3'UTRs will be assessed and their mode of regulation evaluated in reporter assays. Due to the impressive length of the 3'UTR, shortened constructs will be generated to facilitate the identification of crucial elements targeted by regulatory factors. To specifically investigate 3'UTR regulation by any miRNA present in the cell, the conditional Dicer knock-down assay will be applied. In the case of evidence of miRNA regulation, selected miRNAs upregulated in RCC should be gathered from different profiling studies. Due to the repressive effect of miRNAs on 3'UTRs, overexpression of a specific miRNA targeting the 3'UTR should result in the downregulation of *HNF4A*. Such an effect measured by luciferase assays should be abolished upon destruction of the potential target sites in the mRNA.

B Materials and Methods

The following procedures were taken from the methods collection of Sambrook et al. (1989), unless other sources are given as reference.

1 Chemicals, enzymes and solutions

Chemicals and enzymes were purchased from Aldrich, Amersham Biosciences, Bio-Rad, Boehringer Mannheim, Fluka, Gibco, Invitrogen, Merck, New England Biolabs, Pharmacia, Roche, Roth, Serva and Sigma in *pro analysis* quality, unless stated otherwise. Cell culture materials were purchased from TTP and Nunc. Oligonucleotides were obtained from Invitrogen.

2 General DNA and RNA procedures

Standard procedures such as gel electrophoresis, restriction digests, ligations, as wells as preparation of competent cells and bacterial transformation were carried out according to standard protocols (Sambrook et al., 1989). The “QIAquick PCR Purification Kit” (Qiagen) was used to purify DNA after enzymatic reactions. For mini-preparations of plasmid DNA up to 20 µg a method based on alkaline lyses was used. Large quantities of plasmid DNA up to 500 µg were extracted with the „Nucleobond PC500 AX Kit“ (Machery und Nagel).
Sequence analyses were carried out by the group of Prof. Küppers of the Institute of Cell Biology or by the sequencing service of the Institute of Human Genetics of the University Clinic Essen.

3 Oligonucleotides

Primers used for esiRNA generation:

Primer name	Primer sequence (5'-3')
T7	TAATACGACTCACTATAGGGA
T3	TAATACGACTCACTATAGGGAAATTAACCCTCACTAAAGGGA
HNF4A2 U	TAATACGACTCACTATAGGGAACCTGTTGC
HNF4A2 L	TAATACGACTCACTATAGGGAACTTCCTGC

Primers used for qRT-PCR:

Primer name	Primer sequence (5'-3')
GAPDH U	GTCAGTGGTGGACCTGAC
GAPDH L	ACCTGGTGCTCAGTGTAG
hCIDEB-E6 U	AGTACTTTCTTTGGGCCAAGTC
hCIDEB-E6 L	CCAAGCACAGCAAGGACAT
mCideb-E6 U	CAAAACACAGCAAGGACAT
mCideb-E6 L	AGTACTCTTTTAGGGCCAACTC

Primers used for Cideb cloning:

Primer name	Primer sequence (5'-3')
mCideb-cDNA U	CCGGAATTCAATGGAGTACCTTTCAGCCTTCA
mCideb-cDNA L	CCGCTCGAGTTAGGAGTGGAGGTGTCTCTGC

Underlined are the *Eco*RI and *Xho*I restriction sites.

Primers used for 3' RACE PCRs:

Primer name	Primer sequence (5'-3')
Oligo-dT-adapter	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT
Adapter AS	CCACGCGTCGACTAGTACTTT
Proximal PA S	CGGGATCCGGCTGCACTAAAATTCACTTAGGGTCG
Distal PA S	CGGGATCCTTCTTACTCTTCTGTGTTTTAACAAAA

Primers used to amplify the HNF4A 3'UTR:

The upper primers used to amplify parts of the HNF4A 3'UTR are always listed first and the lower primers second. The upper primers are either flanked by a *Spe*I (bold) or *Xba*I (bold and italics) restriction site, while the lower primers contain a *Not*I (underlined) site for ligation into the *Xba*I/*Not*I sites downstream of the *renilla* luciferase into the RL-Con plasmid.

HNF4A 3'UTR (nt)	Primer (5'-3')
1 - 3180	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCTTAGAAAACATATGCGCCATTT
1 - 2769	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCTGTCCCCCCAGCAAC
1 - 2573	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCCCTCCAGAAAGGGGTAGATTC
1 - 1746	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCGAGAAAAGCTGTCAAGAGTCATGA
1 - 630	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCCCCTGCCTGGTGCCT
1 - 449	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCCGGCCGCTGCCCCAAGTGCCAC
1 - 386	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCTAGGAGAGGAGAAGCACCAGG
1 - 378	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCGAGAAGCACCAGGCTAGGG
1 - 264	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCTGAAGGCAGTGGCTTCAAC
1 - 258	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCCAGTGGCTTCAACATGAGAAAA
1 - 249	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCCAACATGAGAAAGTTGTCCAAG
1 - 241	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCGAAAAGTTGTCCAAGGCAGTAGA
1 - 204	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCAAAGTCTTGTTATCCAGAGCAGG
1 - 196	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCGTTATCCAGAGCAGGGCGT
1 - 159	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCGTGGCCCTTAGGCCATG

1 - 151	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCTAGGCCATGTTCTCGGG
1 - 134	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCCCCTTCATCCTTCCCATTC
1 - 126	GG**ACTAGT**TAGCAAGCCGCTGGG
	GCATGCGGCCGCCCTTCCCATTCCTGCTCTG
423 - 875	GG**ACTAGT**CTGGGTCCAATTGTGGCA
	GCATGCGGCCGCTCCCATCTCACCTGCTCTACC
850 - 899	GC***TCTAGA***TGGCTGGTAGAGCAGGTGA
	GCATGCGGCCGCTGGCTCAGGCTGTTCTTTG
850 - 1013	GC***TCTAGA***TGGCTGGTAGAGCAGGTGA
	GCATGCGGCCGCCTCAGCCTGGTGTTCCAGA
850 - 1167	GC***TCTAGA***TGGCTGGTAGAGCAGGTGA
	GCATGCGGCCGCGTCCTCTCCAGCCCCAAG
850 - 1207	GC***TCTAGA***TGGCTGGTAGAGCAGGTGA
	GCATGCGGCCGCCCTCCTGATGTCACTCTGAT
850 - 1259	GC***TCTAGA***TGGCTGGTAGAGCAGGTGA
	GCATGCGGCCGCAGACAGTGCCTGGGAGTAAGG
850 - 1313	GG**ACTAGT**TGGCTGGTAGAGCAGGTGA
	GCATGCGGCCGCGGTTAATAGGGAGGAAGGGAGG
900 - 1013	GC***TCTAGA***AGGCCTAGTGGTAGTAAGAATCTAGC
	GCATGCGGCCGCCTCAGCCTGGTGTTCCAGA
900 - 1167	GC***TCTAGA***AGGCCTAGTGGTAGTAAGAATCTAGC
	GCATGCGGCCGCGTCCTCTCCAGCCCCAAG
900 - 1207	GC***TCTAGA***AGGCCTAGTGGTAGTAAGAATCTAGC
	GCATGCGGCCGCCCTCCTGATGTCACTCTGAT
1014 - 1167	GC***TCTAGA***GTCCTGATCAGCTTCAAGGAGT
	GCATGCGGCCGCGTCCTCTCCAGCCCCAAG
1014 - 1207	GC***TCTAGA***GTCCTGATCAGCTTCAAGGAGT
	GCATGCGGCCGCCCTCCTGATGTCACTCTGAT
1127 - 1207	GC***TCTAGA***TAATGCGGGTGAGAGTAATGAG
	GCATGCGGCCGCCCTCCTGATGTCACTCTGAT
1208 - 1313	GC***TCTAGA***AATAAGCTCCCAGGGCCTG
	GCATGCGGCCGCGGTTAATAGGGAGGAAGGGAGG
1288 - 1460	GC***TCTAGA***TAATCCTCCCTTCCTCCCTATT
	GCATGCGGCCGCCTTCCTAGTTGTGTGAGTTTCAGAA
1288 - 1513	GC***TCTAGA***TAATCCTCCCTTCCTCCCTATT

	GCATGCGGCCGCAAGAGCTCCTGTTCTGATCCAG
1288 - 1597	GC***TCTAGA***TAATCCTCCCTTCCTCCCTATT
	GCATGCGGCCGCTGTAGAAGGGAGCCGGAAG
1288 - 1666	GC***TCTAGA***TAATCCTCCCTTCCTCCCTATT
	GCATGCGGCCGCCAGCCTCAGGCCAATCTT
1288 - 1746	GG**ACTAGT**TAATCCTCCCTTCCTCCCTATT
	GCATGCGGCCGCGAGAAAAGCTGTCAAGAGTCATGA
1336 - 1746	GC***TCTAGA***TTCTCCTCCTCCCTCCCC
	GCATGCGGCCGCGAGAAAAGCTGTCAAGAGTCATGA
1392 - 1513	GC***TCTAGA***TTACAGAAGCTGAAATTGCGTTC
	GCATGCGGCCGCAAGAGCTCCTGTTCTGATCCAG
1392 - 1746	GC***TCTAGA***TTACAGAAGCTGAAATTGCGTTC
	GCATGCGGCCGCGAGAAAAGCTGTCAAGAGTCATGA
1461 - 1746	GC***TCTAGA***TGGCTGAGTCAGGACTTGAA
	GCATGCGGCCGCGAGAAAAGCTGTCAAGAGTCATGA
1725 - 2573	GG**ACTAGT**ATGACTCTTGACAGCTTTTCTCTCT
	GCATGCGGCCGCCCTCCAGAAAGGGGTAGATTC
2574 - 3180	GG**ACTAGT**AGAAACCCATTCCACCTTAATAAC
	GCATGCGGCCGCTTAGAAAACATATGCGCCATTT
2771 - 3180	GG**ACTAGT**AGCGTGGGCACAATTTC
	GCATGCGGCCGCTTAGAAAACATATGCGCCATTT
SV40 PA	GC***TCTAGA***TTCCCTTTAGTGAGGGTTAATGC
	GG**ACTAGT**ATCACCCTAATCAAGTTTTTTGGG

4 Plasmid constructions

The pcDNA5/FRT/TO expression vector (Invitrogen) was used to generate the Flp-In T-Rex 293 cell lines of interest. When co-transfected with the Flp recombinase expression plasmid pCSFLPe1 (Werdien et al., 2001) into the Flp-In host cell line, the pcDNA5/FRT/TO vector containing the gene of interest (GOI) is integrated in a Flp recombinase-dependent manner into the genome. Generation of pcDNA5/FRT/TO containing the myc-tagged open reading frame (ORF) of *HNF4A8* was described previously (Erdmann et al., 2007).

For *Cideb*, a full-length mouse cDNA clone was obtained from RZPD (IRAVp968B0424D6) and the ORF was amplified by PCR using primers (2.4 Oligonucleotides) containing restriction sites for *Eco*RI and *Xho*I. The digested PCR product was first cloned into the

*Eco*RI and *Xho*I sites of the pCS2+MT plasmid (Rupp et al., 1994) to add a myc-tag to the sequence and verified by sequencing. The myc-tagged *Cideb* was excised with *Bam*HI and *Xho*I and cloned into the same sites of pcDNA5/FRT/TO.

The *HNF4A* P2 promoter luciferase constructs were generated previously by introducing different PCR fragments of the P2 promoter sequence into *Xho*I and *Hin*dIII restriction sites of the pGL3-BasicII vector (Promega; Thomas et al., 2001).
The QuikChange II Site-Directed Mutagenesis Kit (Stratagene) was used to introduce point mutations into P2/-285 and obtain P2/-285(-136A>G), P2/-285(-169-C>T) and P2/-285 (-192C>G) (Wirsing et al., 2010).
To obtain *HNF4A* P1 promoter fragments upstream of the firefly ORF in the pGL3 Basic plasmid (Promega), P1 promoter fragments were excised from hHNF4-luc plasmid that was generated previously (Thomas et al., 2002). P1/-1114 was restricted with *Kpn*I and *Hin*dIII, P1/-590 with *Bam*HI and *Hin*dIII, P1/-281 with *Xma*I and *Hin*dIII and P1/-132 with *Bgl*II and *Hin*dIII and all fragments were ligated to corresponding sites within the pGL3 basic plasmid.
The CMV promoter was excised from the pCSGFP2 plasmid (Wild et al., 2000) with *Sal*I and *Hin*dIII and cloned into the *Xho*I and *Hin*dIII sites of pGL3 basic.

The *HNF4A* 3'UTR was amplified using a human BAC clone (RPCIB753B08466Q; imaGenes) as a template and primers containing flanking *Spe*I or *Xba*I and *Not*I sites (2.3 Oligonucleotides). The restricted *HNF4A* 3'UTR and all shortened fragments were cloned into *Xba*I/*Not*I sites of pRL-Con (Schmitter et al., 2006) and subsequently sequenced.
The sequence 631-3180 of the corresponding construct was excised from construct 1-3180 with *Xba*I/*Not*I and ligated into the same sites of the RL-Con plasmid. To delete the sequence containing negative element A and B and obtain constructs 1-844+1720-3180 and 631-844+1720-3180, two *Eco*RI sites present in the HNF4A 3'UTR sequence were used. The latter construct was also used to generate construct 631-849+1718-850+1719-3180, by re-introducing the excised *Eco*RI-fragment and selecting for clones containing this sequence in 3'-5' direction. To get constructs 1-636+850-1207 and 1-636+1288-1666, a construct containing the 5' 843 nt of the HNF4A 3'UTR was cut with *Xba*I/*Not*I and ligated into the *Xba*I/*Not*I restricted PCR products from 850-1207 and 1288-1666. To determine if negative element A and B function on RNA level, a *Xba*I/*Spe*I cleaved PCR product containing the SV40 PAS was introduced into the *Xba*I site upstream of negative element A and B constructs. Both orientations of the insert were identified by sequencing, resulting in construct 5'-3' PAS + 850-1207, 3'-5' PAS + 850-1207, 5'-3' PAS + 1288-1666 and 3'-5' PAS + 1288-1666.

5 Cell culture

All procedures with eukaryotic cells were performed under sterile conditions at a laminar flow hood.

5.1 Growth and maintenance of cell cultures

All cell lines were grown in DMEM or RPMI-1640 medium (Gibco-BRL) supplemented with 10% heat inactivated fetal calf serum (FCS), penicillin/streptomycin (100 U/ml) and 2 mM glutamine at 37°C under 8% CO_2 atmosphere and a relative humidity of 95%.
The host cell line Flp-In T-Rex 293 was cultured in DMEM supplemented with 15 µg/ml blasticidin and 100 µg/ml zeocin. In case of the stable cell lines derived from the host cell line, zeocin was substituted with 50 µg/ml hygromycin B. Dicer-kd/2b2 cells (Schmitter et al., 2006) were grown in DMEM supplemented with 10 µg/ml blasticidin and 50 µg/ml zeocin (Invitrogen). Expression of an anti-Dicer short hairpin was induced with 1 µg/ml of doxycycline (dox). HEPG2 cells were maintained in DMEM and HK120 cells (Stilla Frede, Institute of Physiology, University clinic Essen) were grown in RPMI-1640 medium. The INS-1 #5.3-19 cell line and INS-1 (HNF1B #1A) (Thomas et al., 2004) cells were cultured in RPMI-1640 medium supplemented with 1 mM sodium pyruvate, 10 mM HEPES, 50 µM mercaptoethanol, 10 µg/ml blasticidin and 200 µg/ml zeocin.
All cells were routinely passaged at approximately 80% confluence. The culture medium was removed, cells washed with 10 ml PBS and detached by incubation in 1 ml Trypsin/EDTA for 2-3 minutes in the incubator. Trypsin was inactivated by addition of 9 ml culture medium. Cells were diluted about 1:10 in fresh medium.

5.2 Cryoconservation

For long term storage, cell pellets were resuspended in 2 ml of cold freezing medium (culture medium without antibiotics and additional 10% FCS and 10% DMSO) after centrifugation for 5 minutes at 900 rpm. 1 ml aliquots were transferred into cryo tubes (1.8 ml). After cooling down to -80°C for 24-48 hours, the cells were stored in liquid nitrogen (-196°C).
Cell cultures were replaced from frozen stocks after 20-30 passages. Cells were thawed in a water bath at 37°C and then diluted with 9 ml cold medium. After centrifugation for 5 minutes at 900 rpm, the cell pellet was resuspended in fresh medium. After 24 hours medium was exchanged and antibiotics were added.

5.3 Cell counting

To determine the number of cells per unit volume of a suspension, a counting chamber (hemocytometer) was used. Prior to counting, cells were trypsinized and resuspended in fresh medium. Cells were counted in each of the four corner squares. The average was multiplied with 1 x 10^4 to obtain the cell number per milliliter cell suspension.

6 Generation of cell lines with the Flp-In T-Rex system

6.1 Flp-In T-Rex 293 cells

The Flp-In T-Rex 293 host cell line (Invitrogen) contains a single integrated FRT site which is recognized by the Flp-recombinase and stably expresses the tetracycline repressor. To obtain a cell line conditionally expressing the GOI, the host cell line was co-transfected 1:1 with the pcDNA5/FRT/TO plasmid containing the GOI and a hygromycin resistance gene and the Flp recombinase expression vector pCSFLPe1 (Werdien et al., 2001). 3 x 10^5 cells were seeded in 6- wells and transfected the next day with a total of 1.6 µg plasmid DNA. 100 µl of lipofectamine (Invitrogen) were diluted 1:17.5 in Optimem (Invitrogen) in a polystyrene (PS) tube. The DNA was diluted in Optimem as well and added to the tube drop-by-drop. While incubating for 15 minutes at room temperature, the cell culture medium was exchanged with 1.5 ml Optimem. The transfection mix was added to the cells and after four hours in the incubator, the medium was replaced with fresh DMEM including blasticidin, but no zeocin.
One day after the transfection, the cells were trypsinized and transferred to a 10 cm dish. To select for cells including the GOI, 24 hours later, fresh medium including hygromycin (and blasticidin) was added. After 10-14 days the selection process was completed. The hygromycin resistant cell colonies were pooled and expanded to obtain a polyclonal cell line. As soon as the cells reached confluence, aliquots of the new cell lines were stored in liquid nitrogen.
To verify that the GOI had integrated into the FRT site, each cell line was tested for the lack of β-galactosidase activity due to disruption of the functional *lacZ-zeocin* fusion gene. Cells expressing β-galactosidase cleave the substrate X-gal and transform it into a blue product easily visible by microscopy, while cells lacking that enzyme remain clear. The generated cell lines were seeded in 6-well dishes, washed 24 hours later with PBS and fixed in PBS containing 1% formaldehyde and 0.2% glutaraldehyde. Cells were washed in PBS again and incubated at 37°C for 1-24 hours with PBS containing 5 mM potassium ferricyanide, 5 mM

potassium ferrocyanide, 2 µM MgCl2 and 1 mg/ml X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside). After the incubation period, the cells were analyzed by light microscopy. Each cell line was only used for further experiments when containing less than 5% of blue cells.

6.2 Induction of cell lines with doxycycline

To induce expression of the stably integrated GOI, cells were cultivated with medium containing doxycycline (1 µg/ml). Doxycycline was used instead of tetracycline due to its longer half time of 48 hours versus 24 hours. Since doxycycline is dissolved in ethanol, control cells were treated with ethanol. If incubation times exceeded two days, medium including fresh doxycycline was exchanged regularly.

7 Analyzing cell morphology and cell proliferation

In the course of the experiments, cell morphology was monitored daily using a phase contrast microscope (Diavert, Leitz). Phenotypical changes of the cells were documented with digital fotography (Nikon Coolpix 4500).

The MTS assay (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega) was used to analyze cell proliferation. This colometric method is based on the metabolic activity of cells and thus determines the number of viable cells in proliferation. The tetrazolium compound MTS is bioreduced into the aqueous soluble formazan by dehydrogenase enzymes found in metabolically active cells, in the presence of the electron coupling reagent PMS. The absorbance of the formazan product is measured at 490 nm in a photometer and is directly proportional to the number of living cells in culture.
Cells were seeded in a 96-well plate at a density of 2-10x10^3 cells in 100 µl medium per well. Doxycycline or ethanol was added after 24 hours and the cells were incubated for 3-6 days. 20 µl MTS reagent was added to each well. After 60 minutes incubation at 37°C, OD_{490} was measured with a microplate photometer (Bio-Rad, Model 550). The measured values from the wells just containing medium, were used to determine the background. Each experiment was performed in triplicate.

8 Immunofluorescence microscopy

Immunofluorescence microscopy was used to visualize the presence and localization of target proteins in the cell. 1 x 10^5 to 3 x 10^5 cells were seeded per well in a 6-well dish on top of cover glasses. For fixation, cells were washed with PBS, fixed with methanol for 10 minutes at room temperature and washed again twice with PBS. To block unspecific binding of the antibody, the cells were incubated with PBS/10% goat serum for 60 minutes at 4°C. The primary antibody targeting the myc-tag (9E10 monoclonal) was diluted 1:4 in DMEM and incubated for 60 minutes at room temperature. The secondary antibody (anti-mouse Cy3; Jackson ImmunoResearch) was diluted 1:200 in PBS/10% goat serum for 60 minutes at 4°C protected from light. To visualize the nuclei, cells were incubated for 5 minutes at room temperature with Hoechst A 33342 (1:1000 in H_2O, Sigma) to stain the DNA and subsequently mounted with Vectashield (Vector-Laboratories). The preparations were analyzed with a fluorescence microscope (DM IRE2, Leica) and documented with an attached digital camera (DC 500, Leica) and the image analysis software Qfluoro.

9 Proteins

9.1 Total cell protein extract and quantification

To obtain whole cell protein extracts, the cells were detached from the petri dish by using trypsin and centrifuged for 5 minutes at 900 rpm at room temperature. The pellet was washed with 1 ml icecold PBS and transferred into a 1.5 ml microcentrifuge tube. After additional centrifugation (14,000 rpm, 1 min, 4°C) the cells were resuspended in icecold RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.1% SDS, 1% Natriumdeoxycholat, 1% Triton X-100) containing protease inhibitors (Sigma; P8340 1:500) and lysed 30 minutes on ice. Cell debris was separated by centrifugation (50,000 rpm, 10 min, 2°C) and the supernatant containing the whole cell protein extract was stored at -80°C.

The Bradford assay (Bradford, 1976) is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The concentration of whole cell protein extracts was determined using the Bio-Rad Protein-Assay reagent. 2-10 µl protein solution were diluted in 1600 µl H_2O in a cuvette and mixed with 400 µl Bio-Rad Protein-Assay reagent. After incubation for 5 minutes at room temperature, the solution was measured in a

Spectrophotometer (S2000 WPA) at OD_{595}. The OD_{595} of each sample was compared to a standard curve prepared with BSA (2-20 µg). RIPA buffer, in which the proteins were diluted, served as a control.

9.2 Discontinuous SDS polyacrylamide gel electrophoresis (SDS-Page)

Protein samples were separated electrophoretically on a denaturing SDS-polyacrylamide gel according to Laemmli (1970). Depending on the size of the proteins, a 7.5% or 10% resolving gel was used in combination with a 5% stacking gel in a vertical Mini-Protean Gel chamber (Bio-Rad). 2x Laemmli buffer (Tris-HCL pH 6.8, 4% SDS, 20% glycerol, 0.2% bromphenol blue, 10% β-mercaptoethanol) was added to each sample in appropriate volumes and samples were denatured at 95°C for 5 minutes before application to gel slot. Electrophoresis was performed in 1x SDS running buffer under 100 V. The prestained protein ladder "Precision Plus protein Standards, Dual color" (Bio-Rad) was used as a standard.

9.3 Western blot and protein detection

A semi-dry blotting chamber was used to transfer the separated proteins from the polyacrylamide gel to a nitrocellulose membrane. All components were soaked in 1x transfer buffer (8 mM Tris, 40 mM glycine, 0.0375% SDS, 20% methanol) for 2 minutes before stacking them between the anode and cathode plate of the blotting chamber (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad) in the following order: five Whatman papers, nylon membrane, gel, five Whatman papers. The transfer was performed for one hour at 2 mA/cm^2. After the transfer, the membrane was blocked to prevent non-specific binding of antibodies to the membrane by incubating with blocking agent (Amersham) diluted 1:20 in blocking buffer (150 mM NaCl pH7.5, 100 mM Tris) for one hour at room temperature. The membrane was further incubated over night at 4°C with the mouse monoclonal primary antibody against the myc-tag (9E10, lab specific) at a dilution of 1:5 in PBS-T (PBS, 0.1% Tween-20). After the primary antibody, the membrane was incubated with the horseradish-peroxidase-conjugated anti-mouse secondary antibody (NXA 931, Amersham) at a dilution of 1:5000 in PBS-T for one hour at room temperature. After each incubation, the membrane was washed three times with PBS-T at room temperature. Immunoreactivity was detected using the ECL Kit (Amersham Bioscience) according to manufacturer´s instructions. The membrane was wrapped in a plastic film and put in a cassette for exposure of the film (Hyperfilm™ ECL™, Amersham Bioscience) for 5 sec to 50 min, depending on signal intensity.

10 Microarray analysis

Microarray analyses were performed in collaboration with PD Dr. Ludger Klein-Hitpass (BioChip Lab, Institute of Cell Biology, University Clinic Essen).

10.1 Microarray chips

The Affymetrix Genechip HG_U133_2.0_Plus is a high density oligonucleotide microarray which detects 54,000 probe sets representing about 38,500 genes. Oligonucleotides, usually 25-mers, are directly synthesized onto a glass wafer by a combination of photolithography and solid phase chemical synthesis technology. Each gene sequence is represented by eleven pairs of oligonucleotide probes, present in millions of copies. A pair consists of a perfect match (PM) probe that is entirely complementary to the gene sequence and a corresponding mismatch (MM) probe that contains a single base substitution in the middle of the sequence, reducing binding of the corresponding transcript. This setup helps to determine the background and nonspecific hybridization that contributes to the signal measured for the PM probe. To obtain the absolute or specific intensity value for each probes set, the hybridization intensities of the MM probes are substracted from those of the PM oligonucleotides.

10.2 RNA isolation for microarray analysis

Total RNA was extracted from induced (+dox) and uninduced (-dox) HEK293 HNF4A8# 11 and HNF4A8 #14 cells using peqGold RNAPure (PeqLab) according to the manufacturer's instructions. For a 10 cm dish, 3 ml peqGOLD RNA pure were used and the isolated RNA was dissolved in 30 µl RNase-free water. The RNA was further purified using the RNeasy Mini Kit (Qiagen) according to the standard protocol including on column DNase digestion with the RNase-free DNase set (Qiagen). After determining the concentration in a ND-1000 Spectrophotometer (NanoDrop Technologies), the RNA was stored at -80°C.

10.3 Synthesis of cDNA, marking and hybridization

mRNA, comprising about 0.2-0.4% of total RNA was reverse transcribed into single-stranded cDNA using a T7-d(T)21 primer. The complementary cDNA strand was synthesized by DNA polymerase I, DNA ligase and RNase H. Using the T7 RNA polymerase recognizing the

introduced T7 promoter sequence, the double-stranded cDNA was used as a template to synthesize biotinylated antisense cRNA by *in vitro* transcription. Labeled cRNA was purified, fragmented and hybridized to the chip. Non-complementary cRNAs were removed by washing and the arrays were stained. Fluorescence intensity emitted by labeled cRNA/cDNA upon laser treatment was measured and quantified using a GeneArray Scanner (25000, Agilent). All steps were carried out according to the Affymetrix Gene Expression Analysis Technical Manual.

10.4 Data analyses

The Affymetrix Microarray Suite Version 5.0 software was used to process array images and determine signal intensities and detection calls, which are defined as present (P), absent (A) or marginal (M) for each probe set. To compensate for variations in the amount and quality of the cRNA samples and other experimental variables of non-biological origin, scaling across all probe sets of a given array to an average intensity of 1000 was conducted. Differentially expressed genes between induced and uninduced cells were determined by comparing the values of each probe set in a pairwise manner using the Affymetrix Microarray Suite 5.0 software, which calculates the significance (change *P*-value) of each change in gene expression based on a Wilcoxon ranking test. To identify target genes, the data was filtered by applying specified cut-offs using the Affymetrix Data Mining Tool 3.0 Software.

11 Quantitative real-time PCR

11.1 RNA isolation and cDNA synthesis for qRT-PCR

Total RNA was isolated from cells using peqGold RNAPure (PeqLab) according to the manufacturer's instructions. To prevent false results due to DNA contamination, the RNA samples were digested with DNase using the RNase-free DNase Set (Qiagen). The RNA concentration was determined in a ND-1000 Spectrophotometer (NanoDrop Technologies) and aliquots were frozen at -80°C. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) including random hexamers.

11.2 qRT-PCR

To obtain relative expression data of several genes, qRT-PCR was performed using the POWER SYBR Green PCR Master Mix from Applied Biosystems. This dye binds to dsDNA during amplification and upon excitation emits light. As PCR product accumulates, fluorescence increases. A dissociation curve stage after the PCR amplification was included to make sure only one sharp peak is detected at the melting temperature of the amplicon and no primer dimers are detected. The gene specific primers used are listed in section 2.3. Each reaction of 20 µl included the SYBR Green Master Mix, primers at a concentration of 0.3 µM and 2-4 ng cDNA. A 10 minute incubation period at 95°C was followed by 40 cycles comprised of 15 s at 95°C (denaturation) and 60 s at 60°C (annealing-extensions). Amplification and detection of the cDNA was carried out with the 7900HT Sequence Detection System (Applied Biosystems) and the corresponding software.
Gene expression was relatively quantified with the $2^{-\Delta\Delta Ct}$ method (2 power of [(Ct target - Ct reference) of calibrator - (Ct target - Ct reference) of sample] (Livak and Schmittgen, 2001). Hence, the averaged values of the reference gene (*GAPDH*) were substracted from the averaged values of the target gene to obtain ΔCt. To calculate ΔΔCt the ΔCt values of the cells treated with doxycycline were substracted from the ΔCt of the control cells (-dox) that served as the calibrator. Each cDNA was measured at least twice in one approach and control reactions without reverse transcriptase were included.

12 Gene inactivation using RNAi

RNAi is used to silence gene expression by using small RNAs complementary to target mRNAs (Echeverri and Perrimon, 2006). In this study the cost-efficient endonuclease-prepared short interfering RNA (esiRNA) method was applied (Kittler and Buchholz, 2003; Kittler et al., 2004; Kittler et al., 2007).

12.1 esiRNA generation

An esiWay silencer was used to generate *CIDEB* specific esiRNAs (p3000E01609, RZPD). The *CIDEB* sequence within the esiWay silencer plasmid (about 550 bp) is flanked by T7 and T3 promoter sequences for amplification by PCR. The T7 sequence was attached to the T3 primer used together with the T7 primer for the PCR reaction to be able to perform the *in vitro* transcription later on. The same primers were used to amplify the *tomato* sequence, coding for a red fluorescent protein, which had been cloned into the pBluescript II SK+ vector

in our lab previously (Roose et al., 2009). The *HNF4A* sequence was amplified using the pcDNA5/FRT/TO HNF4A2 vector as a template and gene specific primers linked to the T7 sequence. All primers are listed in section 2.3. PCR reaction was performed in volumes of 50 µl with the Pwo-DNA polymerase (Peqlab) in a Gene AmpPCR System 2400 thermocycler (Perkin Elmer). An aliquot of 4 µl was used to confirm the expected size of the amplicon on a gel.

The *in vitro* transcription was performed using the MEGAscript Kit (Ambion) according to manufacturer´s instructions, but in half the recommended volume. The reaction including 4 µl of the PCR product was incubated over night at 37°C. The RNA was annealed after an initial denaturation step at 95°C for 3 minutes and slowly cooled down to room temperature. The dsRNAs were digested for 30 minutes at 37°C, using the ShortCut RNase III (Biolabs) to restrict long dsRNAs to small RNAs, 18-25 bp of size. This digestion step was a modification to the original protocol (Kittler and Buchholz, 2003) as well as the following purification step using the RNeasy Kit (Qiagen) as described by Byrd and Watzl (Lab Times online, Lab Trick4: Money saving siRNA purification: http://www.labtimes.org/tricks/ index.html). The correct sizes of the RNA fragments were confirmed on gels after the *in vitro* transcription, RNA digestion and purification step. For the last two cases, a 5% gel for small fragments (Roth) was used. The esiRNA concentration was determined in a ND-1000 Spectro-photometer (NanoDrop Technologies).

12.2 esiRNA dependent cell proliferation assays

24 hours before transfection, 2 x 10^3 HNF4A2 #4 (Lucas et al., 2005) cells were seeded in a 96-well plate in DMEM medium without antibiotics. Transient transfections were carried out using ExtemeGene transfection reagent (Roche) according to manufacturer´s instructions. A reaction mix contained 0.1 µg esiRNA and 1µl transfection reagent. Four hours after the transfection process antibiotics were added to the cells as well as 1 µg/ml doxycycline for induction of *HNF4A2* expression or an equal amount of ethanol. After three days the medium was exchanged and after four days cell proliferation rate was determined using the MTS assay (Promega).

13 3' RACE PCRs

Total RNA was isolated from HEPG2 and HK120 cells as described above (11.1 RNA isolation and cDNA synthesis for qRT-PCR). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) together with an oligo-dT-adapter primer (2.3 Oligonucleotides).

PCR was performed (FailSafe PCR System, EPICENTRE) using a sense gene specific primer for the proximal and distal PA of *HNF4A* together with an antisense adapter primer (2.3 Oligonucleotides). PCR products were analyzed on an agarose gel and then cloned into pBluescript and sequenced or sequenced directly.

SYBR-Green real time PCR was performed as described above (11.2 qRT-PCR). Templates were determined in triplicate and the housekeeping gene *GAPDH* served as a reference. To check for DNA contamination, control reactions without reverse transcriptase were performed. The primers used were the same as for the standard PCR. To determine the abundance of different *HNF4A* 3'UTRs, $2^{-\Delta Ct}$ was calculated using *GAPDH* as a reference.

14 Transient transfections and luciferase assays

For each assay, 1 x 10^4 to 2.5 x 10^4 cells were seeded into a 96-well plate in 100 µl DMEM medium without antibiotics, 24 hours before transfection. The transfection mix was prepared in PS tubes and included a total of 40 ng DNA and 0.2 µl FuGene HD (Roche) diluted in 5 µl OptiMEM per well. The DNA mix was comprised of *renilla* reporter plasmids and firefly plasmids used for normalization of transfection efficiencies or firefly was used as a reporter and the *renilla* plasmid RL-Con served to control for transfection efficiency. The total amount of DNA was adjusted to 40 ng with Rc/CMV (Invitrogen) if necessary. After a 15 minute incubation period at room temperature, the mix was carefully added to the cell medium. 24 hours after transfection, cells were lysed by addition of 25 µl 1x lysis buffer (Promega) per well followed by incubation at room temperature with continual rocking for 15 minutes. A 5-15 µl sample of the lysate was transferred into a white 96-well plate to avoid crossover luminescence from neighboring wells. Firefly and *renilla* luciferase activities were measured sequentially with a luminometry (Centro LB 960, Berthold) using the Dual-Luciferase Reporter Assay Kit (Promega) according to the manuals instruction, but adding only 50 µl of the reagents. Specifics and alterations to this procedure are described below.

For *HNF4A* P2 promoter mutation analyses, HEK293 (HNF1B) (Senkel et al., 2005) and INS-1 (HNF1B #1a) (Thomas et al., 2004) cell lines, both containing a tetracycline inducible

HNF1B transgene were used. 30 ng of the P2 specific firefly promoter constructs were co-transfected with 0.01 ng pRL-Con for normalization of transfection efficiencies. Four hours after transfection, *HNF1B* expression was induced by the addition of 1 µg/ml tetracycline.

For Dicer knock-down experiments, expression of the short hairpin targeting *Dicer* (Schmitter et al., 2006) was induced with 1 µg/ml of doxycycline in total for three or seven days. To determine regulation of the *HNF4A* promoters by miRNAs, 8 ng of firefly promoter constructs were co-transfected with 0.01 ng pRL-Con. For miRNA dependent *HNF4A* 3'UTR analyses, transfection assays were performed using 0.08 ng of *renilla* reporter plasmids and 0.02 ng of the firefly construct. Four hours after transfection, addition of doxycycline was repeated and 48 hours after transfection, luciferase activities were determined. The normalized values for each construct obtained for uninduced cells, treated with ethanol, were always set to 100% and used for standardization.

In case of transfecting different 3'UTR fragments, the DNA mix was comprised of 0.05-0.08 ng RL reporter plasmid to obtain equal molar amounts and 0.02 ng firefly luciferase construct (pGL3). Normalized *renilla* activities in cells transfected with pRL-Con was always set to 100% and used for standardization.

To determine the impact of specific miRNAs on the *HNF4A* 3'UTR, 0.08 ng of the *renilla* reporter plasmids were co-transfected with 0.02 ng of the firefly construct and 50 ng of miRNA expression plasmids (pSM-122 (Lin et al., 2008), pCMV-miR-21 (Zhu et al., 2007) or pri-miR-34a (Lodygin et al., 2008)). As a negative control 50 ng Rc/CMV or 50 ng pSM-155 (Du et al., 2006) were used instead of the miRNA expression plasmid and the obtained normalized values were used for standardization (100%).

15 miRNA expression profile

RNA was isolated from HEK293 cells by using the mirVana^{TM} RNA isolation Kit (Ambion) according to the manufacturer's instructions. RNA samples (20 ng) were reverse transcribed (TaqMan MicroRNA RT Kit, Applied Biosystems) using eight different 48plex stem-loop RT primer pools. The cDNAs were quantified by real-time PCR using the corresponding 8x48 individual miRNA Taqman Assays in duplicate reactions (10 µl) containing 0.1 ng of cDNA, 1x Universal Master Mix and 1x assay. Data were analyzed by the ΔCT method using RNU48 as a normalization control. Microarray analyses were performed in collaboration with PD Dr. Ludger Klein-Hitpass (BioChip Lab, Institute of Cell Biology, University Clinic Essen).

16 *In silico* analyses

Target sites for 20 miRNAs (Table 2) were predicted within the 3180 nt *HNF4A* 3'UTR with RNA22 (Miranda et al., 2006) using 1, 7, 14 and -20 for unpaired bases, seed/nucleus in nucleotides, minimum number of paired-up bases and maximum folding energy in heteroduplex, respectively. The proximal miR-34a target site in the 5' 449 nt of the *HNF4A* 3'UTR was predicted with TargetScan (Lewis et al., 2003). The UTRdb database (Grillo et al., 2009) was used to identify regulatory motifs within the *HNF4A* 3'UTR. MIRb and MIRc were predicted using the RepeatMasker function from the UCSC Genome Browser of Human Feb. 2009 Assembly (http://www.genome.ucsc.edu/cgi-bin/hgGateway).

C Results

1 Search for proliferation relevant target genes regulated by HNF4A

Overexpression of *HNF4A2* causes a decrease in cell proliferation and morphological changes. An approach to narrow down the large number of predicted target genes of HNF4A involved in various processes, to those responsible for the proliferation decrease is based on a microarray analysis with an HNF4A isoform that has no impact on cell proliferation. All genes regulated by this isoform should not be relevant for the cell proliferation decrease and could thus be eliminated from the numerous HNF4A2 target genes determined by previous microarray analyses in HEK293 cells (Lucas et al., 2005; Grigo et al., 2008).

1.1 Generation and characterization of HEK293 cells conditionally expressing *HNF4A8*

HNF4A8, expressed from the P2 promoter is the alternative isoform to P1 specific *HNF4A2* and thus lacks the N-terminal activation domain (Sladek and Seidel, 2001). To analyze the effect of HNF4A8 on cell proliferation in HEK293 cells, a cell line conditionally expressing *HNF4A8* was generated. Using the Flp-In T-Rex 293 cell system (Invitrogen) and the pcDNA5/FRT/TO plasmid containing the myc-tagged *HNF4A8* sequence (Erdmann et al., 2007), three independent cell lines, HNF4A8 #9, HNF4A8 #11 and HNF4A8 #14, were generated. All three HNF4A8 cell lines are fully functional and show a comparable expression pattern to other established HNF4A cell lines as determined by the following assays. Insertion of the cassette containing the GOI into the FRT site disrupts the functional *lacZ-Zeocin* transcriptional unit. Lack of β-galactosidase activity, validated the integration of *HNF4A8* at the right locus (data not shown). To test for proper HNF4A8 expression upon addition of doxycycline, western blot analyses and immunofluorescence microscopy using a myc-specific antibody were performed. After induction with doxycycline for 24 hours, HNF4A8 was expressed in all three cell lines to a similar amount, while no protein was detected in uninduced cells (Fig. 3A). Loading two different amounts of protein lysate for each induced cell line resulted in a correspondent change in signal detection. Immunofluorescence analysis validated the doxycycline dependent expression of HNF4A8 in about 99% of the cells in all three cell lines and also confirmed the location of this transcription factor in the nucleus (Fig. 3B).

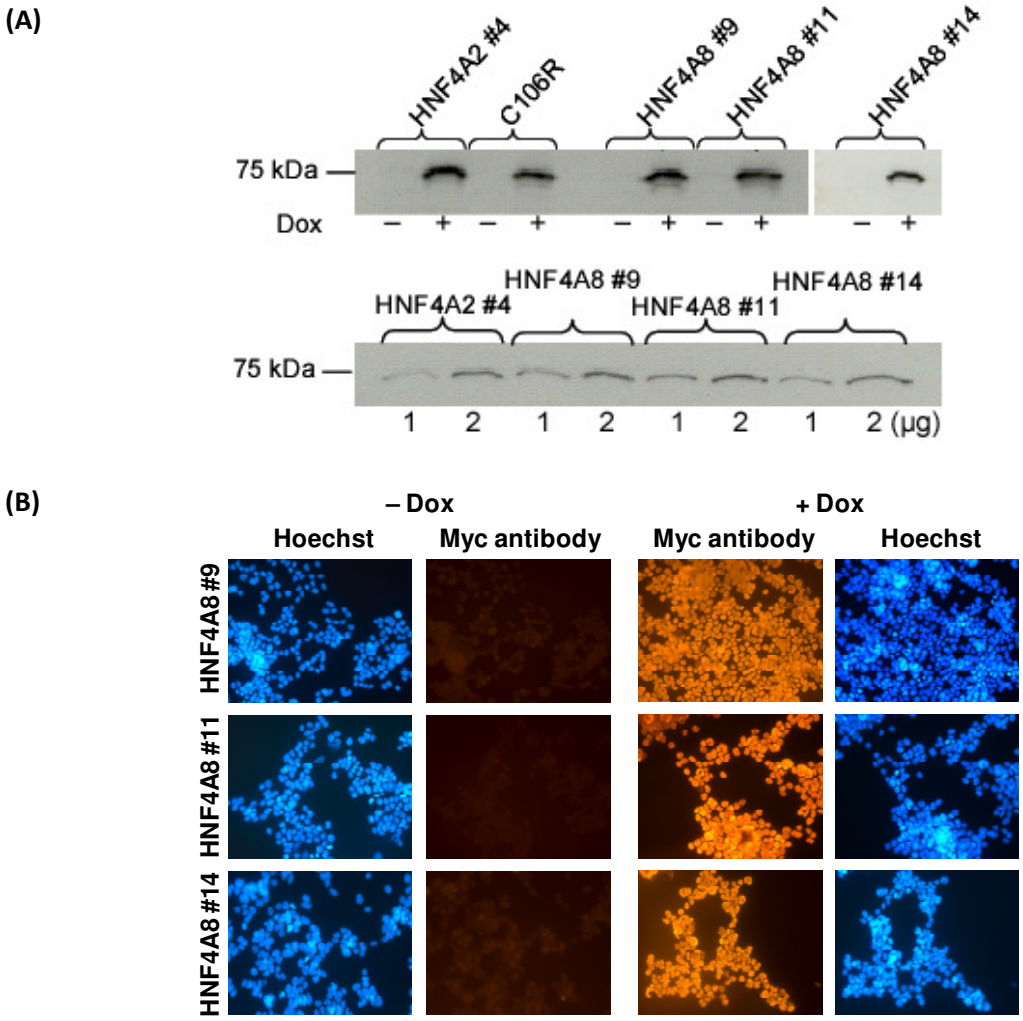

Figure 3: Establishment of HEK293 cells conditionally expressing *HNF4A8*.
Cells were treated with 1 µg/ml doxycycline (+) or ethanol (-) and expression of the different HNF4A isoforms was detected using the monoclonal antibody 9E10 that recognizes the myc-tag. **(A)** The upper part of the western blot image compares HNF4A expression of cells treated with doxycycline or ethanol for three days, while in the lower part two different amounts of protein extract of cells induced for 24 hours were probed. The artificial HNF4A mutant C106R has a point mutation in the second zinc-finger of the DNA-binding domain and acts as a loss-of-function mutation with a weak dominant negative effect (Taylor et al., 1996). **(B)** Hoechst staining and immunofluorescence are given for three independent HNF4A8 cell lines treated with doxycycline or ethanol for 24 hours.

To determine if HNF4A8 regulates cell proliferation as known for HNF4A2, the proliferation of induced (+dox) and uninduced (-dox) cells was compared by measuring the metabolic activity using the MTS assay (Fig. 4A). For each cell line the values of the untreated cells were used for standardization (100%). Overexpression of *HNF4A2*, used as a positive control, clearly reduced cell proliferation after three and even more pronounced after six days. Induction of HNF4A8 #9 inhibited growth as well, but just after six days and only to about 90%. The metabolic activity of the other two HNF4A8 cell lines was highly comparable to the artificial mutant C106R and resulted in part in a minor increase in activity which was significant for C106R and HNF4A8 #14 after three days of induction.

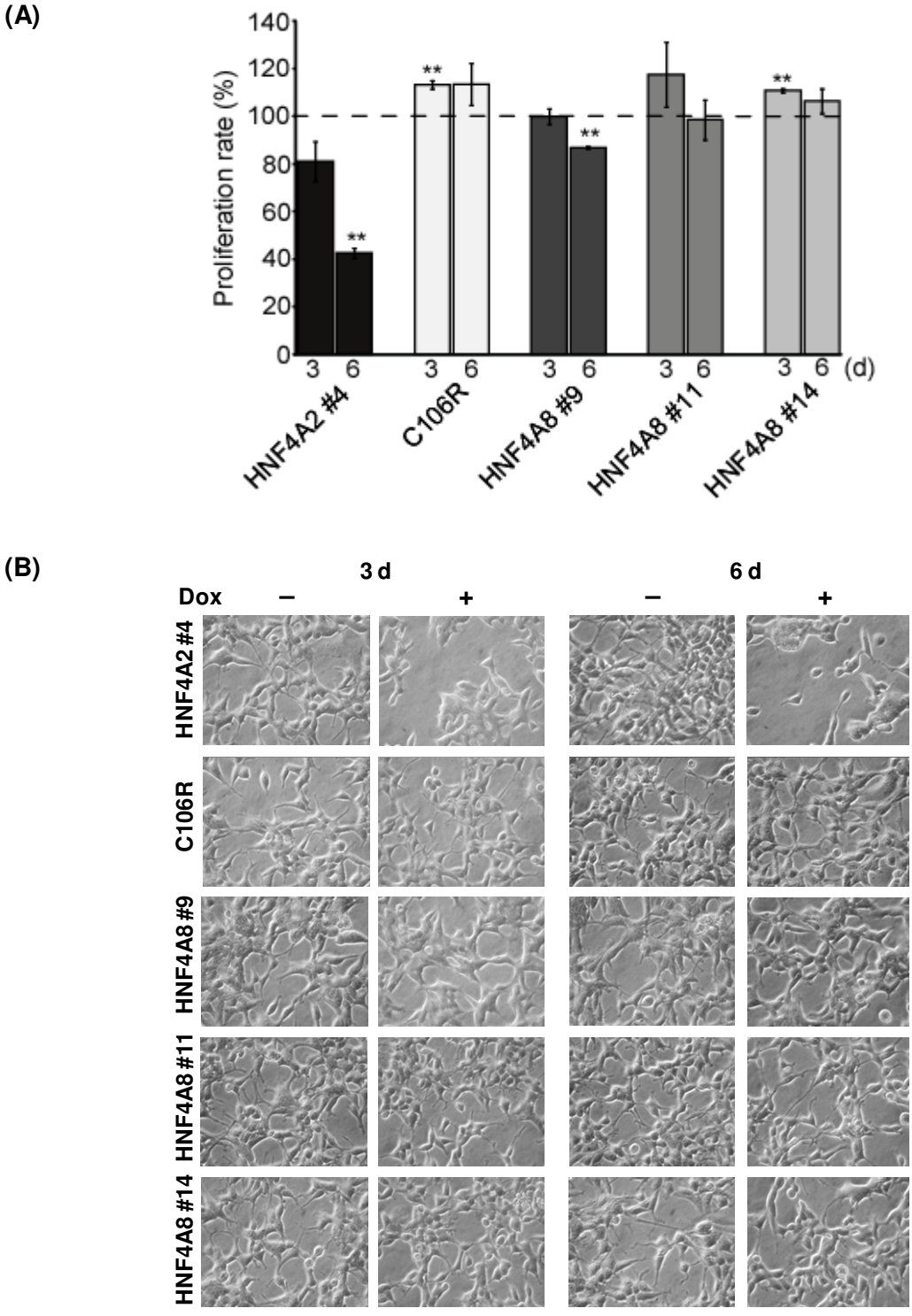

Figure 4: Expression of *HNF4A8* has no impact on cell proliferation rate and morphology.
The cell line HNF4A2 #4 and mutant C106R served as positive and negative control, respectively. **(A)** Proliferation of cell lines induced with 1 µg/ml doxycycline for three and six days to express the given *HNF4A* isoform was determined using the MTS assay (Promega). The values were standardized to untreated cells grown under the same conditions. The results are means±SD of three independent experiments performed in triplicate. *P* values were determined using a one-sample *t* test. *P* values of < 0.05 and of < 0.01 are indicated by * or **, respectively. **(B)** Cells were analyzed by phase-contrast microscopy after treatment with 1 µg/ml doxycycline (+) or ethanol (-) for three and six days.

Overexpression of *HNF4A2* has been shown to change morphology in HEK293 cells (Lucas et al., 2005). While uninduced cells exhibit a small, polygonal-shaped morphology and form a closely packed monolayer at confluence, induced cells change to a rounded phenotype, clump together building aggregates and fail to grow in an epithelium-like monolayer. Comparable to the negative control C106R, cell morphology was not altered in the three HNF4A8 cell lines upon *HNF4A8* expression as assessed by phase-contrast microscopy after three and six days of treatment with doxycycline or ethanol (Fig. 4B).

1.2 Comparing microarray analyses of HNF4A8 with HNF4A2

In contrast to HNF4A2, HNF4A8 is clearly not affecting cell proliferation or morphology upon overexpression in the two HEK293 cell lines HNF4A8 #11 and #14. To exclude target genes of HNF4A not involved in proliferation control, the expression profile upon *HNF4A8* overexpression was determined using the Affymetrix GeneChip HGU 133_Plus_2.0 that probes approximately 38,500 human protein coding genes (54,000 probe sets). RNA isolated from uninduced cells and cells treated with doxycycline for 24 hours was compared from the two HNF4A8 cell lines. Target probe sets were identified using the following filter conditions. The probe sets had to be induced (I) or decreased (D), the candidates had to score a change *p*-value < 0.0045 for induced probe sets or *p* > 0.9955 for decreased probe sets and had to include at least one present call in the uninduced versus induced sample pair.

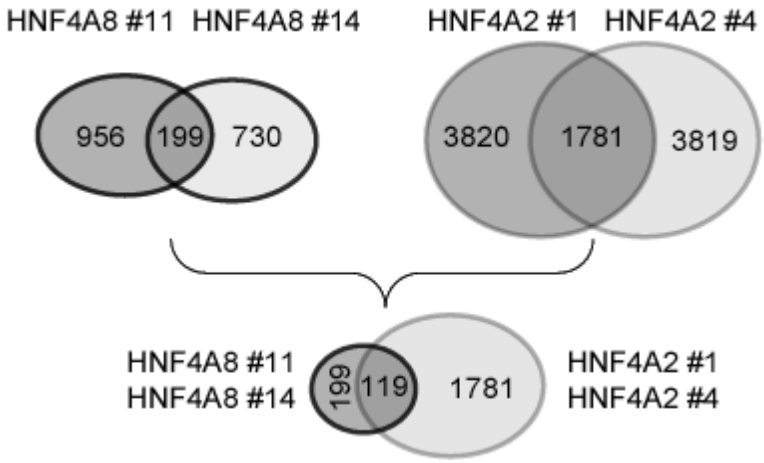

Figure 5: HNF4A8 regulates only few target genes in comparison to HNF4A2.
Microarray analyses were performed with the two independent HNF4A8 cell lines #11 and #14. The expression profile of cells treated with 1 µg/ml doxycycline for 24 hours was compared to untreated cells (ethanol). The 199 probe sets consistently regulated by HNF4A8 were compared to the 1781 probe sets determined in two HNF4A2 cell lines (Grigo et al., 2008). 119 probe sets were regulated by both HNF4A isoforms. All microarrays were performed with the Affymetrix HGU 133_Plus_2.0 GeneChip using the same filter conditions (increase: $p < 0.0045$; decrease: $p > 0.9955$).

Microarray analysis revealed 956 and 730 differentially expressed probe sets for HNF4A8 #11 and HNF4A8 #14, respectively and 199 probe sets were collectively regulated by HNF4A8 (Fig. 5). An overlap of 1781 probe sets was determined between target genes regulated by HNF4A2 #1 (3820 probe sets) and HNF4A2 #4 (3819 probe sets) by previous microarray analyses under the same conditions (Grigo et al., 2008). In conclusion, 119 probe sets were consistently regulated by both HNF4A isoforms, HNF4A2 and HNF4A8. Since some genes are represented by several probe sets on the chip, 119 probe sets correspond to 111 genes. Several differences were observed between the HNF4A8 and HNF4A2 dependent microarray analyses. While about two-thirds of the genes were upregulated (126 versus 55) by HNF4A8, the number of genes downregulated (628) upon HNF4A2 expression was almost as high as the increased (783) gene number. Even more striking is the four to five times higher number of target genes identified for HNF4A2 in comparison to HNF4A8 in the individual cell lines. The overlap of targets between HNF4A2 #1 and HNF4A2 #4 of almost 50% is clearly greater than for the two HNF4A8 cell lines with about 25%. This difference is quite remarkable considering that the two cell lines were established using the same host cell line at the same time, should have *HNF4A8* integrated at a defined locus and had a highly similar phenotype in the conducted assays. Despite the low number of collectively regulated genes by the two HNF4A8 cell lines, the overlap of genes regulated by both isoforms is relatively high with over 60% of genes regulated by HNF4A8. However, only 111 of 1411 HNF4A2 target genes were ruled out to have a significant impact on cell proliferation control or morphology using this approach. Although still left with 1300 potential HNF4A target genes involved in proliferation control, one gene (*CIDEB*) seemed worthwhile to pursue in more detail.

1.3 CIDEB is involved in the HNF4A2 dependent decrease in cell proliferation

CIDEB, a gene described to induce apoptosis, was highly upregulated in the two HNF4A2 cell lines as determined in two independent microarray analyses (Lucas, 2005; Grigo, 2007). The HNF4A2 dependent upregulation of *CIDEB* could previously not be validated by qRT-PCR using the TaqMan Low Density Array (Applied Biosystems) and *CIDEB* was thus neglected (Lucas, 2005; Grigo, 2007). Interestingly, *CIDEB* expression as determined in the present microarray analyses was not altered by overexpression of *HNF4A8* or the *HNF4A* mutants C106R and R154X, which do not decrease cell proliferation (Fig. 6). Since it is possible, that the primer pair used to detect *CIDEB* in the low density array was not functional, new primers were generated. qRT-PCR using the same RNA as for the expression profile, validated the microarray data for the HNF4A isoform specific regulation of

CIDEB (Fig. 6). Some differences in transcript detection between the two methods are likely based on the higher sensitivity of qRT-PCR. In contrast to the qRT-PCR data, in the microarray analyses *CIDEB* expression was absent in the *HNF4A* mutants, uninduced HNF4A2 #1 cells and both HNF4A8 cell lines. Upregulation of *CIDEB* upon *HNF4A2* expression in both cell lines was higher in the microarray analyses than in the qRT-PCR data. However, comparing the expression level of *CIDEB* in the induced HNF4A2 #1 and HNF4A2 #4 cell lines for each method, *CIDEB* showed a higher upregulation upon *HNF4A2* expression in HNF4A2 #1 cells by microarray analyses and in HNF4A2 #4 cells by qRT-PCR analyses. This observation is likely based on the absent call of *CIDEB* in uninduced HNF4A2 #1 cells in the microarray data, since the fold induction from an absent call is not as precise as from a present call.

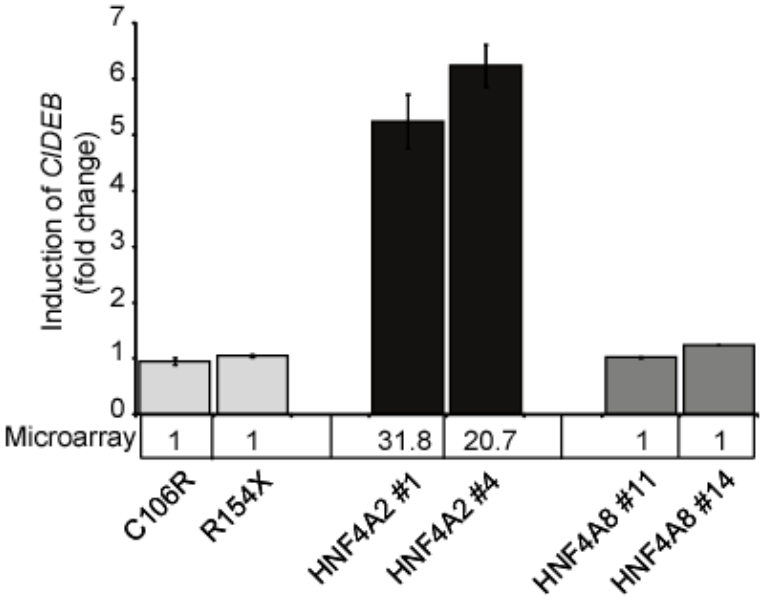

Figure 6: *CIDEB* is upregulated upon *HNF4A2* expression.
CIDEB induction was compared between cells induced with 1 µg/ml doxycycline or ethanol for 24 hours in cell lines expressing the different types of *HNF4A* as indicated. The *HNF4A* mutant cell line R154X expresses a truncated HNF4A protein that lacks most of the ligand binding domain (Lausen et al., 2000; Laine et al., 2000). The fold induction from the microarray analyses for *CIDEB* is given in the bottom row. Values obtained in qRT-PCR were normalized to the house keeping gene *GAPDH.* The results are means±SD of one experiments performed at least in duplicate.

RNA interference was used to determine if *CIDEB* is not only a target gene of HNF4A2, but also involved in the HNF4A2 dependent proliferation control. The endoribonuclease-prepared short interfering RNA (esiRNA) method was used to generate a pool of specific esiRNAs targeting various parts of the target mRNA to inhibit its expression (Kittler et al., 2004). According to the hypothesis that CIDEB is involved in the proliferation decrease triggered upon *HNF4A2* overexpression, inhibiting *CIDEB* with esiRNAs should interrupt the signal cascade, resulting in an increased proliferation rate. Transfection of untreated cells not

expressing *HNF4A* (-dox) with negative control esiRNA lacking a human gene target (*tomato*) or with HNF4A esiRNA did not affect proliferation (Fig. 7). Thus, esiRNAs lacking endogenous target mRNAs do not exhibit any side effects. In contrast to the uninduced cells, transfection of cells expressing *HNF4A* (+dox) with tomato or HNF4A esiRNA resulted in a significant difference in cell proliferation decrease. Although this rescue effect was significant, the decrease in cell proliferation was not entirely abolished upon transfection of HNF4A esiRNA. Possibly not all cells were transfected and/or the esiRNAs were not active over the entire four day time period. However, this technique has been extensively used in a different study and the effect was highly reproducible (Grigo et al., 2008). Application of esiRNA targeting *CIDEB* rescued the inhibition of cell proliferation triggered by induction of *HN4A2* expression for four days to a comparable degree as detected for the HNF4A esiRNA itself. Hence, CIDEB seems to be part of the signal cascade triggered by *HNF4A2* overexpression, resulting in a decrease in cell proliferation.

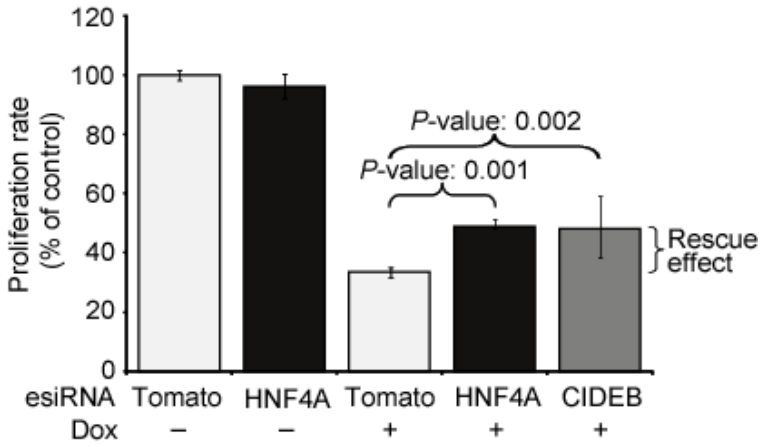

Figure 7: CIDEB is involved in the HNF4A2 dependent decrease in cell proliferation.
Cells were transfected with the indicated esiRNAs, four hours before addition of ethanol or 1 µg/ml doxycycline to induce *HNF4A2* expression. As controls, esiRNA targeting *tomato* or *HNF4A* were used. Cell proliferation was assayed after four days using the MTS assay (Promega). The value given by adding tomato esiRNA and ethanol was used for standardization. The standardized value of cells transfected with tomato esiRNA and expressing *HNF4A2* (+) was used to determine the rescue effect caused by HNF4A and CIDEB esiRNA in induced cells. The results are means±SD of one experiment run in triplicate with one exception. For *CIDEB* the results are means±SD of three experiments run in triplicate involving three independent esiRNA preparations.

1.4 CIDEB only functions within a network of HNF4A2 target genes

To determine if CIDEB is a key player in the proliferation decrease mediated by HNF4A2 and sufficient to cause a decrease in cell proliferation independently of *HNF4A2* expression, a HEK293 cell line expressing *Cideb* upon demand was generated using the Flp-In technology. To be able to differentiate between the endogenous human CIDEB and the stable integrated

Cideb, an expression construct encoding the mouse sequence including a myc-tag at the N-terminus was introduced into the cells. The two independent cell lines Cideb #1 and Cideb #2 lacked β-galactosidase activity (data not shown) and expressed Cideb upon addition of doxycycline for 24 hours as determined by western blot analysis using a myc-specific antibody (Fig. 8A).

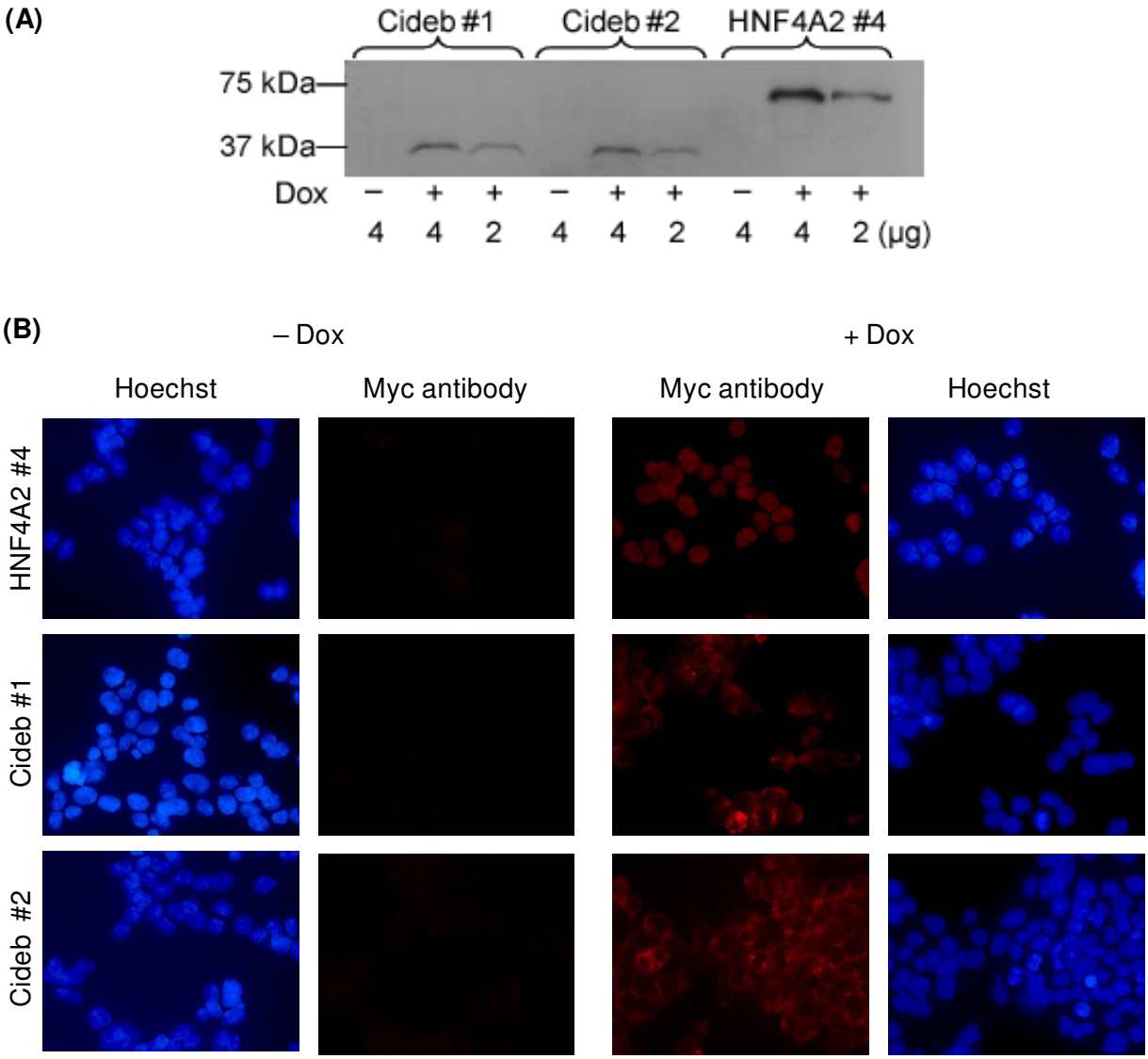

Figure 8: Establishment of HEK293 cells conditionally expressing *Cideb*.
Expression of Cideb or HNF4A was detected with the monoclonal antibody 9E10 targeting the attached myc-tag. Cells were treated with 1 µg/ml doxycycline (+) or ethanol (-) for 24 hours. HNF4A served as a positive control. **(A)** Two different amounts of protein extract were probed on the gel for the induced cells in the western blot analysis. **(B)** The images obtained after Hoechst staining and immunofluorescence analyses are given for the two Cideb cell lines.

As visualized by immunofluorescence analysis, all cells expressed Cideb in the two cell lines after doxycycline treatment for 24 hours, while uninduced cells lacked Cideb expression. Furthermore, the localization of Cideb in cytosolic corpuscles was clearly visible in contrast to HNF4A being detected in the nucleus (Fig. 8B). Mitochondria localization of CIDEB has been

described to be essential for induction of apoptosis (Chen et al., 2000). However, morphological analysis of stained nuclei (Hoechst) of cells overexpressing Cideb in comparison to uninduced cells, showed no signs of apoptosis (data not shown).

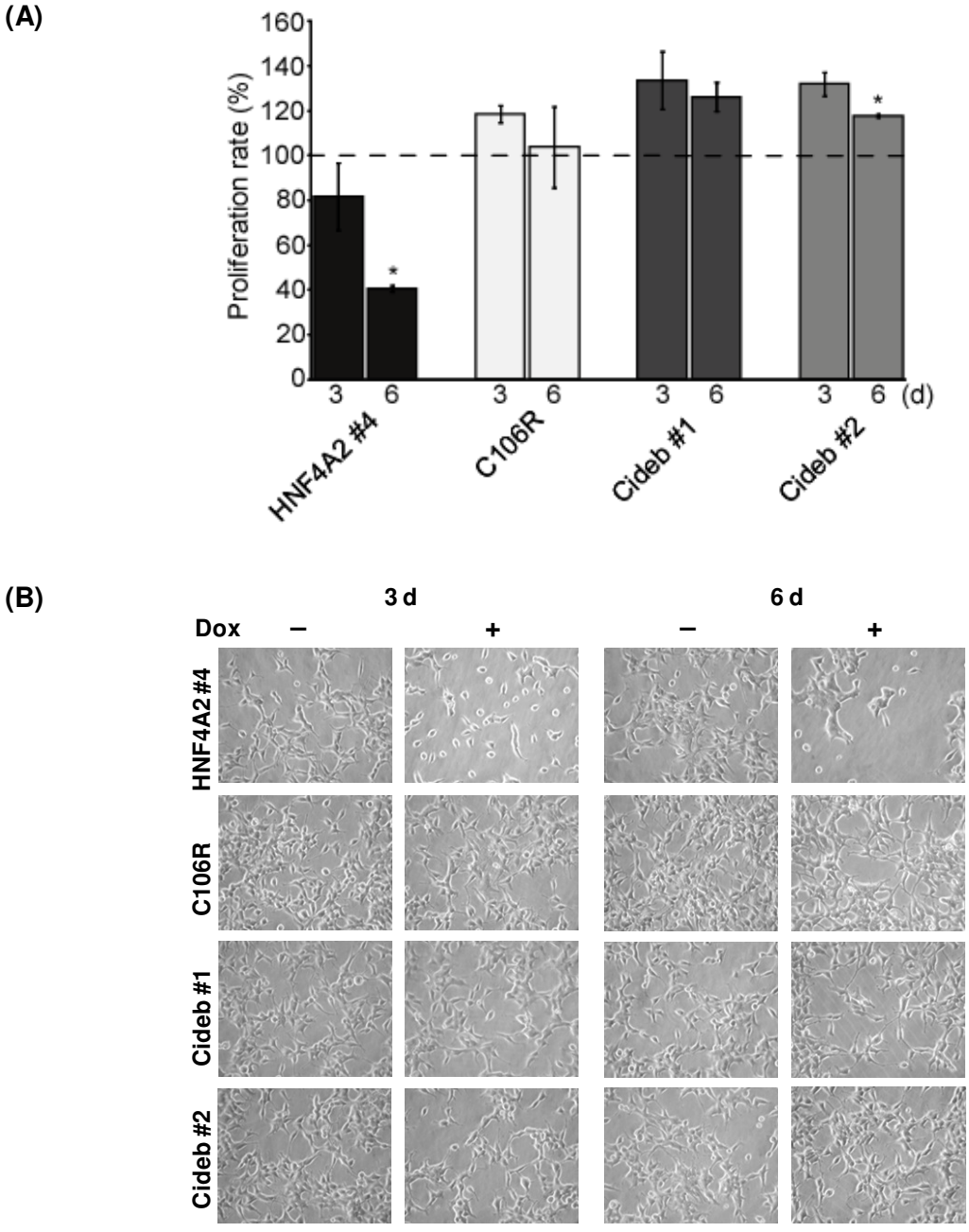

Figure 9: Expression of *Cideb* does not decrease cell proliferation or changes morphology. The cell line HNF4A2 #4 and mutant C106R served as positive and negative control, respectively. **(A)** Proliferation was measured using the MTS assay (Promega). Gene expression was induced by addition of 1 µg/ml doxycycline for three and six days. Cells treated with ethanol and grown under the same conditions were used for standardization. The results are means±SD of two independent experiments performed in triplicate. *P* values were determined using a one-sample *t* test. *P* values of < 0.05 and of < 0.01 are indicated by * or **, respectively. **(B)** Morphology of cells treated with 1 µg/ml doxycycline (+) or ethanol (-) for three and six days were analyzed by phase-contrast microscopy.

The MTS assay was used to determine the effect of *Cideb* overexpression on cell proliferation in the two Cideb cell lines. The metabolic activity of cells treated with

doxycycline for three or six days was standardized to the values obtained for the uninduced cells of each cell line (100%). In each assay HNF4A2 and the mutant C106R were included as positive and negative control, respectively. Similar to the negative control, overexpression of *Cideb* in the two cell lines for three or six days resulted in a minor in part significant increase in cell proliferation (Fig. 9A).

In agreement with the proliferation data, overexpression of *Cideb* did not change cell morphology after three or six days of induction in comparison to untreated cells (Fig. 9B). HNF4A2 #4 and the mutant C106R were included as positive and negative control, respectively. Hence, expression of *Cideb*, independent of HNF4A2 expression, does not decrease cell proliferation.

The expression level of *Cideb* might not be sufficient to cause an effect in the two Cideb cell lines. Due to the lack of a functional CIDEB specific antibody targeting both, the mouse and human sequence, comparing the exogenous Cideb expression level in the Cideb cell lines with the endogenous CIDEB expression triggered by *HNF4A2* overexpession on protein level was not possible.

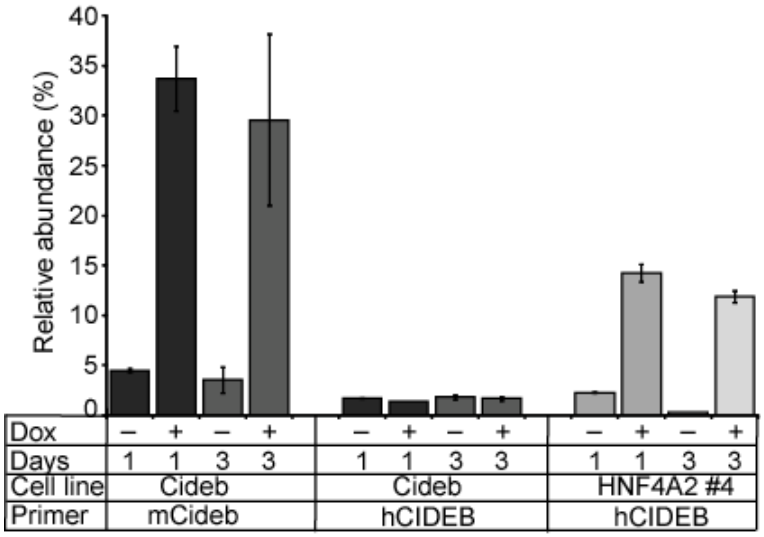

Figure 10: Relative expression level of *CIDEB*.
CIDEB expression was determined by qRT-PCR in Cideb and HNF4A2 #4 cells treated for one or three days with doxycycline (+) or ethanol (-). The values were normalized to the house keeping gene *GAPDH*. Two different primer pairs were used to distinguish between the endogenous *CIDEB* (human, HEK293 cell derived) and exogenous *Cideb* (mouse, transfected) introduced into the Cideb cell lines. The results are means±SD of one experiment performed in duplicate involving Cideb #2 and doxycycline or ethanol treatment for 1 day. Values determined after three days of incubation include results performed in duplicate with Cideb #1 and #2 cells. mCideb, primer targeting the mouse sequence; hCIDEB, primer targeting the human sequence.

To approach this issue at least on RNA level, qRT-PCR was performed (Fig. 10). Primers targeting the mouse *Cideb* sequence introduced into the Cideb cell lines were used to analyze the transgene. No transcripts were detected in the HNF4A2 #4, HNF4A8 and C106R

cell lines with those primers, proofing that they do not target the endogenous human *CIDEB*, but are specific for the mouse sequence (data not shown). Addition of doxycycline to the Cideb cell line for one and three days resulted in a six to seven fold increase in transgene *Cideb* expression. Using primers targeting the human sequence, endogenous *CIDEB* was shown to be present at a relative abundance of about 2% compared to the house keeping gene *GAPDH* in Cideb and untreated HNF4A2 #4 cells. Upon addition of doxycycline, endogenous *CIDEB* expression remained at the same level in Cideb cells, but increased in HNF4A2 #4 cells. Thus, induced expression of the *Cideb* transgene does not seem to influence endogenous *CIDEB* expression. The relative abundance of exogenous *Cideb* in the Cideb cell lines was more than twice as high as the endogenous *CIDEB* level upon overexpression of *HNF4A2*. Hence, at least the RNA expression level of *Cideb* is not too low to cause an effect in the Cideb cell lines.

2 Transcriptional regulation of *HNF4A* via the P2 and P1 promoter

The regulation of *HNF4A* itself is crucial for proper function. At the transcriptional level, regulation of *HNF4A* is imparted by two promoters, P2 and P1, which mediate cell-specific activity (Thomas et al., 2001). The transcriptional regulation of both promoters was investigated with regards to dysregulation leading to human diseases.

2.1 Mutations in the P2 promoter impair the function of the promoter *in vitro* and co-segregate with diabetes

The -192C>G mutation in the *HNF4A* P2 promoter was linked to diabetes in several families by two independent studies, but an impact of the mutation on the promoter activity could not be confirmed by luciferase assays (Ek et al., 2006; Raeder et al., 2006a). From our colleagues in Exeter, Bratislava and Auckland we learned of two additional mutations in the P2 promoter of the *HNF4A* gene including a -136A>G mutation in a Slovak family and a -169C>T mutation in a New Zealand family. These mutations were not identified in >800 control chromosomes (500 of which were of European Caucasian origin). The -136A>G mutation co-segregated with diabetes in two family members with normal body mass index and early diabetes onset (Fig. 11A). The -169C>T mutation co-segregated with diabetes in all but one family member (maternal aunt) who has features of T2DM (Fig. 11B). Details of clinical data have been published recently (Wirsing et al., 2010).

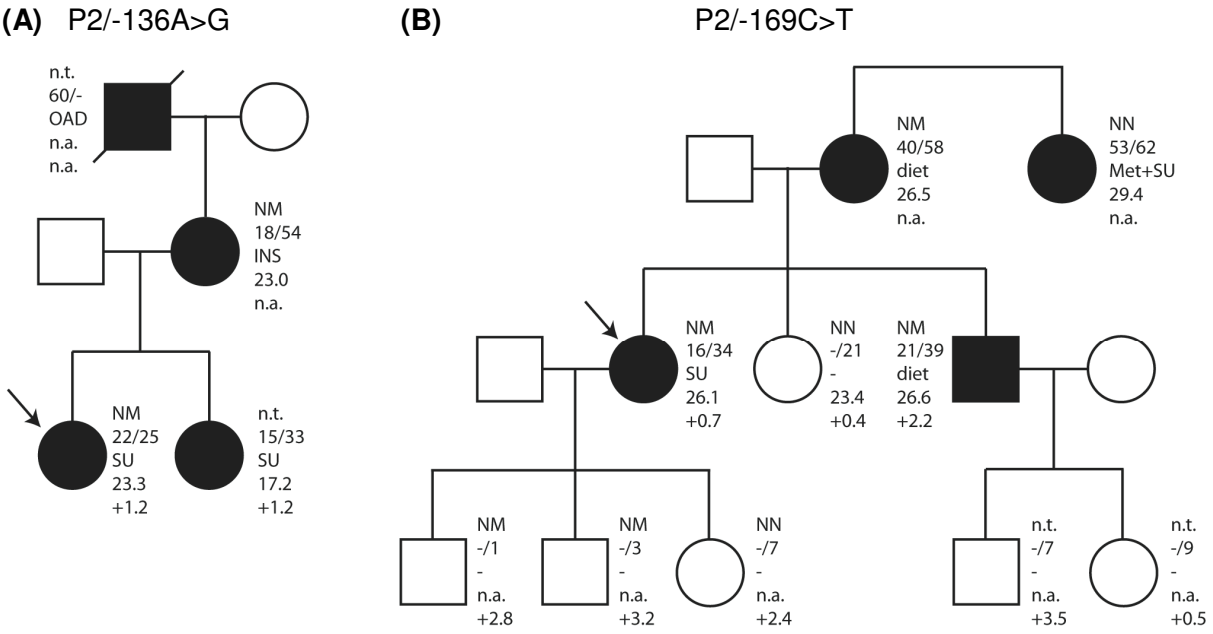

Figure 11: Pedigrees of the families with P2/-136A>G (A) and P2/-169C>T mutations (B). Squares represent male; circles represent female; open symbols are normal glucose tolerant; and filled symbols are diabetic. Probands are indicated by an arrow. The text beside each individual represents: mutation carrier status (NM, *HNF4A* P2 mutation positive; NN, wild-type; and n.t., not tested); age at diagnosis if diabetic/current age, current diabetes treatment (diet; Met, metformin; SU, sulfonylurea; OAD, oral antidiabetic drug – type unknown; INS, insulin); body mass index; and birthweight standard deviation score. n.a., not available. Data as given in Figure 1 of Wirsing et al. (2010).

The -136A>G mutation affects the region identified as the HNF6/OC2 binding site in the murine promoter (Briancon et al., 2004) and -169C>T mutates the HNF1 binding site of the P2 promoter (Thomas et al., 2001; Hansen et al., 2002). The latter site can be targeted by HNF1A and HNF1B since the DNA binding sequence specificity is almost identical for both transcription factors (Tronche and Yaniv, 1992). The previously described mutation -192C>G affects binding of an unknown factor enriched in human pancreatic islets and rat INS-1 cells (Ek et al., 2006). All three mutations alter nucleotides that are conserved from human to platypus (Fig. 12). To reveal the functional importance of these alterations, mutations for -136A>G, -169C>T and -192C>G were introduced into a firefly luciferase reporter construct containing the highly conserved P2 promoter region from -285 to -1 upstream of the translational start site (P2/-285; Fig. 12). Their effects were analyzed in transient transfection experiments in the HEK293 (HNF1B) cell line (Senkel et al., 2005) and INS-1 (HNF1B) cells (Thomas et al., 2004) allowing for homogenous induction of *HNF1B* expression. While HEK293 cells lack endogenous *HNF1A* and *HNF1B* expression (Lucas et al., 2005), endogenous *HNF1A* expression is higher than *HNF1B* in the INS-1 cell line (Thomas et al., 2004).

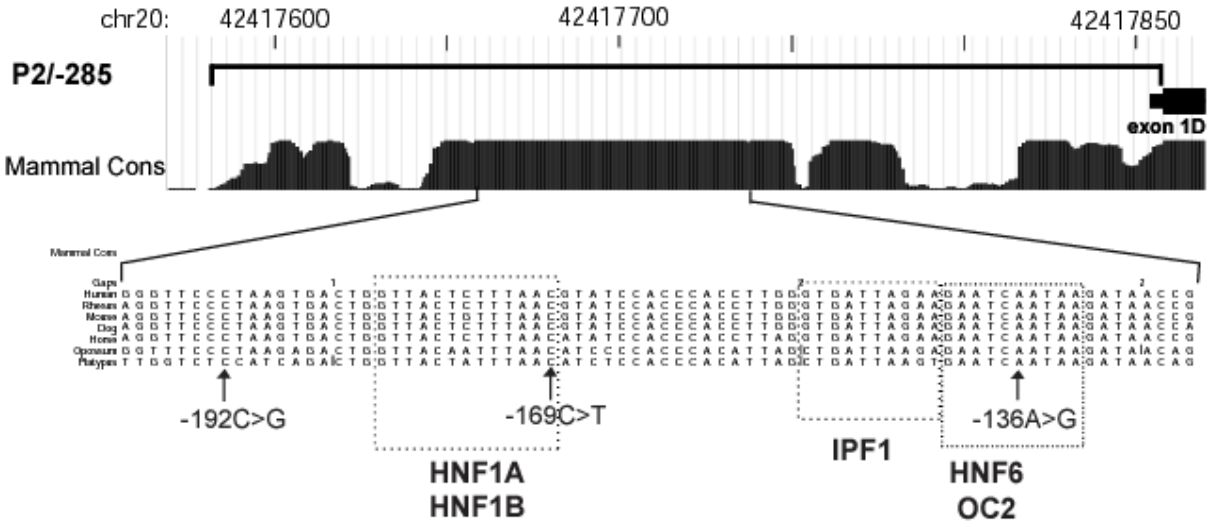

Figure 12: Schematic representation of the P2 promoter region of the human *HNF4A* gene.
The screen shot taken from the UCSC Genome Browser (assembly March 2006) displays the genome position from 42417570 to 42417866 of chromosome 20 and the degree of conservation across 28 species indicated by black areas. The bracket locates the region included in the P2/-285 promoter luciferase construct from -285 to -1 relative to the ATG located in exon 1D (Thomas et al., 2001). The nucleotide sequence alignment of a highly conserved region and the position of the novel mutations -136A>G and -169C>T and the late-onset diabetes mutation -192C>G (Ek et al., 2006; Raeder et al., 2006a) is shown below. The binding sites for HNF1A/HNF1B (Thomas et al., 2001; Hansen et al., 2002), IPF1 (Thomas et al., 2001) and HNF6/OC2 (taken from the mouse data (Briancon et al., 2004)) are boxed. Data published in Wirsing et al. (2010).

In HEK293 cells the basal activity of the P2/-285(-136A>G) and P2/-285(-169C>T) mutated constructs was significantly lower than the basal activity of the wild-type promoter construct P2/-285 (Fig. 13, upper part). Induction of *HNF1B* expression resulted in a fourfold activation of the wild-type promoter, but all three mutated constructs were activated at a significantly lower level. Mutation of the HNF1 binding site (-169C>T) resulted in the lowest transactivation by HNF1B. Comparing the activity in the presence of HNF1B, all three mutated constructs revealed a lower activity, although the effect of the -192C>G mutation was marginal. In INS-1 cells, the constructs P2/-285(-169C>T) and P2/-285(-192C>G) had significantly impaired basal activity compared with the wild-type promoter (Fig. 13, lower part). Following tetracycline induction of *HNF1B* expression, the activity of all of the constructs except P2/-285(-169C>T) decreased to about 50% of their uninduced activity. Comparing the activity in the presence of HNF1B, all three mutated promoter constructs had reduced activity compared to the wild-type promoter.

In conclusion the two novel mutations, -136A>G and -169C>T and the previously identified late-onset diabetes mutation, -192C>G are linked to diabetes and impair the function of the *HNF4A* P2 promoter *in vitro*.

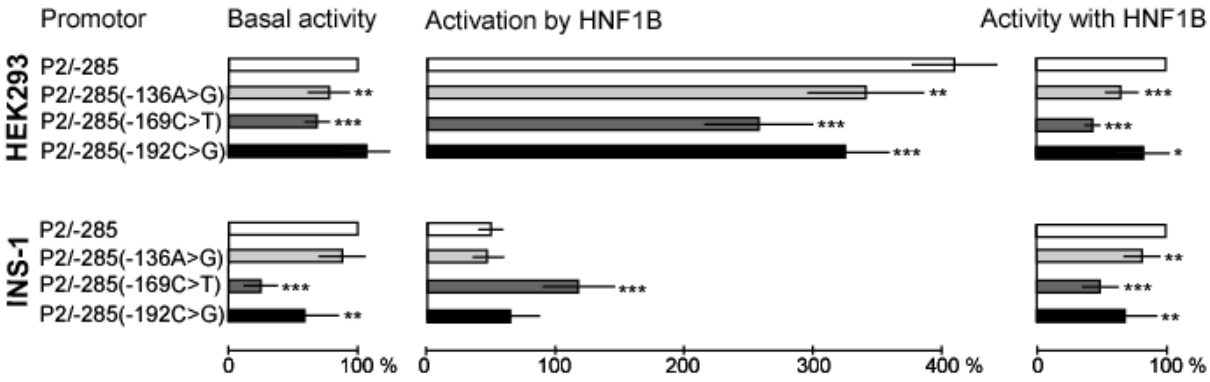

Figure 13: *In vitro* analyses of mutations in the P2 promoter of the *HNF4A* gene.
The results of transient transfection assays in HEK293 and INS-1 cells are shown. The activity of the wild-type promoter was about threefold and fiftyfold higher than the value obtained from pGL3-Basic in HEK293 and in INS-1 cells, respectively. For each construct, eight transfection assays were performed involving four independent plasmid preparations. Each luciferase assay was performed in triplicate and a CMV driven *renilla* luciferase was used to control for transfection efficiency. The activity of the wild-type construct was used to standardize the basal activity (no HNF1B). The activation by HNF1B reflects the activation of the constructs by the induction of *HNF1B*. The activity with HNF1B compares the activity of the mutants with the wild-type in the presence of induced *HNF1B*. *P* values were determined using a one-sample *t* test for basal activity as well as for activity with HNF1B and an independent-samples *t* test for activation by HNF1B. *P* values of < 0.05, of < 0.01 and of < 0.001 are indicated by *, ** or ***, respectively.

2.2 The P1 and P2 promoter might be regulated by miRNAs

In addition to transcription factors regulating the activity of promoters, few studies have described an activating effect of agRNAs (Li et al., 2006a; Janowski et al., 2007) and miRNAs on promoters (Place et al., 2008; Majid et al., 2010). Hence, miRNAs that are downregulated in diabetes and RCC might contribute to the downregulation of *HNF4A* in these diseases due to the loss of activation of the P2 and P1 promoter, respectively. Numerous potential miRNA binding sites were predicted within the *HNF4A* P2 and P1 promoter using the RNA22 program (Miranda et al., 2006). To experimentally evaluate the possibility of any miRNA targeting the P2 and P1 promoter, a HEK293 cell line was used in which the Dicer protein can be conditionally knocked-down by doxycycline (Schmitter et al., 2006). Since Dicer is required for miRNA biogenesis, its inhibition results in a lack of miRNAs. In the case of miRNAs targeting and activating a promoter, addition of doxycycline attenuates the activating effect. A reporter plasmid (pGL3 basic) containing the P2 promoter sequence extending from -2200 bp to the nucleotide preceding the translation initiation site (position -1) upstream of the firefly luciferase was transfected into cells depleted of Dicer. In comparison to uninduced cells, luciferase activity was decreased by about 25%, indicating a potential regulation of the *HNF4A* P2 promoter by miRNAs present in HEK293 cells (Fig. 14A). To locate the area of crucial miRNA binding sites, luciferase activity of various 5' deletions constructs was assessed upon Dicer knock-down. However, even deleting the

promoter sequence up to position -135 bp upstream of the ATG codon (transcription start site at position -103 and TATA box starting at -136) still resulted in a highly similar decrease in luciferase activity upon miRNA depletion as for all other P2 constructs. To ensure that the observed effect was promoter specific, the impact of miRNA depletion was analyzed on the CMV promoter, resulting in no decreased activity (Fig. 14A).

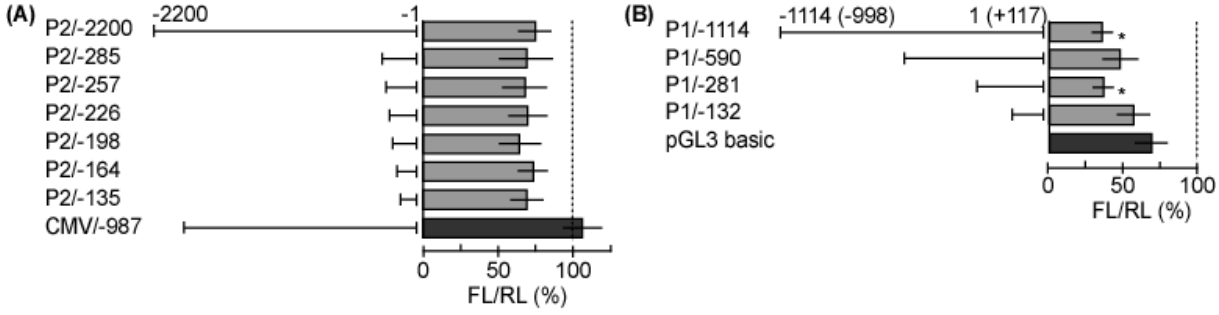

Figure 14: Dicer knock-down experiments to determine whether the *HNF4A* P2 (A) and P1 (B) promoters are regulated by miRNAs.
The P2 and P1 promoter fragments are all cloned into the pGL3 basic plasmid. The longest P2 promoter fragment contains 2200 base pairs upstream of the translation initiation site (excluding the ATG codon). The P1 promoter fragment of 1114 base pairs extends from nucleotides -998 to +117 from the transcription start site. Dicer knock-down was triggered by addition of doxycycline to the Dicer-kd/2b2 cell line (Schmitter et al., 2006) for three and seven days. Firelfly reporter constructs were transiently transfected two days before luciferase activity was measured. At least three transfection assays were performed for each construct, involving two independent plasmid preparations, except for P1/-132 for which just one construct was available. Each assay was performed in triplicate and a CMV-driven *renilla* luciferase was used to control for transfection efficiency. The values obtained for each construct determined in the presence of Dicer (ethanol added) were used for standardization (100%) and are not shown. The *P* values were determined between the promoter constructs and pGL3 basic in induced cells using an independent-samples *t* test. *P* values of < 0.05 are indicated by *.

Analyses of the 1114 bp P1 promoter revealed a highly reduced luciferase activity to 35% upon Dicer knock-down, suggesting an activating effect of miRNAs on the *HNF4A* P1 promoter (Fig. 14B). Further 5' deletion of this promoter, even up to -132 bp still conferred a reduced activity to 56% in comparison to cells expressing mature miRNAs. Although no effect had been detected using the CMV promoter, the activity of the CMV promoter was severalfold higher than the P1 and P2 activity. To rule out possible side effects from the residual sequence of the pGL3 reporter plasmid, which might be masked by the high activity of the CMV promoter in the corresponding construct, the Dicer dependent activity was determined for the pGL3 basic plasmid. Although this plasmid does not contain a promoter sequence, it was slightly active and also conferred a reduced activity upon Dicer knock-down. However, upon miRNA depletion the activity of the P1 constructs containing 1114 bp and 282 bp was significantly reduced by about 50% in comparison to pGL3 basic. Although there is little knowledge about the regulation of promoters by miRNAs and adequate positive and negative controls are missing, the present data including the measurements of the pGL3

basic plasmid make the regulation of the *HNF4A* P2 promoter by miRNAs unlikely, but provides evidence for an activating effect of miRNAs on the P1 promoter.

3 Posttranscriptional regulation of *HNF4A* via the 3'UTR

While transcriptional regulation of *HNF4A* has been extensively studied, the posttranscriptional regulation has been entirely neglected to date. The evident role of 3'UTRs in translation, localization and stability of mRNAs and its impact on various diseases, has proven that it is essential to gain knowledge of the posttranscriptional regulation (Chatterjee and Pal, 2009; Thomas et al., 2010). To obtain a comprehensive understanding of *HNF4A*, the 3'UTR and its regulation was investigated.

3.1 *HNF4A* expresses two alternative 3'UTRs

The RefSeq sequences NM_000457.3 and NM_178849.1 of the human *HNF4A* mRNA encode a 3'UTR of 1724 bp that contains the non-canonical PAS GATAAA 15 nt upstream of the 3' end (Fig. 15A). However, this PAS and the surrounding sequence are conserved in primates only, but not among other mammals. In contrast, the 3'UTR of the murine *Hnf4a* mRNA is 2816 nt in length (RefSeq NM_008261.2) and encompasses the canonical PAS AATAAA (Fig. 15A). This PAS and the surrounding sequence are highly conserved among different mammals including human. To determine which polyadenylation site is functional in human cells, 3' RACE was performed with *HNF4A* mRNA isolated from the hepatoma cell line HEPG2 and the kidney cell line HK120. Sequence analyses of the cDNA revealed that in both cell lines the proximal as well as the distal PAS are used, resulting in cleavage of the mRNA 15 nt and 19 nt downstream of the PAS at position 1724 nt and 3180 nt, respectively.
To determine the abundance of the short and long 3'UTR in the human *HNF4A* RNA of the HEPG2 and HK120 cell lines, qRT-PCR was performed using *GAPDH* as a reference. The amount of the *HNF4A* 3'UTR was about two-fold higher in HEPG2 than in HK120 cells (Fig. 15B). In both cell lines the distal PAS generating the long 3'UTR was used predominantly, representing about 75% and 60% of the *HNF4A* transcripts in the hepatoma and kidney cell line, respectively. These data reveal that in human cells in addition to the predicted *HNF4A* 3'UTR of 1724 nt, a much longer 3'UTR of 3180 nt is expressed.

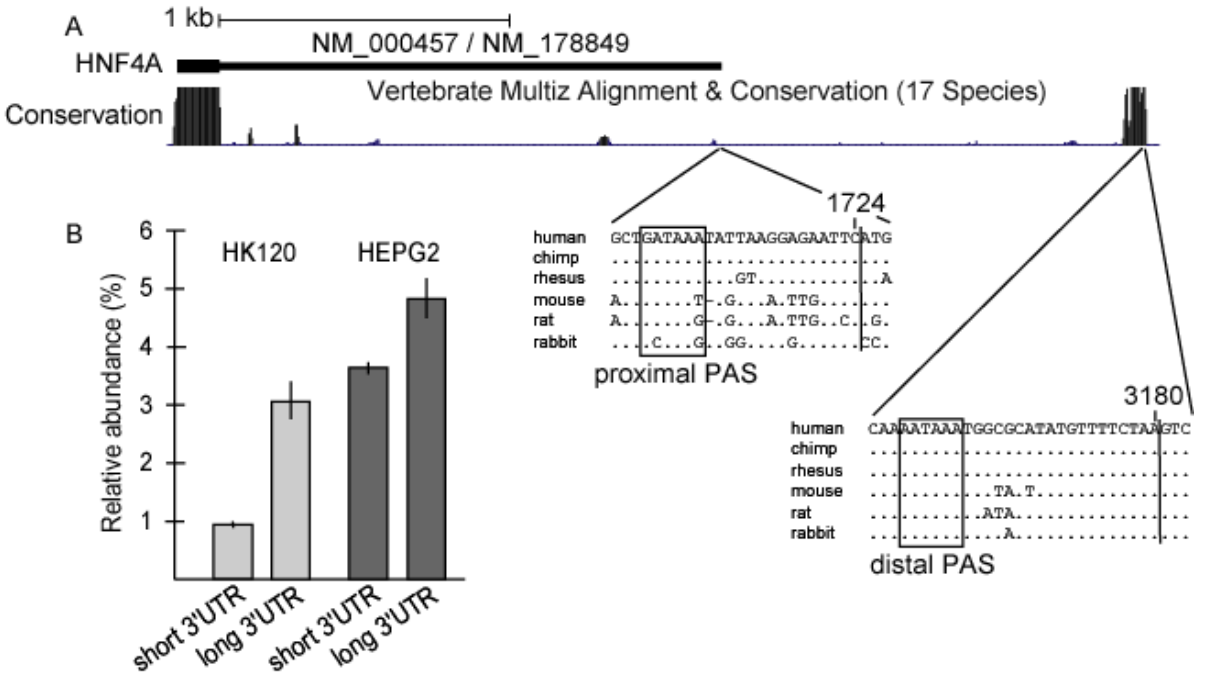

Figure 15: Two distinct PAS in the human *HNF4A* mRNA.
(A) Schematic representation of the *HNF4A* 3'UTR. The screen shot taken from the UCSC Genome Browser (assembly March 2006) depicts the known human *HNF4A* 3'UTR with the RefSeq sequences NM_000457.3 and NM_178849.1 and the genome position from 42,491,540 to 42,494,950 of chromosome 20. The degree of conservation across 17 species is indicated by black areas. The nucleotide sequence alignment of the region surrounding the proximal and distal PAS is shown below. Non-conserved nucleotides in comparison to the human sequence are given for the different species, while dots represent conserved nucleotides. The proximal and distal PAS are boxed and the corresponding cleavage sites, as determined by 3' RACE and subsequent sequencing, are indicated by a vertical line. The last nucleotide of the short and long 3'UTR is marked at position 1724 and 3180, respectively. **(B)** The relative abundance of the short and long *HNF4A* 3'UTRs was determined in comparison to the house keeping gene *GAPDH*. Two independent RNA samples were prepared from each cell line and the qRT-PCR was performed in triplicates. Each column thus represents the mean±SD of six measurements.

3.2 Both 3'UTRs confer a repressive effect

To gain insight into the mode of regulation of *HNF4A* via its 3'UTR, the short and long 3'UTR was cloned downstream of the *renilla* luciferase ORF into the reporter plasmid RL-Con (Schmitter et al., 2006). The effect was analyzed in HEK293 cells using a firefly luciferase reporter as reference (Fig. 16, middle panel). The *renilla* luciferase activity was significantly reduced to about 60% by insertion of the long (1-3180) or short (1-1746) 3'UTR implying the existence of elements conferring a repressive effect.

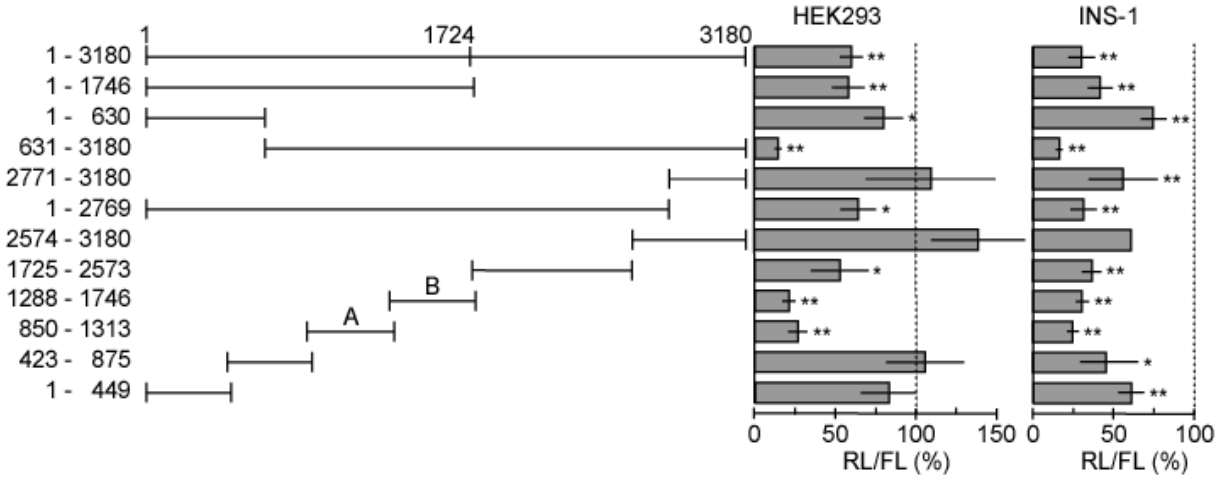

Figure 16: Systematic reporter analyses of the human *HNF4A* 3'UTR.
The results of luciferase assays 24 hours after transient transfections into HEK293 and INS-1 cells are shown. The numbers of the construct names refer to the nucleotide position in the *HNF4A* 3'UTR with 1 being the first nucleotide after the stop codon. Each 3'UTR fragment was cloned downstream of the *renilla* luciferase ORF into the RL-Con plasmid. At least three transfection assays were performed for each construct, involving two independent plasmid preparations. Each assay was performed in triplicate and a CMV-driven firefly luciferase was used to control for transfection efficiency. The activity of the RL-Con plasmid was used for standardization to 100%. *P* values were determined using a one-sample *t* test. *P* values of < 0.05 and of < 0.01 are indicated by * or **, respectively.

To get an indication which *cis*-elements mediate the effect, an *in silico* search was performed. Only few potential *cis*-elements of RNA-binding proteins were found (Tab. 1). The sex-lethal (SXL) binding site consists of a polyuridine tract of eight or more residues and is bound by the SXL RNA-binding protein (Samuels et al., 1994). In *Drosophila melanogaster* this female specific protein functions as the master regulator of somatic sex determination and X-chromosome dosage compensation. It modulates splicing and translation of target pre-mRNAs (Johnson et al., 2010). The K-box (cTGTGATa) has been identified in the 3'UTRs of many Notch pathway target genes in *Drosophila melanogaster* (Lai et al., 1998). Regulation by K-box is spatially and temporally ubiquitous and mediates negative posttranscriptional regulation, mainly causing decreased transcript levels. The Musashi binding element (MBE) is critical for early translational activation in *Xenopus* by interaction with the Musashi binding protein (Arumugam et al., 2010). In mammalian somatic cells, MBE seems to mediate repression of mRNAs at the translational level (Imai et al., 2001). Furthermore, the distal, but not the little conserved proximal PAS was found. Considering the large size of the 3180 nt long *HNF4A* 3'UTR, this raised the question if various other functional binding elements are missing. miRNA binding site prediction in turn revealed several hundred possible target sites. Depending on which miRNA prediction program and setting was applied, potential target sites for various miRNAs varied substantially. To address this issue of uncertain regulatory element prediction, functional assays were used to initially locate the area of crucial regulatory elements.

Table 1: Regulatory sites in the long 3'UTR of *HNF4A*.
Binding sites identified by UTRdb (http://utrdb.ba.itb.cnr.it/; Grillo et al., 2009).

Binding site	Position	Sequence
SXL binding site	2680-2695	TTTTTTTTTTTTTTT
K-box	2940-2947	CTGTGATC
Musashi binding element	910-914	GTAGT
Musashi binding element	2152-2157	ATTAGT
Polyadenylation signal	3156-3180	AATAAATGGCGCATATGTTTTCTAA

In a comprehensive systematic approach, the 3'UTR was dissected and a repressive activity was found in the 5' part (1-630), whereas the 3' part (2770-3180) had no influence (Fig. 16). Furthermore, a distinct activity located in fragment 631-3180 was observed that surprisingly had a much higher repressive effect, while a construct containing the sequence from 1-2769 resulted in a decrease in luciferase activity similar to the one observed for the long 3'UTR. To pin down the area within the *HNF4A* 3'UTR which confers the strong repressive effect, short, mainly overlapping constructs were generated covering the entire 3180 nt of the 3'UTR. Whereas the majority of 3'UTR-fragments showed no or only minor effects, the two constructs comprising the 3'UTR sequences A (850-1313) and B (1288-1746) repressed luciferase activity down to 27% and 21%, respectively. Since in HEK293 cells the *HNF4A* gene is silent (Lucas et al., 2005), the activity of the same 3'UTR fragments was measured in the INS-1 cells expressing *HNF4A* (Thomas et al., 2004). In this cell line similar repressive activities were seen that were even more pronounced (Fig. 16, right panel). Taken together, the *HNF4A* 3'UTR contains several elements that negatively influence luciferase reporter activity.

3.3 Identification of two novel negative elements within the *HNF4A* 3'UTR

Using UCSC Genome Browser the "mammalian interspersed repetitive elements" MIRb and MIRc were identified within sequence A (850-1313) and B (1288-1746), respectively (Fig. 17, left panel). MIRs are tRNA-derived SINEs found in all mammalian genomes, including marsupials (Smit and Riggs, 1995) and have a trend for PAS association (Lee et al., 2008). However, it was excluded that these repetitive elements mediate the repressive effects, as fragments retaining the sequence for MIRb (1208-1313) or MIRc (1392-1513) did not affect luciferase reporter activity in HEK293 and INS-1 cells (Fig. 17, middle and right panel).

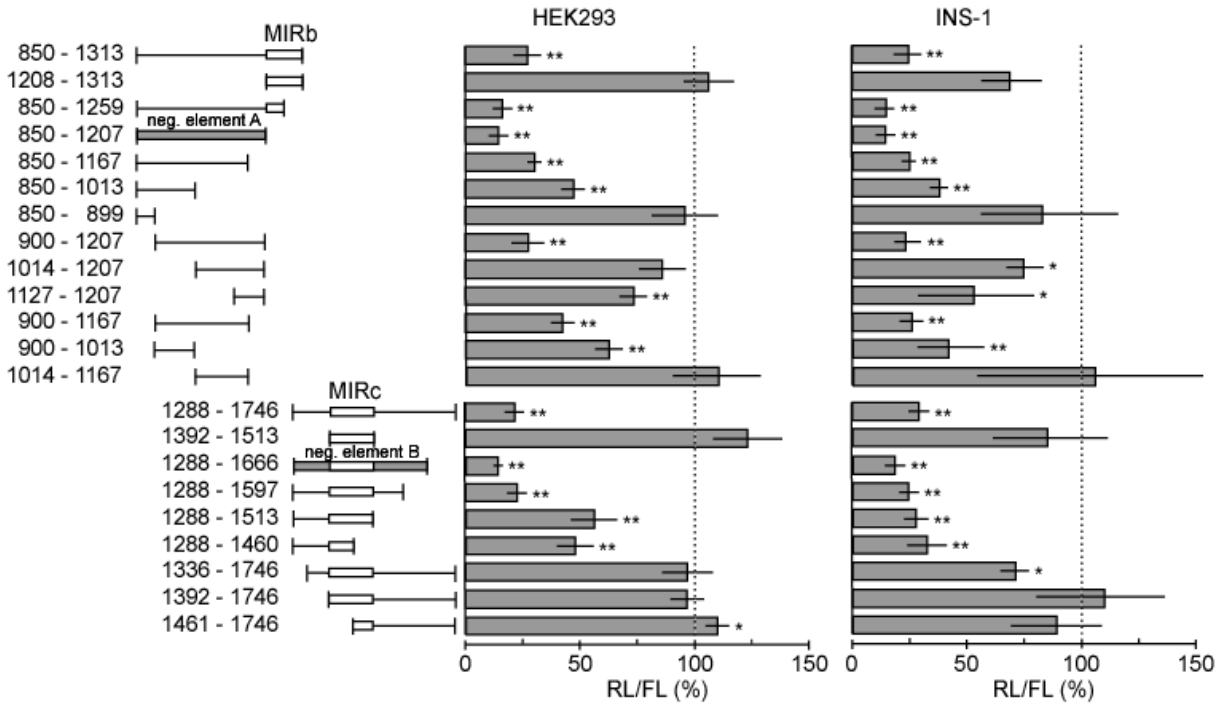

Figure 17: Locating negative elements within sequence A and B of the *HNF4A* 3'UTR.
The results of luciferase assays were derived and evaluated as in Figure 16. The grey boxes in the left panel indicate negative element A or B. The white box illustrates the mammalian interspersed repetitive elements (MIRb or MIRc) as predicted by the USCS Genome Browser.

To locate the functional elements, the sequences were gradually trimmed from the 3' and 5' end. Shortening sequence A on the 3' end to position 1259 and even to 1207 amplified the repressive effect in HEK293 cells to 16% and 15%, respectively. Further constriction on either side revealed a gradual release of the repressive effect, ruling out an essential role of the Musashi element (910-914) on the *HNF4A* 3'UTR (Tab. 1). Instead, the fragment extending from 850-1207 was defined as negative element A. Similarly, shortening sequence B on the 3' and 5' end, negative element B (1288-1666) was defined, as it mediates the highest repressive function and any truncation leads to a partial or even total loss of the repressor activity. A corresponding analysis in INS-1 cells gave similar result (Fig. 17, right panel).

Taken together, two previously unknown elements were located within the *HNF4A* 3'UTR that are separated by about 80 bp. Their size of approximately 400 nt (357 nt and 378 nt) is quite large and based on their position they are present in the short as well as the long 3'UTR of the *HNF4A* mRNA.

The strong activity of negative element A and B was only observed when these elements were excised from the 3'UTR (Fig. 16). Deleting a sequence containing both negative elements from the construct containing the long *HNF4A* 3'UTR did not change the luciferase activity in comparison to the entire 3'UTR (Fig. 18). In contrast, the high repressive effect of

construct 631-3180, was abolished upon deletion or inversion of the sequence containing negative element A and B. This implied a counteracting element in the 5' part of the 3'UTR (1-630). Indeed, insertion of the sequence 1-630 nt upstream of element A or B largely abolished the repressive effect of the negative element A or B. The observation that the repressive effect of element A and B is lost upon inversion is consistent with a regulatory element functioning on the RNA level. To obtain further evidence, the SV40 3'UTR with its PAS was inserted upstream of negative element A or B. In both cases the repressive effect was lost, as expected if the transcript is polyadenylated at the SV40 PAS and thus does not include negative element A or B. However, the abolishment of the repressive effect was not seen, if the SV40 3'UTR was inserted in opposite direction leading to a PAS on the noncoding DNA. Additionally, this experiment excluded the possibility that any sequence introduced upstream of element A or B would abrogate the effect. All described effects were highly similar in HEK293 and INS-1 cells.

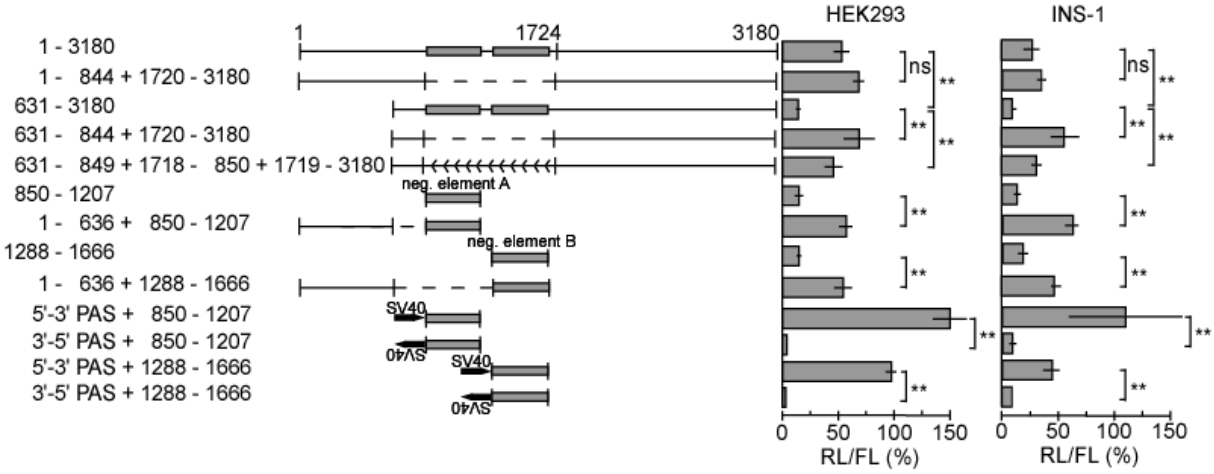

Figure 18: Counteracting the two negative elements.
The negative elements A and B are indicated by grey boxes, the deletion of the negative elements is illustrated by a broken line, whereas the inversion of this element is marked by backwards arrows. The insertion of the SV40 termination signal in sense and antisense is marked. The results of luciferase assays were derived and evaluated as in Figure 16. *P* values were determined between two columns as indicated by brackets. Non-significant changes are marked by ns and refer to *p* values > 0.01.

3.4 The *HNF4A* 3'UTR is regulated by miRNAs

To address the question experimentally whether *HNF4A* is regulated by miRNAs, initially a general approach was applied using the HEK293 cell line in which the Dicer protein can be conditionally knocked-down by doxycycline (Schmitter et al., 2006). Since Dicer is required for miRNA biogenesis, the repressive effect of miRNAs is relieved, if Dicer is downregulated. Using the *renilla* luciferase reporter with the entire 3180 nt *HNF4A* 3'UTR, depletion of Dicer resulted in an increase in luciferase reporter activity by 21% (Fig. 19A). The effect was not as

pronounced as for the RL-Perf reporter containing one perfect let-7a binding site or the RL-3xBulgeB reporter construct containing three bulged let-7a sites (Schmitter et al 2006), that mediated in the present study an increase of 55% and 89%, respectively. However, the significant increase indicated that miRNAs regulate the *HNF4A* 3'UTR.

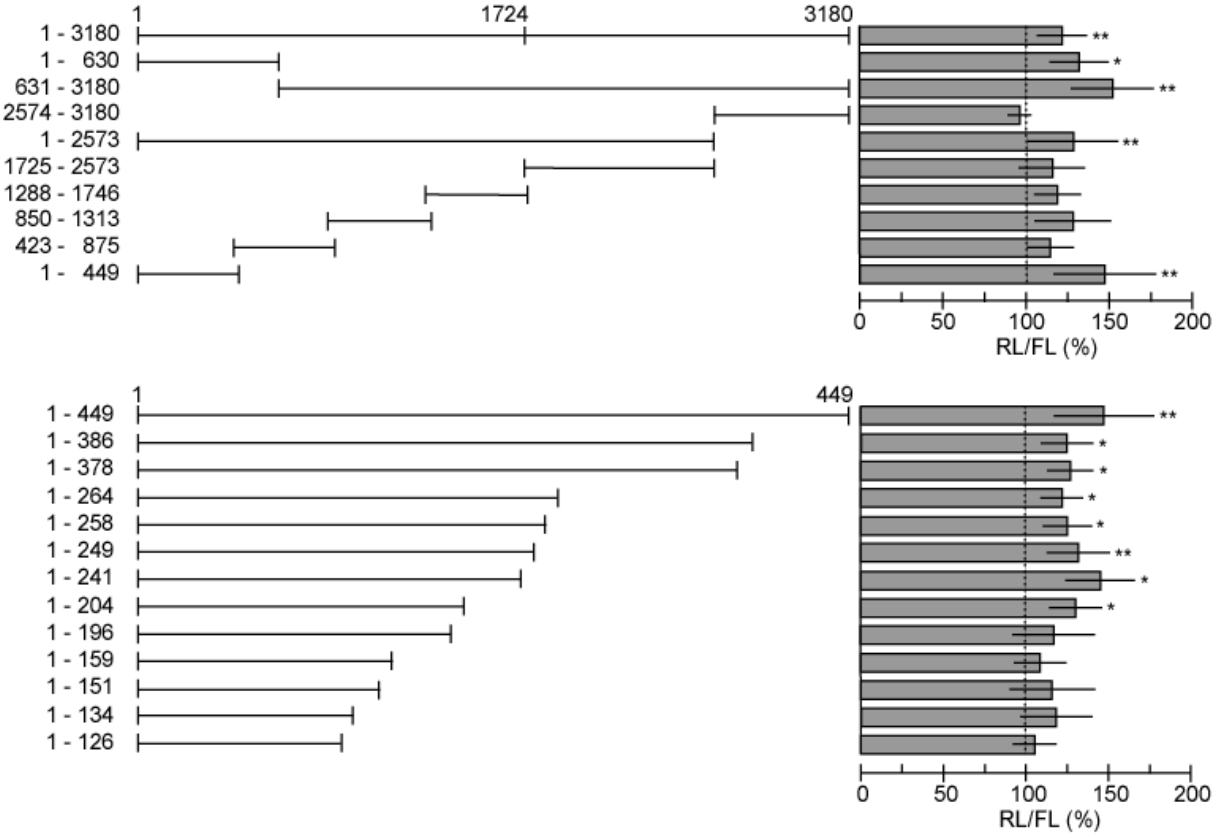

Figure 19: Dicer knock-down indicates that the *HNF4A* 3'UTR is regulated by miRNAs.
To knock-down the Dicer protein, doxycycline was added to the Dicer-kd/2b2 cell line (Schmitter et al 2006) for three or seven days. Two days before luciferase activity was measured, the cells were transiently transfected with reporter constructs. The nomenclature of the constructs is as in Figure 16. At least three transfection assays were performed for each construct, involving at least two independent plasmid preparations. Each assay was performed in triplicate and a CMV-driven firefly luciferase was used to control for transfection efficiency. The activity of each construct measured in the presence of Dicer (ethanol added) was used for standardization (100%) and is not shown. The 3xBulgeB and RL-Con reporter (Schmitter et al 2006) harboring three bulged binding sites for let-7a and lack any binding sites, respectively, were included as a positive and negative control in each experiment (not shown). The *P* values were determined using an independent-samples *t* test. *P* values of < 0.05 and of < 0.01 are indicated by * or **, respectively.

Several miRNAs misregulated in RCC have been identified, while no miRNA expression profiles for diabetic β-cells exist. To determine if *HNF4A* is regulated by miRNAs with regards to a physiological significance, binding sites for miRNAs that are upregulated in RCC were predicted. 20 miRNAs were selected due to the following criteria (Tab. 2). Each miRNA had to be upregulated in RCC according to at least one of the six studies (Gottardo et al., 2007; Kort et al., 2008; Nakada et al., 2008; Jung et al., 2009; Chow et al., 2010; Juan et al., 2010). One site with a perfect seed sequence was required within the 3180 nt of the *HNF4A* 3'UTR,

unless the miRNA was identified in more than four of six studies with a potential target site lacking a perfect seed sequence. The RNA22 program was used to search for binding sites for those 20 miRNAs, since it allows to specifically predict binding sites for miRNAs of interest within the queried 3'UTR sequence (Miranda et al., 2006). Despite the restriction to 20 miRNAs overexpressed in RCC, still 140 potential binding sites for miRNAs were retrieved within the *HNF4A* 3'UTR.

Table 2: Set of miRNAs upregulated in RCC.

The 20 selected miRNAs upregulated in RCC are listed. For each miRNA the studies reporting an increase are given as indicated by 1-6 (1: Gottardo et al., 2007; 2: Kort et al., 2008; 3: Nakada et al., 2008; 4: Jung et al., 2009; 5: Chow et al., 2010; 6:Juan et al., 2010). Eric J. Kort provided the original data on miRNA expression profiling (Kort et al., 2008). The average fold change for a miRNA is used when identified in more than one study. The predicted number of target sites by RNA22 (Miranda et al., 2006) including perfect seed sequences is given with the perfect seed sites also listed in brackets. The CT values were determined in HEK293 cells by qRT-PCR using 384 TaqMan human miRNA assays from Applied Biosystems and a multiplex RT protocol comprising eight different pools of stem-loop RT primers. In cases only the opposite stem loop of the corresponding miRNA was on the TaqMan Array this is indicated. miRNAs not analyzed are marked as not determined (n.d.).

miRNA	References	Fold induction	N of target sites (seed)	CT
miR-7	1	1.25	8 (1)	33.4
miR-18a*	2, 6	4.05	12 (1)	33.1 (miR-18a)
miR-21	2, 4, 5, 6	3.50	7 (0)	34.3
miR-27a	4	1.85	13 (1)	35.3
miR-34a	2, 5, 6	2.95	14 (2)	34.2
miR-106b*	4	2.30	9 (2)	29.8 (miR-106b)
miR-122	2, 3, 4, 6	28.16	9 (0)	> 40
miR-140-5p	4	10.63	4 (2)	31.7
miR-146b	2	1.70	2 (1)	35.0
miR-155	2, 3, 5, 6	5.66	2 (0)	36.6
miR-193a-3p	6	2.20	1 (1)	35.6 (miR-193a-5p)
miR-210	2, 3, 4, 5, 6	11.85	9 (0)	33.1
miR-224	2, 3, 4, 5, 6	7.05	2 (0)	> 40
miR-340*	4	2.41	9 (1)	34.1 (miR-340)
miR-342-3p	4	2.35	4 (1)	29.5
miR-342-5p	2	2.30	10 (1)	29.5 (miR-342-3p)
miR-452*	2, 3, 6	8.50	2 (1)	> 40 (miR-452)
miR-584	4	2.91	14 (1)	n.d.
miR-592	5	4.95	4 (1)	n.d.
miR-1271	4	3.86	5 (1)	n.d.

Hence, to locate the area of crucial miRNA binding sites within the *HNF4A* 3'UTR, several fragments of the 3'UTR were analyzed. Transfection of the 5' end of the 3'UTR (1-630) conferred a significantly increased luciferase reporter activity upon Dicer depletion, but the increase was even more pronounced for a construct containing the remaining sequence from 631-3180 (Fig. 19, upper part). Therefore, the focus was set on the previously described

fragments spanning the entire 3180 nt. Whereas miRNAs did not seem to target the 3' end of the 3'UTR (2574-3180), the remaining fragments mediated a slight increase in reporter activity upon Dicer knock-down. However, only the 5' fragment 1-449 nt showed a highly significant effect. To locate the area of crucial miRNA binding sites even further and possibly identify the exact location by loss of the effect, 12 gradually shortened constructs were analyzed within the 5' 449 nt (Fig. 19, lower part). The significant increase upon miRNA depletion was still present in over 50% of the constructs, but lost for constructs containing 196 or less nucleotides from the 5' end. Hence, miRNA target sites significant for *HNF4A* regulation seem to be located within nucleotides 204-449. No significant loss of effect between two consecutive constructs was observed, possibly reflecting several miRNAs targeting this area with a similar effect. In that case depleting all miRNAs is useful to locate an area of crucial miRNA binding, but a more specific method is needed to pinpoint single miRNA binding sites. In conclusion, the data reveal potential functional miRNA target sites distributed within 2.6 kb of the 3.2 kb 3'UTR.

3.4.1 miR-122 and miR-21 are not key regulators of the *HNF4A* 3'UTR

To experimentally verify miRNAs upregulated in RCC that potentially target the *HNF4A* 3'UTR (Tab. 2), miRNAs were specifically mimicked. miR-122 shows the highest fold induction in RCC and has nine potential binding sites predicted within the 3180 nt *HNF4A* 3'UTR including one located within nucleotides 204-449 at position 249-270 according to RNA22. To ensure optimal experimental conditions, the miR-122 dependent downregulation of the validated reporter plasmid psi-CCNG1 was reproduced (Fig. 20A) using the expression plasmid pSM-122 (Lin et al., 2008). Transfection of the reporter plasmid containing the long *HNF4A* 3'UTR or fragment 631-3180, resulted in a similar significant decrease as obtained for psi-CCNG1, indicating the regulation by miR-122 (Fig. 20A). Further analysis of various shortened constructs including fragment 1-449, revealed a slightly inhibited activity, but no significant effect upon miR-122 expression in most of the cases. Deleting the remaining potential miR-122 binding site in construct 1-249, still caused the same decrease. Hence, to rule out side effects from the residual sequence of the reporter plasmid as observed in the promoter studies, the effect of miR-122 was determined on the RL-Con plasmid. Indeed, a significant decrease was observed, making the regulation of the *HNF4A* 3'UTR by miR-122 unlikely. Furthermore, no regulation of the *HNF4A* 3'UTR by miR-122 was detected in INS-1 cells (Senkel et al., unpublished data).

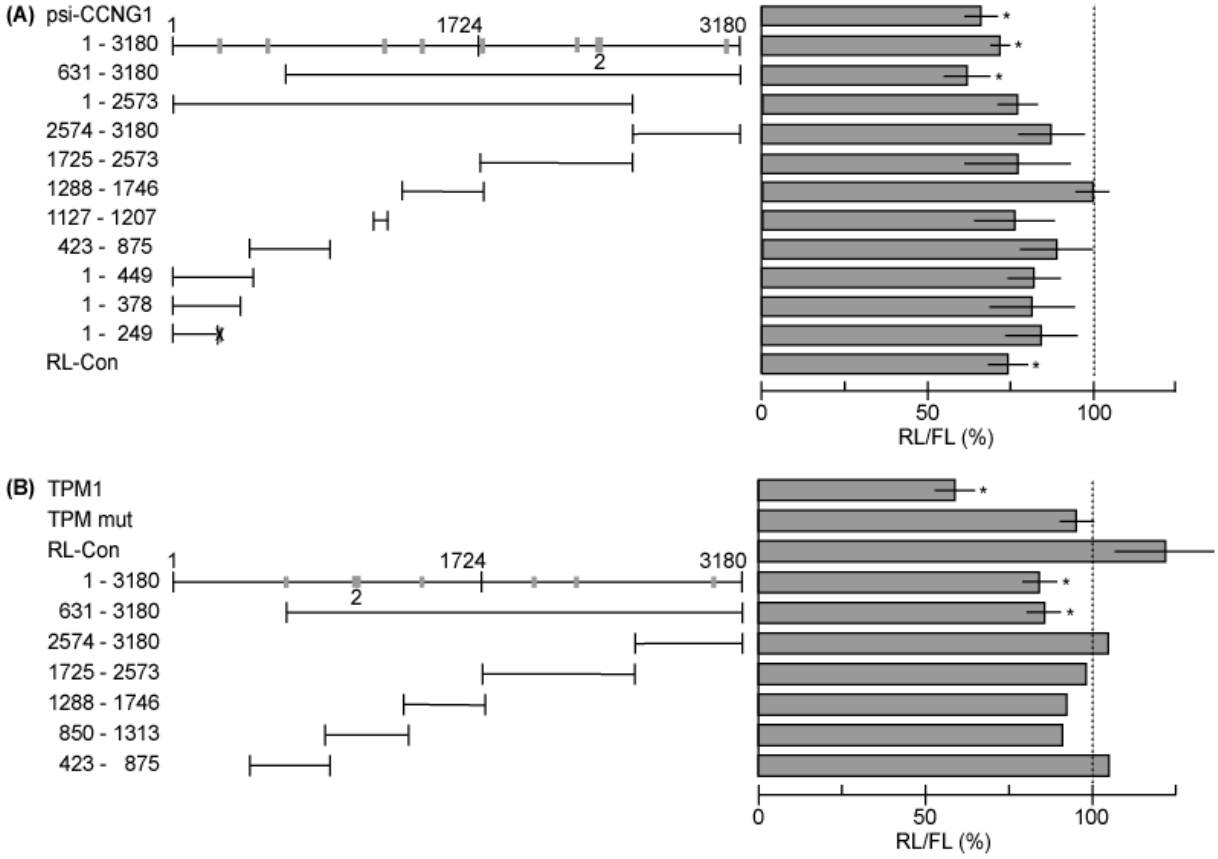

Figure 20: Reporter analyses of miR-122 (A) and miR-21 (B) sites in the *HNF4A* 3'UTR.
HEK293 cells were co-transfected with reporter plasmids and miRNA expression vectors (pSM-122; Lin et al., 2008 and pCMV-miR-21; Zhu et al., 2007) 24 hours before cell collection. At least one transfection assay was performed for each construct, involving two independent plasmid preparations in case of two or more assays. Each assay was performed in triplicate and a CMV-driven firefly luciferase was used to control for transfection efficiency. The activity of each construct in the absence of the miRNA expression plasmid (replaced by pSM-155; Du et al., 2006 or Rc/CMV) was used for standardization (100%) and is not shown. psi-CCNG1 (Lin et al., 2008) and TPM1 (Luc-TPM1-V1-UTR; Zhu et al., 2007) served as positive controls for miR-122 and miR-21, respectively, while TPM1 mut (Luc-TPM1-V1-UTR-d; Zhu et al., 2007) was used as a negative control for miR-21. The firefly activity used to control for transfection efficiency was expressed from the same plasmid as the *renilla* activity in case of psiCCNG1. The positive and negative control for miR-21 expressed firefly luciferase so that the RL-Con plasmid was used to control for transfection efficiency. The grey boxes indicate potential miRNA target sites without a perfect seed sequence and the number of target sites is given underneath the box in case of more than one site. A black cross in the grey box illustrates a destroyed target site. *P* values were determined using a one-sample *t* test. *P* values of < 0.05 are indicated by *.

While miR-122 is usually highly liver specific (Lagos-Quintana et al., 2002), miR-21 expression is conserved in the kidney and data indicates it is involved in kidney disease and functions as an oncogene (Zhu et al., 2007; Saal and Harvey, 2009). Hence, miR-21 shows a moderate expression in HEK293 cells. The upregulation in RCC is less pronounced but frequently detected (Tab. 2). To ensure that miR-21 expression effects target genes despite the moderate endogenous miR-21 level, the decrease of the reporter construct Luc-TPM1-V1-UTR was confirmed in the present study upon miR-21 expression (pCMV-miR-21), while no effect was detected for the TPM1 mutant (Luc-TPM1-V1-UTR-d) in which the miR-21 bindings site is deleted (Zhu et al., 2007; Fig. 20B). Furthermore, an impact of miR-21 on

RL-Con was ruled out. Transfection of the 3180 nt *HNF4A* 3'UTR and the construct 631-3180 resulted in a similarly small but significant decrease (Fig. 20B). Hence, the potential binding site at position 621-642 does not seem to be functional. Analyses of shortened constructs containing at least one potential binding site did not cause a decreased luciferase activity upon miR-21 expression. Thus, miR-21 seems to only have a slight impact on the *HNF4A* 3'UTR by targeting several sites simultaneously.

3.4.2 miR-34a downregulates *HNF4A* by targeting several sites in the 3'UTR

miR-34a has one of the highest numbers of potential target sites (14) within the *HNF4A* 3UTR (Tab. 2). Two sites include a perfect seed sequence and one of them is located within the 5' sequence of 1-449 nt of the *HNF4A* 3'UTR (RNA22). Although miR-34a is moderately expressed in HEK293 cells (Tab. 2), overexpression of pri-miR-34a downregulated the validated miR-34a reporter plasmid pGL3-CDK6-BS2 (Fig. 21A) containing one miR-34a target site (Lodygin et al., 2008). A similar effect was seen in INS-1 cells. An even more pronounced decrease by miR-34a was observed by using reporters including the 5' end construct 1-449 or 1-378 of the *HNF4A* 3'UTR. Destroying the target sequence of the predicted miR-34a binding site in construct 1-249 clearly diminished the decrease, but did not entirely abrogate the effect. Therefore, additional miR-34a binding sites were searched for within the 5' 249 nt and another potential site containing a perfect seed sequence was identified using TargetScan (Lewis et al., 2003). Transfection of constructs 1-159 and 1-151 which both lack the seed sequence of the proximal miR-34a target site (Fig. 21B), resulted in loss of effect in HEK293 and INS-1 cells. Taken together, it is evident that the proximal and distal miR-34a binding site within the 5' 449 nt are functional and their effect is additive.
Examination of the entire 3180 nt 3'UTR revealed 13 additional potential miR-34a binding sites (Fig. 21C). Overexpression of miR-34a with the long 3'UTR luciferase reporter led to a decreased luciferase activity in HEK293 and INS-1 cells similar to the one observed for pGL3-CDK6-BS2 reporter construct. It was ruled out that this decrease was based entirely on the two identified miR-34a binding sites within the 5' 449 nt fragment, as a miR-34a dependent drop in luciferase activity was measured with a construct (631-3180) lacking the 5' sequence. Therefore, several shortened constructs of the *HNF4A* 3'UTR were tested, each containing at least one potential miR-34a binding site. Since the construct 1288-1746 was affected by miR-34a overexpression, it is assumed that this region contains several cooperating miR-34a sites.

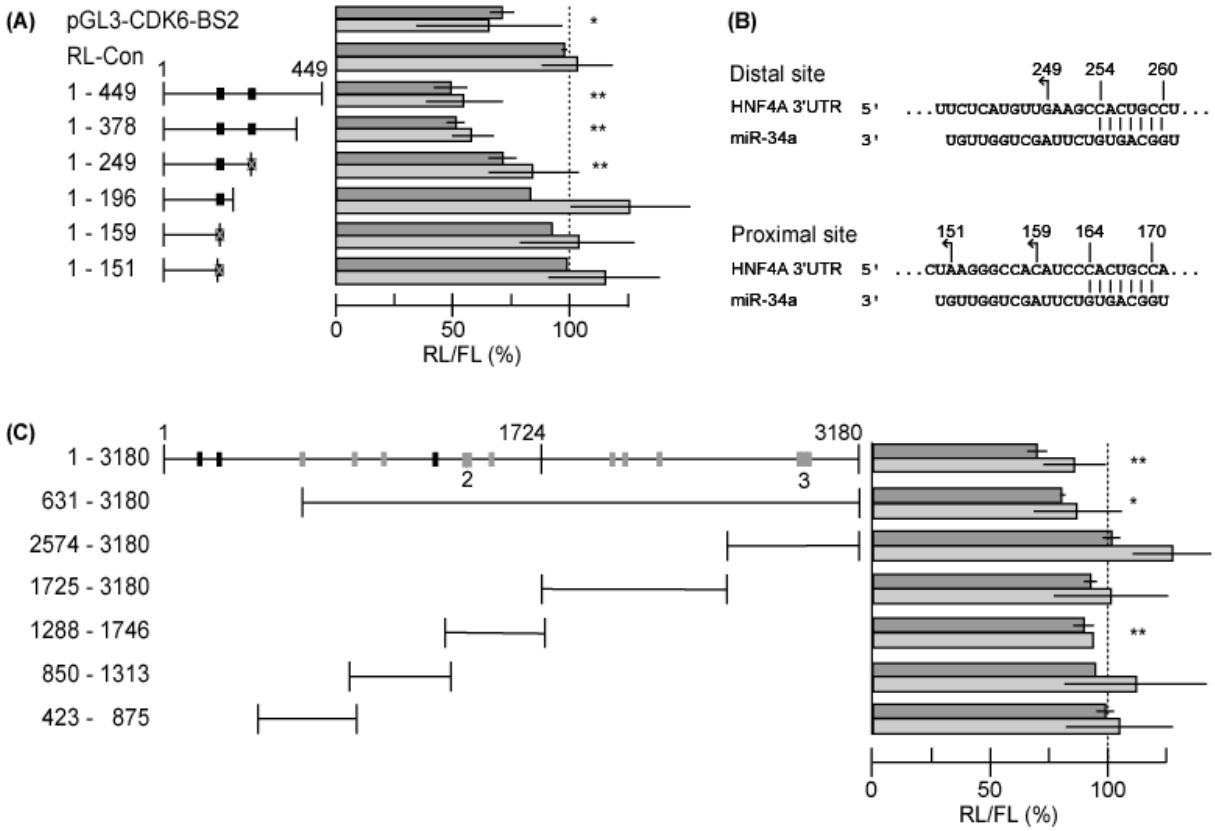

Figure 21: Reporter analyses of miR-34a binding sites in the *HNF4A* 3'UTR.
(A) Reporter plasmids and pri-miR-34a expression plasmids were co-transfected 24 hours before cell collection, into HEK293 (upper dark grey columns) and INS-1 cells (lower light grey columns). At least one transfection assay was performed for each construct, involving two independent plasmid preparations in the case of two or more assays. Each assay was performed in triplicate and a CMV-driven firefly luciferase was used to control for transfection efficiency. The activity of each construct in the absence of pri-miR-34a (replaced by Rc/CMV) was used for standardization (100%) and is not shown. pGL3-CDK6-BS2 (Lodygin et al., 2008) and RL-Con served as positive and negative controls, respectively. Since pGL3-CDK6-BS2 expresses the firefly luciferase, the RL-Con plasmid was used to control for transfection efficiency. The black boxes indicate miR-34a target sites including a perfect seed sequence. A grey cross in the black box displays a partially destroyed target site. *P* values were determined using a one-sample *t* test. Values of HEK293 and INS-1 cells were combined for each construct. *P* values are < 0.05 (*) and < 0.01 (**). **(B)** Schematic diagram of the two potential miR-34a binding sites within the 5' 449 nt of the *HNF4A* 3'UTR. The numbering refers to the first nucleotide after the stop codon as 1. The distal site located at 239-261 nt was predicted by RNA22 and TargetScan and is highly conserved among vertebrates. The proximal site extending from 149-171 nt was only predicted by TargetScan and is little conserved. The two perfect seed matches are indicated by vertical lines between the *HNF4A* 3'UTR and miR-34a sequence. The arrows at position 151, 159 and 249 mark the last nucleotide of the *HNF4A* 3'UTR in the corresponding constructs. **(C)** Analyzing miR-34a binding sites in the long *HNF4A* 3'UTR performed as in panel A. The grey and black boxes indicate miR-34a target sites with and without a perfect seed sequence, respectively. The number of target sites indicated by a box is given underneath the target site in case of more than one site.

D Discussion

1 Search for proliferation relevant target genes regulated by HNF4A

The transcription factor HNF4A is dysregulated in various diseases such as diabetes and cancer. Overexpression of *HNF4A2* results in a clear decrease in cell proliferation and morphological changes in different tissues in contrast to the *HNF4A* mutant C106R (Lucas et al., 2005; Erdmann et al., 2007; Grigo et al., 2008). These facts together with the finding that C106R cannot bind to DNA, but contains the full protein sequence (Taylor et al., 1996), suggests that the effects are mediated by target genes transcriptionally regulated by HNF4A. Previous microarray analyses identified 1411 potential target genes of HNF4A2 in HEK293 cells (Grigo et al., 2008). While in microarray experiments differentially expressed genes are revealed simultaneously among tens of thousands of genes with relative ease, the challenge is to retrieve direct target genes involved in the process of interest. Considering the numerous tissues and biological processes HNF4A is involved in (Bolotin et al., 2009), a significant amount of the potential 1411 HNF4A2 target genes are likely involved in mechanisms other than proliferation control and morphological changes. Selecting genes due to ontological analyses is associated with various limitations such as bias toward well annotated biological processes (Khatri and Draghici, 2005). To circumvent these issues, an expression profile of an HNF4A isoform, such as HNF4A8, with possibly no impact on proliferation control was sought for, to narrow down the 1411 HNF4A2 target genes to a reasonable number of proliferation relevant genes.

1.1 Target genes of HNF4A8 which have no impact on cell proliferation

HNF4A8 only differs in the first exon from *HNF4A2* due to differential promoter usage and hence lacks the activation function AF-1 (Nakhei et al., 1998). Such a difference has been shown to be sufficient to cause functional distinction as reported for example for Hnf4a1 versus Hnf4a7 (Briancon and Weiss, 2006) and is likely based on the distinct capacities of AF-1 and AF-2 to interact with cofactors (Wang et al., 1998; Sladek et al., 1999; Eeckhoute et al., 2003). In contrast to *HNF4A2,* it could be shown in the present study that cell lines conditionally expressing *HNF4A8* have no impact on cell proliferation or morphology in HEK293 cells (Fig. 4A). Even though a single copy of the gene-of-interest is supposed to be

integrated at a defined locus in the Flp-In system, slight functional differences have been observed in the resulting cell lines (Lucas et al., 2005; Grigo et al., 2008). While overexpression in the cell line HNF4A8 #9 caused a small decrease in cell proliferation after six days of induction with doxycycline, a transient increase was observed for HNF4A8 #11 and HNF4A8 #14 after three days (Fig. 4A). The same phenomenon was reported for *HNF4A8* expression in INS-1 cells and seems to be based on an initial increase in metabolic activity measured by MTS assay, as no increase in cell number was detected by cell counting (Erdmann et al., 2007). In INS-1 cells, overexpression of *HNF4A8* reduces cell proliferation and changes morphology, although cell reduction has a later onset and is not as pronounced as for *HNF4A2* and cell morphology changes are distinct (Erdmann et al., 2007). Those differences in INS-1 cells, which express endogenous *Hnf4a* (Huang et al., 2008) in contrast to HEK293 cells (Jiang et al., 2003) are likely based on a distinct cofactor environment and subsequently on a diverse set of target gene. This assumption of tissue-specific functioning is supported by a very limited overlap of HNF4A target genes determined by microarray analyses in hepatocytes (Naiki et al., 2002), insulinoma cells (Thomas et al., 2004) and HEK293 cells (Lucas et al., 2005).

In this study, microarray analyses revealed about 25% of consistently regulated target genes in both HNF4A8 #11 and HNF4A8 #14 cell lines (Fig. 5). A limited number of concordantly regulated genes of about 30% (Lucas, 2005) and about 50% (Grigo, 2007) has been previously observed for two independent HNF4A2 cell lines. Hence, the slight functional differences of the independent Flp-In cell lines containing the same GOI seem to be reflected by, in part, a distinct set of target genes. The amount of 181 consistently regulated target genes by HNF4A8 identified in this study is about eight times smaller than 1411 genes regulated by HNF4A2 in HEK293 cells (Grigo et al., 2008). This is in accordance with previous findings in INS-1 cells, identifying about three times less target genes for HNF48 in comparison to HNF4A2, although the difference is not as pronounced (Erdmann et al., 2007). Those data can be explained by the findings that HNF4A8 is a weaker transactivator than HNF4A2 due to the lack of AF-1 in the former isoform (Nakhei et al., 1998; Torres-Padilla et al., 2002; Eeckhoute et al., 2003; Ihara et al., 2005) and is supported by the identification of AF-1 dependent target genes (Briancon and Weiss, 2006). The majority of HNF4A8 target genes identified in this study are upregulated (~70%) in HEK293 cells, supporting the assumption of HNF4A as a mainly positive regulator due to the following data published previously. About 90% (Lucas et al., 2005) and 55% (Grigo et al., 2008) of genes were upregulated by *HNF4A2* in HEK293 cells. In INS-1 cells about 70% of upregulated genes were detected upon *HNF4A2* expression (Thomas et al., 2004). Another study using INS-1 cells detected about 80% and 90% positive regulation for target genes upon *HNF4A2* and *HNF4A8* expression, respectively (Erdmann et al., 2007). Microarray analyses of human

hepatoma cells (HuH-7) additionally transfected with rat *Hnf4a2* cDNA identified the majority of target genes (~90%) to be upregulated as well (Naiki et al., 2002).
Similar to the 70% overlap of HNF4A8 target genes with HNF4A2 regulated genes in INS-1 cells (Erdmann et al., 2007), about 60% of genes identified for HNF4A8 in the present study were also regulated by HNF4A2 in HEK293 cells (Fig. 5). But in contrast to INS-1 cells, where both isoforms cause a decrease in cell proliferation and morphological changes, in HEK293 these genes are assumed to have no impact on cell proliferation decrease or morphology. However, the elaborate approach to narrow down the number of 1411 potential HNF4A2 target genes to a reasonable number of proliferation relevant genes failed to do so in this study. Of only 181 target genes identified for HNF4A8, 111 genes were also regulated by HNF4A2 and thus deemed not to be relevant for proliferation, still leaving 1300 potential HNF4A target genes involved in proliferation control.

1.2 The multifaceted target gene CIDEB

Despite the vast amount of potential target genes, *CIDEB* was analyzed in more detail. Out of all tested isoforms and *HNF4A* mutants it was exclusively upregulated upon *HNF4A2* expression (Fig. 6) which has a strong impact on proliferation decrease. Furthermore, it has been shown in HEK293 cells that HNF4A targets the internal *CIDEB* promoter and enhances its activity (Da et al., 2006). CIDEB is a member of the cell death-inducing DNA fragmentation factor-α-like effector (CIDE) family that contain an evolutionary conserved CIDE-N domain sharing sequence similarity with DNA fragment factor 40/45 (DFF40/45; Inohara et al., 1998). Hence, all initial publications described a cell death-inducing activity for CIDEB (Inohara et al., 1998; Lugovskoy et al., 1999; Chen et al., 2000; Erdtmann et al., 2003; Reed et al., 2004). In previous experiments *HNF4A2* was ruled out to induce apoptosis in HEK293 cells by propidium iodine staining in FACS, annexin staining and measuring caspase 3/7 activity (Lucas et al., 2005). This is in contrast to INS-1 cells where overexpression of *HNF4A* induced apoptosis (Erdmann et al., 2007). However, apoptosis triggered by *Cideb* in mammalian cells could not be inhibited by caspase inhibitors, suggesting a caspase independent mechanism (Inohara et al., 1998). This potentially provides an alternative mechanism via CIDEB causing a decrease in cell number upon *HNF4A2* expression that would not be contradictory to the lack of *HNF4A2* induced caspase 3/7 activity in HEK293 cells (Lucas et al., 2005). qRT-PCR validated the *HNF4A2* dependent increase in *CIDEB* expression in the present study (Fig. 6). This method is known to be more sensitive than microarray analysis and thus only qRT-PCR revealed constitutive expression of *CIDEB* in HEK293 cells independent of *HNF4A* expression. This is in contrast to previous data detecting no *CIDEB* mRNA in HEK293 cells likely due to application of less

sensitive northern blot analyses (Inohara et al., 1998). The short 1.3 kb *CIDEB* transcript that is activated by HNF4A is the major transcript and expressed at high levels in adult and fetal liver and at lower levels in various tissues including kidney (Inohara et al., 1998; Liang et al., 2002). However, the primer pair used in this study targeted exon six and thus did not differentiate between the short and long transcript.

RNAi experiments performed in the present study indicated that CIDEB has an impact on the HNF4A2 dependent decrease in cell proliferation (Fig. 7). The cost-efficient esiRNA method has been shown to be comparable to optimized siRNAs in its silencing effect (Kittler and Buchholz, 2003). The advantage lies in the pool of esiRNAs used for one target gene, since the individual concentration of each esiRNA is believed to be too low to cause off-target effects, but the sum of many esiRNAs targeting the same gene is high enough for efficient genet silencing (Kittler et al., 2007; Mathey-Prevot and Perrimon, 2007). Although, in the present study, the rescue effect was only partial using *HNF4A* and *CIDEB* specific esiRNAs, it was highly reproducible even with different sets of esiRNAs. The reliability of this assay is supported by a previous report in which only about 20% of selected, upregulated HNF4A2 target genes showed a highly significant rescue effect ($p>0.01$) including *p21* (*CDKN1A*) that was sufficient to cause a decrease in cell proliferation independent of *HNF4A2* expression (Grigo et al., 2008).

Flp-In cell lines conditionally expressing Cideb (Fig. 8) confirmed the previously reported location of this protein in cytosolic corpuscles (Liang et al., 2002). A more detailed study reported that Cideb is localized to the endoplasmic reticulum and lipid droplets (Ye et al., 2009), while mitochondria localization as well as dimerization seems to be required for CIDEB induced apoptosis (Chen et al., 2000). Indeed, the observed corpuscular distribution of Cideb in the cytoplasm in the present study could reflect mitochondria location, but no signs of apoptosis such a chromatin condensation were observed in the Cideb cell lines upon induction (data not shown), in contrast to previous transient transfection studies (Inohara et al., 1998; Chen et al., 2000; Liang et al., 2002; Erdtmann et al., 2003). Notably, Cidea, initially recognized for its ability to trigger apoptosis just like Cideb (Inohara et al., 1998) does not induce cell death in liver of mice (Viswakarma et al., 2007) and no difference in apoptosis was observed in brown adipocytes between wild-type and *Cidea*-deficient mice (Gong et al., 2009).

In the present study, overexpression of *Cideb* in the two cell lines did not decrease cell proliferation as observed for *HNF4A2*, but instead resulted in a small increase after three days of induction which diminished after six days (Fig. 9A). This might be due to an initial increase in metabolic activity as described for *HNF4A8* in INS-1 cells (Erdmann et al., 2007), but the cell number was not counted to confirm this hypothesis for *Cideb*. Possibly, cell line

dependent factors are responsible for the insufficiency of to cause a decrease in cell proliferation. For example, CIDEB induced apoptosis has been shown to be dose-dependent (Inohara et al., 1998; Liang et al., 2002). However, qRT-PCR ruled out that insufficient *Cideb* expression is the limiting factor, since the transgene is expressed more than twice as high as the endogenous *CIDEB* gene upon *HNF4A2* expression (Fig. 10). Notably, the RNA level does not necessarily correlate with the protein amount due to posttranscriptional regulation. Endogenous and exogenous CIDEB protein level could not be compared, as commercially available antibodies were not specific enough to detect the protein unambiguously.
In contrast to the lack of Cideb protein in untreated cells as determined by western blot and immunofluorescence analyses using a myc-tag antibody, *Cideb* RNA was detected by qRT-PCR even in uninduced Cideb cells. The two protein specific methods are less sensitive than qRT-PCR, although it cannot be ruled out that the RNA is not efficiently translated into protein. Obviously, the two Cideb cell lines are leaky, an issue of the Flp-In system which has been recently reported (Senkel et al., 2009). If increased expression levels of CIDEB trigger apoptosis and the system is leaky, no such cell line could be generated. Possibly the established Cideb cells have acquired genetic traits during the selection process to cope with elevated *Cideb* expression levels, which makes them insensitive to increased *Cideb* expression.

Figure 22 summarizes some of the HNF4A dependent effects on cell function mediated by targets genes with emphasis on *CIDEB* as determined in the present and previous studies. While HNF4A8 has no impact on cell proliferation in HEK293 cells as demonstrated in this study, HNF4A2 mainly activates target genes resulting in a decrease in cell proliferation (Grigo et al., 2008). *p21* is sufficient to inhibit cell proliferation independent of *HNF4A2* expression. *CIDEB*, extensively analyzed in this study, only contributes to the proliferation decrease in cooperation with other factors within the signal cascade triggered by *HNF4A2*. The same conclusion was already drawn in previous experiments for *ANK3*, *ALDH6A1*, *BPHL*, *DSC2*, *EFHD1*, *EPHX2*, *NELL2*, *MME*, *PROS1*, *SEPP1*, *TGFA* and *THEM2* (Grigo et al., 2008). It is noteworthy that *SEPP1*, *NELL2* and even *p21* are activated by HNF4A8 to a highly similar extent as by HNF4A2. Hence, it is not possible to conclude that all target genes of HNF4A8 have no impact on cell proliferation due to the observation that *HNF4A8* does not influence proliferation. Those findings further corroborate the assumption that not a single gene, but the complex interplay of numerous factors determines the overall function. For example, *p21* was implicated as a target gene of HNF4A2 relevant for proliferation decrease (Grigo et al., 2008). Another study revealed a dichotomy between differentiation and proliferation due to interactions between Sp1, HNF4A1 and c-Myc proteins and the *p21* promoter (Hwang-Verslues and Sladek, 2008). In the absence of c-Myc, HNF4A1 activates

p21 by interaction with Sp1, causing a block in cell cycle and decreased cell proliferation. In the presence of c-Myc, activation of *p21* by HNF4A1 is reduced due to several mechanism such as displacement of HNF4A1 from Sp1, resulting in increased proliferation.

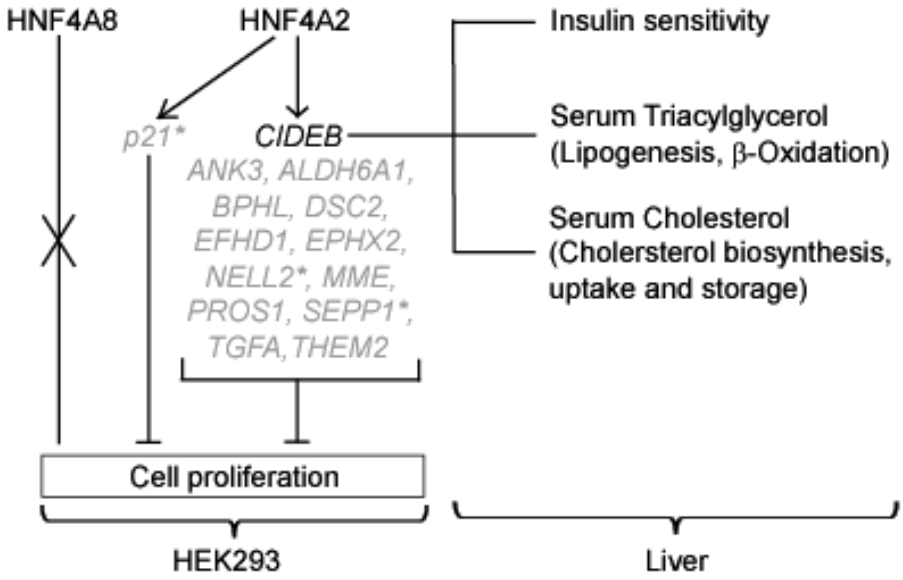

Figure 22: Impact of HNF4A via target genes such as *CIDEB* on various cell functions. Overexpression of *HNF4A2*, but not *HNF4A8* inhibits cell proliferation in HEK293 cells mediated by several target genes. Most target genes including *CIDEB* are only functional within the HNF4A2 dependent network, while *p21* is sufficient to cause a decrease in cell proliferation. Genes depicted in grey were identified previously (Grigo et al., 2008). Genes activated by HNF4A8 as well are indicated by an asterix. In the liver, *Cideb* has been shown to influence metabolic processes (Gong et al., 2009; Li et al., 2010).

In accordance with one gene mediating multiple functions under different circumstances, CIDE proteins including CIDEA, CIDEB and Fsp27 (CIDEC) have been recently associated in the development of metabolic disorders such as diabetes, liver steatosis and obesity. *Cideb* knock-out mice are lean and resistant to diet-induced obesity and liver steatosis. This phenotype is caused by increased insulin sensitivity despite lower levels of plasma insulin and reduced serum triacylglycerol (TAG) due to decreased hepatic lipogenesis and increased fatty acid β-oxidation (Li et al., 2007b; Gong et al., 2009; Fig. 22). Furthermore, a recent report observed decreased cholesterol biosynthesis in *Cideb* knock-out mice that showed increased hepatic cholesterol uptake and storage upon high cholersterol diet (Li et al., 2010; Fig. 22). The essential function of HNF4A in the liver has been thoroughly investigated and includes the regulation of genes involved in the control of lipid homeostasis (Li et al., 2000; Hayhurst et al., 2001; Naiki et al., 2005). HNF4A regulates the majority of apolipoproteins and is thus assumed to play a role in atherosclerosis (Li et al., 2000; Bolotin et al., 2010). Cideb was shown to interact with apoB (Ye et al., 2009) and is suggested to contribute to atherosclerosis as well (Li et al., 2010). HNF4A2, which upregulates *CIDEB*, is the main isoform in the adult liver, while expression of *HNF4A8*, which has no impact on

CIDEB expression as shown in this study, is absent (Torres-Padilla et al., 2001). A recent report used an integrated approach of genome-wide techniques, *in silico* predictions and functional assays to identify direct target genes of HNF4A and confirmed *CIDEB* as an HNF4A target gene in the liver (Bolotin et al., 2009). Taken together, *CIDEB* seems to be a crucial target gene of P1 specific HNF4A isoforms due to its involvement in proliferation control and energy homeostasis in a cell type dependent manner (Fig. 22).

2 Transcriptional regulation of *HNF4A* via the P2 and P1 promoter

2.1 Impact of mutations in the P2 promoter on gene expression

An overview of the regulation of *HNF4A* as determined mainly in this study and its implication on diabetes and cancer is given in Figure 23. Transcriptional regulation is mediated by transcription factors and miRNAs targeting the *HNF4A* P2 and P1 promoter, respectively. The posttranscriptional control is achieved via binding sites for miRNAs and regulatory factors within the two 3'UTRs identified for *HNF4A*.

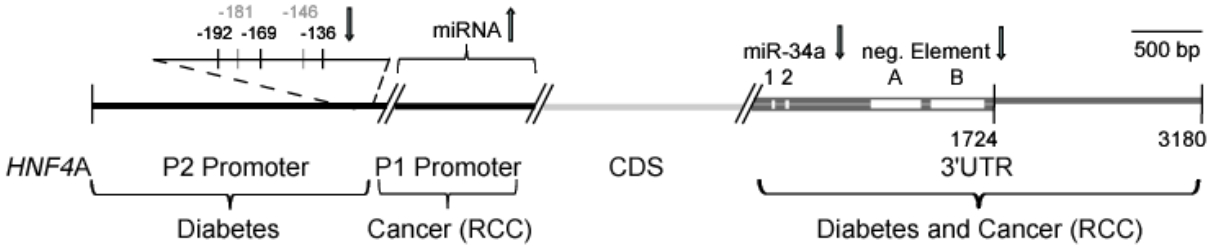

Figure 23: Transcriptional and posttranscriptional regulation of *HNF4A*.
Mutations identified within the *HNF4A* P2 promoter that are linked to diabetes and impair the P2 promoter activity *in vitro* as indicated by an arrow pointing downwards, are depicted. P2/-136 and P2/-169 were characterized in the present study (Fig. 13). P2/-146 (Thomas et al., 2001), P2/-181 (Hansen et al., 2002) and P2/-192 (Ek et al., 2006; Raeder et al., 2006a) were reported previously, but the latter mutation was shown to reduce promoter activity in this study (Fig. 13). miRNAs potentially activate the *HNF4A* P1 promoter as displayed by an upwards pointing arrow. Downregulation of those miRNAs might contribute to the dysregulation of *HNF4A* in cancer such as RCC. Both 3'UTRs with a length of 1724 bp and 3180 bp confer a negative posttranscriptional regulation of *HNF4A* mediated by at least two binding sites for miR-34a and negative element A and B. Downwards pointing arrows describe the negative effects of miR-34a and factors targeting negative element A and B on *HNF4A* regulation. Upregulation of miR-34a, as detected in RCC for example or/and other factors likely cause downregulation of *HNF4A* relevant for cancer or diabetes progression.

The families presented in Figure 11 are consistent with *HNF4A* associated diabetes, including either neonatal hypoglycaemia (-136A>G) or increased birth weight (-169C>T).

Diabetes was generally adequately controlled by sulphonylurea (SU) or diet alone. Mutations affecting the P2 promoter or P2-derived isoforms are typically diagnosed later (median age, 31 years) compared with mutations that affect all isoforms of *HNF4A* (median age, 24 years; Harries et al., 2008). Most of the P2 promoter mutation carriers presented in this study were diagnosed before 25 years of age, suggesting that these mutations are relatively severe or, alternatively, this could represent case-finding bias leading to mutation suspicion and testing only in younger affected individuals. In contrast, the -192C>G mutation is linked to a later age of onset (mean age of diagnosis, 45 years) and few carriers were diagnosed before 25 years of age (Ek et al., 2006; Raeder et al., 2006a).

Both novel *HNF4A* P2 promoter mutations, -136A>G and -169C>T, decrease the basal activity of the promoter in transient transfection assays in HEK293 cells (Fig. 13). Based on gene expression profiling, HEK293 cells do not express *HNF1A*, *HNF1B*, *HNF6* or *OC2* (Lucas et al., 2005), implying that the mutations at -136 and -169 affect the binding of additional factors (Fig. 12). In contrast, -192C>G does not affect the basal activity in HEK293 cells, but decreases the basal activity in INS-1 cells (Fig. 13), supporting previous evidence that this mutation affects binding of a cell-specific factor enriched in INS-1 cells (Ek et al., 2006). All three mutations have decreased activity in the presence of HNF1B compared with the wild-type promoter in both kidney and β-cell lines. The -169C>T mutation, affecting the consensus sequence of the HNF1 binding site, shows the most marked reduction in activity with HNF1B. Since the other mutations are unlikely to affect binding of HNF1B directly, it is most likely that the reduced activity with HNF1B reflects a co-operative action of transcription factors at the *HNF4A* P2 promoter. An interplay of various tissue-specific transcription factors has also been assumed in previous P2 promoter analyses, since transactivation by CDX-2 is reduced for the mutated P2/-146T>C site, although it does not seem to interact directly with it (Thomas et al., 2001). In the present study in INS-1 cells, a consistent decrease in promoter activity was observed upon *HNF1B* induction for all of the constructs except P2/-285(-169C>T), which has a mutated HNF1 binding site. The decrease in activity is as expected, since the HNF1 binding site is recognized by HNF1A and HNF1B, but INS-1 cells express more *HNF1A* than *HNF1B* (Thomas et al., 2004). Thus endogenous HNF1A is outcompeted by the weaker transactivator HNF1B (Ryffel, 2010). A mutation upstream in the same HNF1 binding site at position -181G>A has been analyzed in more detail previously and revealed a decreased activation due to a reduced affinity for HNF1A (Hansen et al., 2002). However, regulation by HNF1B was not determined. It is most likely that -169C>T affects binding of HNF1A in the same way.

Previous experiments by two independent groups failed to show an impaired performance of the *HNF4A* P2 promoter mutation -192C>G in transfection assays in the MIN6 cell line and in CaCo2 cells (Ek et al., 2006), but also in INS-1 cells (Ek et al., 2006; Raeder et al., 2006a).

While promoter variants are known to affect promoter activity in a cell-specific manner as reported for the -146T>C mutation that caused a decreased activity in INS-1 cells but not in hepatoma cells (FT0-2B; Thomas et al., 2001), the lack of effect in INS-1 cells requires another explanation. Possibly, the use of a *renilla* luciferase construct as an internal control in the present experiments may have facilitated the detection of small changes. In addition, it is likely that the use of cell lines containing *HNF1B* as a stably integrated conditional transgene mimics the *in vivo* situation more efficiently.

The present data establish that three *HNF4A* P2 promoter point mutations co-segregating with diabetes affect highly conserved nucleotide positions and, more importantly, impair the function of the mutated promoters in transfected cells. This makes it most likely that these mutations cause the diabetic phenotype. Taking into account the -146T>C (Thomas et al., 2001) and -181G>A mutations (Hansen et al., 2002), there are now five mutations known in the P2 promoter that affect the performance of *HNF4A* in pancreatic β-cells and result in diabetes (Fig. 23).

2.2 Impact of miRNAs on the P2 and P1 promoter

So far it could only be assumed that binding of miRNAs to promoter regions follows the same guidelines as established for miRNAs targeting 3'UTRs, since corresponding data is lacking. A recent study examined computational methods for predicting miRNA binding sites within promoter sequences and indicates that those regulatory sequences are as good candidates for miRNA regulation as 3'UTRs (Younger et al., 2009). On average they identified about 30 miRNA seed matches per promoter sequence analyzed (-200 to -1 relative to transcription start site) and suggest that minimum free energy as well as high complementarity between the miRNA and target sequence are useful criteria to prioritize miRNA target predictions within promoters, similar as known for 3'UTRs. *In silico* prediction using the online RegRNA software (available at http://regrna.mbc.nctu.edu.tw; Huang et al., 2006) revealed numerous potential miRNA binding sites within the *HNF4A* P2 promoter. Although this software was designed to predict regulatory motifs and miRNAs in RNA sequences, it predicted the miR-373 binding site within the *E-cadherin* and *CSDC2* promoters. For both promoters the activating function was experimentally validated (Place et al., 2008). However, it is not possible to upload any miRNA sequences in order to obtain a specific map of potential miRNA binding sites for miRNAs of interest within the sequence analyzed. Using RNA22 (Miranda et al., 2006), various potential binding sites were predicted within the *HNF4A* promoter regions for miRNAs that are downregulated in RCC.

An interplay of ubiquitous and cell-specific factors targeting various regulatory elements within promoter sequences as well as the chromatin context, regulate the activity of gene promoters and influence experimental procedures (Maston et al., 2006). While promoter regulation by transcription factors has been studied extensively, knowledge about promoter modulation by miRNAs and inevitable experimental limitations are scarce. Certainly, various properties for promoter regulation by transcription factors and miRNAs apply to both. In accordance with that, the effect of miR-373 on *E-cadherin* and *CSDC2* is cell type specific (Place et al., 2008) as described for many transcription factors for example on the *HNF4A* promoters (Bailly et al., 2009). In contrast to transcription factors, small dsRNAs complementary to gene promoters have been shown to modulate transcription of target genes by recruiting members of the Ago family to RNA transcripts that originate from the target promoter in either sense or antisense direction (Janowski et al., 2006; Kim et al., 2006; Han et al., 2007; Schwartz et al., 2008). So far it is only assumed that miRNAs might regulate gene promoters by targeting such transcripts as well (Kim et al., 2008; Younger et al., 2009). In that case, generation of promoter transcripts might be disrupted by introducing only parts of the promoter sequences into a reporter plasmid. Induction of gene promoters by miR-373 was identified by analyzing the effect of this miRNA on the endogenous target gene and not by reporter analyses (Place et al., 2008). However, a recent study claims the regulation of the interleukin genes *IL24* and *IL32* by miR-205 via targeting specific sites in the promoter sequences (Majid et al., 2010). Besides demonstrating the miR-205 dependent regulation of the endogenous genes, they observed an activating effect of miR-205 on a co-transfected vector containing the 2.2 kb *IL24* promoter sequence in luciferase assays. Hence, it seems to be possible to address miRNA regulation on at least large promoter fragments in transient transfection assays. Notably, this experiment was just shown for *IL24*, but not for *IL32* and even more importantly, the predicted miR-205 binding sites within the promoter sequences were not mutated to confirm the sequence specific effect of miR-205 on the *IL24* and *IL32* promoters. Despite promising data, indirect effects of miR-205 on *IL24* and *IL32* promoter activation cannot be entirely excluded. The experimental limitations might explain the lack of research in this field. However, a recent study analyzed conserved short sequence (< 8 nt) located within 100 bp up- and downstream of the transcription start site. They postulate that the majority of the common sequences are frequently found within mature miRNAs and stem-loop sequences (Putta and Mitra, 2010) and hence provide further evidence of a possible widespread impact of miRNAs on promoter sequences.

Due to the high degree of uncertainty in this field, in this study a general approach was applied by knocking down Dicer, to evaluate regulation of the two *HNF4A* promoters by any miRNA. Deletion of Dicer decreases or abrogates the production of mature miRNAs (Hutvagner et al., 2001; Grishok et al., 2001). Although depletion of the Dicer protein is not

complete in the cell line after addition of doxycycline (Schmitter et al., 2006), a significant relief of reporter constructs containing binding sites for let-7a in the 3'UTRs (RL-Perf, RL-3xBulge) was verified in contrast to RL-Con, lacking any binding site (data not shown). This data confirmed the miRNA specific functionality of the assay, but was restricted to 3'UTR sequences, since positive controls for promoter sequences are lacking.

The activity measured in this study for the gradually shortened *HNF4A* P2 and P1 promoter fragments in uninduced cells was differential, likely due to interruption of transcription factor binding sites as described previously for the P2 promoter (Thomas et al., 2001). However, in the current analyses the focus was set only on miRNA regulation by comparing activities between cells in the presence and absence of miRNAs for each construct. The decrease in activity of the P2 promoter upon Dicer knock-down (Fig. 14A), implies depletion of a miRNA functioning as an activator. However, this finding is controversial, since the decrease is not significant in comparison to the empty control vector pGL3 basic and is not abolished upon gradual deletion of the promoter sequence even up to constructs P2/-135 which essentially removes the promoter (transcription start site at -103) and mainly consists of 5'UTR sequence (~100 bp). In contrast, the CMV-promoter was not affected by miRNA depletion, which suggests a promoter specific effect. However, its activity is several hundred-fold higher in comparison to the P2 promoter and small alterations in its activity would thus be lost in detection due to the high expression. A potential influence of miRNAs on the P2 promoter mutations described in the previous section was not analyzed. Even in case of P2 regulation by miRNAs, the present data would indicate that miRNA targeting takes place downstream of -135 relative to the translational start site, but the identified P2 promoter mutations were all located upstream from this position.

In general, the P1 promoter was more active than the P2 promoter. The former promoter seems to be predominantly active in the embryonic kidney and P1 specific Hnf4a1 was detected at a higher level in comparison to Hnf4a7 in murine kidney (Kanazawa et al., 2009). Regulation of the P1 promoter by miRNAs as determined in this study seems more likely since the effect varies to some extent with different fragments and is significantly reduced in comparison to pGL3 basic in case of P1/-1114 and P1/-281 (Fig. 14B). Interestingly, the activity of those two constructs in uninduced cells is comparable and higher than for P1/-590 and P1/-132. However, since no preferential miRNA targeting was observed for any area within the 200 bp upstream of the transcription start site of promoters surveyed (Younger et al., 2009), any predicted miRNA within the P1/-281 construct (117 bp are 5'UTR sequence) is a potential candidate.

Data obtained in this study by specifically mimicking or inhibiting certain miRNAs as described in this section is not shown. miR-187, miR-199b-5p and miR-200c are

downregulated in RCC according to at least two different studies (Kort et al., 2008; Nakada et al., 2008) and were predicted to have 5(2), 8(5) and 3(1) target sites within the sense strand of the *HNF4A* P1/-1114 (P1/-281) promoter as determined by RNA22. The approach to specifically inhibit certain miRNAs by using chemically synthesized, single-stranded, modified RNAs (Qiagen), was successfully established as inhibiting let-7a resulted in a significant increase in luciferase activity for the reporter constructs containing binding sites for let-7a in the 3'UTRs (RL-Perf, RL-3xBulge). No changes in activity were observed for RL-Con serving as a negative control. However, positive and negative controls specific for miRNA promoter analyses were lacking. Inhibiting miR-187, miR-199b-5p and miR-200c did result in a pronounced decrease in luciferase activity as expected if abolishing their activating effect on the promoter. However, the decreased activity was gradually lost with increasing inhibitor concentrations ranging from 50 nM to 250 nM and even turned into an increased activity at high concentrations. Notably, the same phenomenon was observed for miR-20a which is not predicted to target the P1/-281 promoter fragment and thus seems to be an unspecific effect. Mimicking miR-200c, using chemically synthesized, dsRNAs from Qiagen at concentration ranging from 1 nM to 100 nM had no effect on P1/-281 expression. Generating and transfecting a pre-miR-200c expression plasmid, the decrease on the validated pMIR-REPORT vector containing parts of the zinc-finger E-box binding homeobox 1 (*ZEB1*) 3'UTR could be reproduced (Burk et al., 2008). One of the three potential miR-200c binding sites located upstream of position -281 in the P1 promoter exhibits high complementarity to the miRNA sequence including just four mismatches. Since high complementarity has been suggested to be a promising criteria for miRNA binding sites within promoter sequences (Younger et al., 2009), the effect of the pre-miR-200c expression plasmid was also tested on the P1/-590 and P1/-1114 constructs. For all three P1 promoter constructs a decrease in luciferase activity was observed, disagreeing with an activating function of miR-200c on the *HNF4A* P1 promoter. Taken together, the attempt to specifically inhibit or mimic certain miRNAs potentially targeting the *HNF4A* promoter failed due to ambiguous results under different conditions and the lack of reliable controls.

Taken together, a tumor-suppressive role has been suggested for P1 driven *HNF4A* (Lazarevich et al., 2004; Lucas et al., 2005; Tanaka et al., 2006; Niehof and Borlak, 2008). Cancer dependent downregulation of specific miRNAs usually targeting and activating the *HNF4A* P1 promoter as indicated in Figure 23, potentially decreases *HNF4A* expression and thus contributes to the progression of certain cancers. This hypothesis would explain why so far no mutations in the ORF have been identified causing the downregulation of *HNF4A* function in RCC (Lausen et al., 2000; Dalgliesh et al., 2010). Such a mechanism has most recently been described involving loss of transcriptional activation of the interleukin tumor

suppressor genes *IL24* and *IL32* in prostate cancer due to silencing of miR-205 targeting specific sites in their promoter sequences (Majid et al., 2010).

3 Posttranscriptional regulation of *HNF4A* via 3'UTRs

In addition to *HNF4A* gene regulation involving complex networks of *cis*-acting elements and *trans*-acting factors that work on the transcriptional level, Figure 23 summarizes the negative posttranscriptional regulation via two *HNF4A* 3'UTRs mediated by *cis*-elements targeted by miRNAs and other regulatory factors as determined in this study. Whereas on the transcriptional level promoter and enhancer elements with their corresponding DNA binding proteins have been well characterized (Mitchell and Tjian, 1989; Kadonaga, 2004), posttranscriptional control involving the 3'UTR of mRNAs has been largely neglected. This ignorance is quite surprising, as in many cases the 3'UTR of a given mRNA exceeds the length of the ORF substantially (Mignone et al., 2002) as exemplified also by *HNF4A* (Fig. 15A). Furthermore, only 39 motifs recognized by RNA-binding proteins are deposited in the database UTRdb (Grillo et al., 2009), whereas 457 transcription factor binding sites are available in JASPAR 2010 (Portales-Casamar et al., 2010).

3.1 Posttranscriptional regulation by RNA-binding proteins

Investigating the 3'UTR of human *HNF4A* for its regulatory potential, a non-canonical and canonical PAS was detected leading to a short (1.7 kb) and long (3.2 kb) 3'UTR, respectively (Fig. 15A). About 29% of mRNAs contain more than one PAS and the distal PAS tend to be canonical signals (Beaudoing et al., 2000). Using *in silico* predictions (Tab. 1) only the highly conserved, distal PAS was identified. In accordance with data showing that non-canonical signals are processed less efficiently than the canonical PAS (Beaudoing et al., 2000), the long 3'UTR is generated predominantly in HEPG2 and HK120 cells (Fig. 15B).
In the present study, a systematic analysis of both 3'UTRs by reporter assays, significantly reduced luciferase reporter activity in HEK293 and INS-1 cells (Fig. 16), as described for other 3'UTRs in previous studies (Cok and Morrison, 2001; Mawji et al., 2004; Johnson et al., 2005; Moncini et al., 2007; Sun et al., 2010). Only few potential *cis*-acting elements could be identified in the *HNF4A* 3'UTR by *in silico* studies (Tab. 1). The SXL-binding site is targeted by a sex-lethal (SXL) female-specific RNA-binding protein identified in *Drosophila melanogaster* (Kelley et al., 1995). In addition to its function, it is not likely to impact the *HNF4A* 3'UTR since no significant difference was observed between constructs 2574-3180

including the element and 2771-3180 lacking it. A K-box element was predicted at the 3' end of the *HNF4A* 3'UTR (2940-2947). Constructs comprising this area show the highest luciferase activity of all constructs tested in HEK293 cells and also higher activities than the majorities of constructs in INS-1 cells (Fig. 16), but K-box is described to mediate negative regulation (Lai et al., 1998). Furthermore, this element was found to be complementary to the 5' end of many miRNAs (Lai, 2002). In Dicer knock-down experiments, construct 2574-3180 including the potential K-box element showed no sign of regulation by miRNAs (Fig. 19). Taken together, it is very unlikely that the K-box element is functional in *HNF4A*. Using the UCSC browser, two elements named MIRb and MIRc were located right within sequence A and B, respectively (Fig. 17). However, those elements were not conserved in the mouse sequence, did not decrease luciferase activity as reported for sequence A and B and are thus unlikely to function as destabilizing elements. Instead, by deletion analyses, the two novel elements A and B were identified, conferring the highest repressive effect in both cell lines (Fig. 17). The Musashi binding element located within negative element A (910-914) might be functional, but is unlikely to be a crucial element, since the large size of negative element A cannot be restricted on either side without diminishing the effect (Fig. 17). Taken together, none of the predicted regulatory elements seems to be essential for posttranscriptional regulation of *HNF4A*, at least under the present physiological conditions, but instead several elements such as negative element A and B and the proximal, non-canonical PAS are functional, but were not identified by *in silico* predictions. Those findings point out the need for more accurate prediction programs and also the importance of functional assays. To exclude that transcriptional elements located in the 3'UTR interfere in the assay, elements A and B were shown to act on RNA level, as the antisense sequences are not functional and a SV40 transcriptional stop codon upstream of the element destroys their function (Fig. 18). The size of elements A and B (~400 nt) is much larger than a binding site of a RNA-binding protein or a miRNA. The single-stranded RNA likely adopts a secondary structure possibly associated with RNA-binding proteins. Such elements have been found for instance in the 3'UTRs of the *Vg1* mRNA (Gautreau et al., 1997) and *bicoid* mRNA (Seeger and Kaufman, 1990; Macdonald, 1990), where they are involved in the cytoplasmic localization of the mRNA.

The pronounced negative effect of element A and B is masked within the *HNF4A* 3'UTRs due to an element located within the 5' sequence 1-630 nt (Fig. 18). Depending on the level of transacting proteins that potentially target these distinct elements, the regulation of *HNF4A* via its 3'UTR could be altered. A similar complex regulation mediated by several, distinct functioning 3'UTR elements has also been described in *Cox-2* (Cok and Morrison, 2001) *Endothelin-1* (Mawji et al., 2004) and *CDK5R1* (Moncini et al., 2007) mRNAs. Interestingly, an interaction of two quite large elements (~100 bp and ~300 bp) separated by about 2 kb

within the insulin-like growth factor II (*IGF-II*) 3'UTR has been described. Specific parts of the two elements can form a stable stem structure that is involved in the formation of RNA-protein complexes. Those complexes are dependent on growth conditions and regulate *IGF II* mRNA levels involving endonucleolytic cleavage of the mRNA within downstream element II (Scheper et al., 1995; Scheper et al., 1996). It is conceivable that such direct or/and indirect interactions through a bridging ribonucleoprotein complex between different elements of the *HNF4A* 3'UTR take place as well. However, due to the very limited number of studies addressing the comprehensive interactions of different parts of the 3'UTR, the majority of phenomena observed are not clearly understood and a distinct biological function remains elusive. Further complexity has been revealed recently by identifying interplays between RNA-binding proteins and miRNAs. Binding of those proteins to mRNAs has been shown to facilitate or counteract miRNA function on 3'UTRs which is in part dependent on the mRNA or cellular context (Krol et al., 2010).

3.2 Posttranscriptional regulation by miRNAs

Although several target prediction algorithms are available, the majority of programs such as miRanda (John et al., 2004), PicTar (Krek et al., 2005) and TargetScan (Lewis et al., 2003), analyze the 3'UTRs and miRNAs contained in their databases. Since the 3180 nt *HNF4A* 3'UTR is not included, the program RNA22 was used, which allows for the analyses of 3'UTRs and miRNAs of interest. Furthermore, this program does not rely on cross species site conservation or a perfect seed sequence, but allows for GU pairing (Miranda et al., 2006). The former two parameters have been shown not to be essential for functional miRNA binding sites, while GU pairing is tolerated (Grimson et al., 2007; Baek et al., 2008; Hammell et al., 2008; Chi et al., 2009; Wu et al., 2010). Due to the less stringent criteria of RNA22, too many potential miRNA targets were found in the *HNF4A* 3'UTR. Therefore, the analysis was restricted to the 20 miRNAs upregulated in RCC (Tab. 2), predicting 140 potential miRNA binding sites within the long *HNF4A* 3'UTR. The false-positive rate of target prediction is quite high (Bentwich, 2005; Rajewsky, 2006; Jiang et al., 2009; Wu et al., 2010) and therefore potential target sites can only be used as a guide (Sethupathy and Collins, 2008). In a recent study for example, out of a pool of 266 miRNAs predicted to target the *p21* 3'UTR by four different prediction programs including TargetScan, PicTar, miRanda and RNA22, only 28 miRNAs significantly reduced luciferase activity (Wu et al., 2010). To locate the area of crucial miRNA binding within the *HNF4A* 3'UTR in the present study a HEK293 cell line with a conditional Dicer knock-down (Schmitter et al., 2006) was used. Potential miRNA targets could be located within the 5' 449 nt of the *HNF4A* 3'UTR with a high probability for sequence 204-449, since the significant increase upon miRNA depletion was lost for

constructs containing 5' 196 or less nucleotides (Fig. 19). In accordance with findings describing the effects of miRNAs on proteins as quite modest (Baek et al., 2008; Selbach et al., 2008), derepression upon Dicer knock-down was moderate for the *HNF4A* 3'UTR, but significant. Dicer knock-down is quite elegant and attractive, but it reveals only targets of miRNAs present in HEK293 cells and those that are dependent on Dicer (Cifuentes et al., 2010). In addition, cell death upon long term Dicer knock-down may lead to secondary effects. Thus, complementary experiments measuring the effect of specific miRNAs were needed.

Regulation of the *HNF4A* 3'UTR by miR-122 as presented in Figure 20A was excluded, since the luciferase activity for *HNF4A* 3'UTR constructs was not significantly reduced in comparison to RL-Con, upon expression of miR-122. The decrease in luciferase activity for RL-Con upon miR-122 expression, confirms the importance of including such controls in miRNA dependent luciferase assays. miR-122 makes up 70% of all miRNAs in the adult liver (Lagos-Quintana et al., 2002; Chang et al., 2004) and has been identified as a significant regulator of hepatic lipid metabolism (Esau et al., 2006). mRNAs showed differential sensitivities to miR-122 levels and the degree of target mRNA modulation was at the most 3.5-fold (Esau et al., 2006). The importance of the stoichiometry of target to miR-122 was already noted previously (Chang et al., 2004). The reporter expression level had to be lowered to observe an effect which then increased with rising levels of miR-122. *HNF4A* is an essential gene in the liver and expressed at a high level (Hayhurst et al., 2001). If *HNF4A* is regulated by miR-122 in the liver, it is likely that *HNF4A* is only responsive to very high miR-122 expression levels. miR-122 is highly overexpressed in RCC (~28-fold). Possibly, the level of miR-122 expression reached in the present study was not sufficient to cause an effect, considering that miR-122 expression is absent in HEK293 cells (Tab. 2). In a recent report Seitz suggested that many predicted miRNA binding sites do not function to repress their targets, but are pseudotargets that sequester a miRNA to prevent it binding to the authentic target (Seitz, 2009). Potentially *HNF4A* functions as such a "sponge" for miR-122 in the liver.

In the present study, miR-21 had a minor, but significant effect on the *HNF4A* 3'UTR which was dependent on several binding sites (Fig. 20B). *HNF4A* might not be responsive to increased, exogenous miR-21 expression levels due to the moderate miR-21 expression in HEK293 cells, which might be sufficient for *HNF4A* regulation. In that case a different threshold applies to *TPM1* since miR-21 overexpression resulted in a clear decrease in luciferase activity (Fig. 20B). However, synergy of miRNA action has been described in previous reports (Doench et al., 2003; Chang et al., 2004; Grimson et al., 2007) and simultaneous repression of an mRNA by different miRNA species was shown to be additive

(Doench and Sharp, 2004). Possibly, miR-21 additionally requires other miRNAs for regulating the *HNF4A* 3'UTR efficiently. miR-21 is a ubiquitious, very well-studied miRNA (Krichevsky and Gabriely, 2009). It is one of the most abundant miRNAs in a large variety of cancers analyzed, including high expression in most cancer cell lines of various origins, but miR-21 is also upregulated in other human proliferative disorders. The oncogenic role is supported by several experiments overexpressing and inhibiting miR-21, resulting for example in enhanced and decreased cell proliferation, migration and invasion in cultured human hepatocellular cancer cells, respectively (Meng et al., 2007). In this specific case, the tumor suppressor phosphatase and tensin homolog (*PTEN*) was identified as a target of miR-21 by luciferase assays and contributed to some of the miR-21 effects. Other studies confirmed the miR-21 dependent regulation of *PTEN in vitro* and *in vivo* by using miR-21 specific mimics, inhibitors and expression plasmids as analyzed by western blot analyses and in part by luciferase assays (Zhang et al., 2009; Roy et al., 2009). The predicted miR-21 binding site within the *PTEN* 3'UTR does not contain a perfect seed sequence (Zhang et al., 2009). Although this miR-21 site was not mutated to confirm the site specific regulation, the amount of data by different studies makes the functionality of this seedless miR-21 site quite likely. Another gene that is regulated by miR-21 via a seedless target site is *RASGRP1* (Wickramasinghe et al., 2009). The predicted miR-21 site was not mutated either, but the luciferase construct contained only the miR-21 site from the *RASGRP1* 3'UTR and five additional nucleotides on each site. Inhibition of miR-21 in MCF-7 cells resulted in increased luciferase activity for the *RASGRP1* construct. Interestingly, miR-21 regulation of *PTEN* is cell-specific, as it was also detected in a colon cancer cell line (Asangani et al., 2008) and vascular smooth muscle cells (VSMCs; Ji et al., 2007), but not in MCF-7 breast cancer (Frankel et al., 2008), A549 non-small cell lung cells (Blower et al., 2008) or glioma cells (Gabriely et al., 2008). This data provides further evidence, that strong *HNF4A* regulation by miR-21 might require additional factors that are lacking in HEK293 cells. In addition to RCC (Tab. 2), miR-21 has been shown to be upregulated in human kidney disease of various causes (Saal and Harvey, 2009). In cultured podocytes and tubular epithelial cells apoptosis was inhibited by miR-21, while inhibition of miR-21 in *TGF-β1* transgenic mice enhanced apoptosis in podocytes. Hence, miR-21 is suggested to have a protective role in diabetic nephropathy. Proinflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, cause defective insulin secretion and apoptosis of pancreatic β-cells and hence play a role in diabetes development (Donath et al., 2008). miR-21 expression is induced by IL-1β and TNF-α in MIN6 cells and human pancreatic islets and elevated miR-21 expression levels were also detected in islets of NOD mice, a well-established type 1 diabetes model (Giarratana et al., 2007), during development of pre-diabetic insulitis (Roggli et al., 2010). It was further shown that inhibiting miR-21 prevented the decrease in glucose-induced insulin

secretion triggered by IL-1β. Interestingly, miR-34a was associated with β-cell failure in the same fashion (Roggli et al., 2010). Possibly, miR-34a and miR-21 act synergistically to regulate the *HNF4A* 3'UTR, which might play a role in diabetes and cancer.

In the present study, miR-34a overexpression experiments validated two bindings sites including perfect seed sequences within the 5' 449 nt of the *HNF4A* 3'UTR as determined in HEK293 and INS-1 cells (Fig. 21A). A significant decrease in luciferase activity was also observed in HK120 cells for the 5' 449 nt upon expression of miR-34a (data not shown), hence confirming the miR-34a dependent regulation of *HNF4A* in three different cell lines. Although the proximal miR-34a binding site is still present in the construct including the 5' 196 nt, the luciferase activity was not significantly reduced upon miR-34a overexpression. The accessibility of a target site influenced by flanking sequences and specific RNA- or protein-based cofactors seem to be major determinants of 3'UTR responsiveness to a miRNA (Bartel, 2009; Sun et al., 2010). Possibly, sequences downstream of the miR-34a site at position 149-171 nt that are necessary for miRNA binding and function were deleted in construct 1-196 nt or changes in the secondary structure resulted in loss of site accessibility. Deleting the 5' sequence including the two miR-34a sites in construct 631-3180 resulted in no significant changes in the Dicer knock-down experiments in which all miRNAs are depleted (Fig. 19). This likely reflects the complex regulation of *HNF4A* by an interaction of various miRNAs. Hence, Dicer knock-down experiments seem to be useful to locate areas of extensive miRNA regulation, but not for a detailed analysis of specific miRNA binding sites. Both miR-34a sites located within the 5' 449 nt contributed equally to repression, a characteristic of independent and noncooperative action termed multiplicative effect (Grimson et al., 2007; Fig. 21A). Recently, miR-34a regulation of the *HNF4A* mRNA has been reported independently in HepG2 cells (Takagi et al., 2010). The present data extends this report that described only the distal miR-34a binding site of the 3'UTR to be involved in repression of *HNF4A*. A recent paper by Sun and colleagues described that the repression of the *RhoB* 3'UTR by miR-223 varied with the reporter construct used, including either just the miRNA binding site, long fragments or the entire 3'UTR (Sun et al., 2010). Hence, it is important to validate miRNA function in different constructs including the full-length 3'UTR sequence to substantiate that this target site is functional *in vivo*. In the present study, miR-34a dependent repression of the long 3180 nt *HNF4A* 3'UTR was verified and by applying the assay in a renal and pancreatic cell type (Fig. 21C), miR-34a function was established in distinct cofactor environments. Since the remaining 13 potential miR-34a binding sites were functioning within construct 631-3180 nt and three even in construct 1288-1746, multiple control elements in the 3'UTR of *HNF4A* are deduced that are targeted by miR-34a. This is further supported by the recent finding of a miR-34a target site within the ORF of *HNF4A* (Takagi et al., 2010).

miR-34a has primarily been characterized as a tumor suppressor, as it is inactivated in several tumors and transcriptionally activated by p53. In addition, ectopic miR-34a expression induces apoptosis, cell cycle arrest or senescence (Medina and Slack, 2008; Hermeking, 2010). In contrast, miR-34a is upregulated in RCC (Tab. 2), hepatocellular carcinoma (Pineau et al., 2010), breast cancer (Iorio et al., 2005), squamous cell lung carcinoma (Gao et al., 2010) and in chronic lymphocytic leukemia (Asslaber et al., 2010). Thus, it appears that mir-34a acts as a tumor suppressor or an oncogene, depending on the cell type specific targets and regulatory mechanisms. This observation has been established for several other miRNAs (Spizzo et al., 2009). In fact, miR-34a was clearly increased in stress induced renal carcinogenesis of the rat and inhibition of miR-34a significantly decreased cell proliferation in a rat RCC cell line, but also in HeLa and MCF-7 cells (Dutta et al., 2007). Furthermore, the oncogenic potential of miR-34a was implied by its upregulation in RCC and the correlated decrease of the tumor suppressor *SFRP1* whose loss has been observed in a majority of RCC (Liu et al., 2010). Noteworthy, functional assays were not applied to validate the regulation of *SFRP1* by miR-34a experimentally. An experimental link of miR-34a and endogenous *HNF4A* mRNA has been verified in HepG2 cells where overexpression decreased *HNF4A* mRNA (Takagi et al., 2010). Therefore, it can be speculated that the upregulation of miR-34a potentially causes the downregulation of *HNF4A* in RCC (Fig. 23), resulting in increased cell proliferation through misregulation of at least 14 HNF4A target genes involved in proliferation control (Grigo et al., 2008).

Although few miRNA expression profiles have been performed, for example in murine pancreas (Baroukh et al., 2007) and MIN6 cells (Lovis et al., 2008; Tang et al., 2009) under different conditions, no profiles from human β-cells exist that might shed some light on the contribution of miRNAs to diabetes development. However, elevated levels of plasma free fatty acid are believed to be a predisposing factor for the development of T2DM (Prentki and Nolan, 2006). Interestingly, miR-34a was increased in the mouse β-cell line MIN6B1 and pancreatic islets of rats upon prolonged treatment with palmitate. miR-34a levels were also elevated in the islets of diabetic db/db mice and overexpression of miR-34a decreased glucose-stimulated insulin secretion (Lovis et al., 2008). In addition, miR-34a was the most upregulated miRNA in livers of streptozotocin-induced type 1 diabetic mice and ob/ob mice (model of nonalcoholic fatty liver disease (NAFLD) and hyperglycemia) and is suggested to be linked to the regulation of glucose metabolism (Li et al., 2009). In a recent report, the impact of miR-34a on β-cell failure caused by proinflammatory cytokines was identified in MIN6 cells, human pancreatic islets and islets of NOD mice during development of pre-diabetic insulitis as mentioned above (Roggli et al., 2010). In the present study overexpression of miR-34a in INS-1 cells resulted in a negative regulation of the *HNF4A* 3'UTR mediated by several sites (Fig. 21). In view of the above mentioned observations, this

interaction might contribute to the downregulation of *HNF4A* linked to type II diabetes (Silander et al., 2004; Love-Gregory and Permutt, 2007) and MODY1 (Ryffel, 2001; Gupta and Kaestner, 2004; Fig. 23).

Considering the remarkable lengths of the two *HNF4A* 3'UTRs, other miRNAs likely regulate *HNF4A*. The impact of miR-20a, miR-210 and miR-361-5p on the full-length *HNF4A* 3'UTR as well as shortened constructs was tested using mimics and/or inhibitors (Qiagen), but no consistent effects were determined (data not shown). Established target sites were introduced into reporter plasmids as positive controls. However, even those positive controls were not or only slightly affected by mimicking or inhibiting the corresponding miRNA. One explanation might be the lack of sequence surrounding the target site *in vivo*. The studies in which the miRNA binding sites were confirmed used larger 3'UTR fragments. Possibly, optimal experimental conditions were failed to achieve as well. Interestingly, pronounced effects were obtained for reporter constructs containing target sites completely complementary to the entire length of the miRNA for miR-20a, miR-34a and miR-122. Furthermore, using mimics (Qiagen, Dharmacon) for miR-34a, decreases in luciferase activity were absent or much less pronounced than the ones determined with the miR-34a expression plasmid (Fig. 21A, C). A recent study revealed the importance of pre-miRNAs on miRNA function, as the pre-miRNA loop nucleotides were responsible for distinct activities of miR-181a-1 and miR-181c that only differ in one nucleotide in the mature sequence (Liu et al., 2008). Considering the complex regulation of miRNA processing, an influence on the function of mature miRNAs is not surprising (Krol et al., 2010). Hence, the method applied to mimic or inhibit miRNAs seems to highly influence the outcome of the experiment. In the present study the use of primary-miRNA expression plasmids produced more consistent results.

In conclusion the experiments show that *HNF4A* is not only regulated on the transcriptional level via its P1 (Hatzis et al., 2006) and P2 (Wirsing et al., 2010) promoters and the enhancer (Hatzis et al., 2006), but also on the posttranscriptional level via its two 3'UTRs. Regulation of *HNF4A* expression inevitable influences the numerous target genes and their functions.

E Summary

HNF4A is a susceptibility gene for diabetes and is considered a tumor suppressor in certain cancers including RCC. Although several different *HNF4A* mutations have been linked to diabetes, in the majority of cases including RCC the reason for HNF4A dysregulation is unknown. In this study, regulation of proliferation relevant target genes of this transcription factor as well as transcriptional and posttranscriptional mechanisms that regulate the expression of *HNF4A* itself were investigated with the intention to illuminate how disruption of those processes could impact on diabetes and RCC.

An inducible *HNF4A8* expression cell line was established, which in contrast to *HNF4A2* has no impact on cell proliferation decrease and morphology. To deduce proliferation relevant genes from the 1411 potential HNF4A2 target genes identified previously, an HNF4A8 dependent microarray was performed. 111 from only 191 HNF4A8 target genes deemed to be irrelevant for proliferation control were also controlled by HNF4A2 and excluded, leaving 1300 potential HNF4A2 target genes. qRT-PCR validated that the apoptosis and metabolism gene *CIDEB* is highly upregulated by HNF4A2 in contrast to HNF4A8 and HNF4A mutants. The impact of CIDEB on proliferation control was reasoned to be dependent on a network triggered by HNF4A2 as determined by RNAi and Cideb inducible cell lines.

The two novel mutations -136A>G and -169C>T identified in the P2 promoter of patients with symptoms of *HNF4A* monogenic β-cell diabetes together with a previously reported -192C>G promoter mutation linked to late-onset diabetes in several families, were shown to impair the function of the *HNF4A* P2 promoter *in vitro* using a luciferase reporter assay system. Furthermore, evidence of miRNAs enhancing gene expression by targeting the *HNF4A* P1 promoter was provided by Dicer dependent luciferase reporter assays.

To elucidate the so far unrecognized posttranscriptional regulation of *HNF4A*, the predicted 1.7 kb *HNF4A* 3'UTR was validated and an additional 3.2 kb long, predominantly used 3'UTR was identified. Both 3'UTRs conferred a repressive effect in HEK293 and INS-1 cells, which was even more pronounced in two distinct, previously unknown elements of about 400 nt located within the 3'UTR as determined by luciferase assays. These negative elements A and B were counteracted by an element located within the 5' 630 nt. Dicer knock-down reporter assays inferred negative regulation of the 3'UTRs by miRNAs. More detailed overexpression experiments of selected miRNAs upregulated in RCC revealed a modest effect of miR-21 dependent on several sites within the *HNF4A* 3'UTR. miR-34a negatively regulated *HNF4A* by targeting at least two sites located at the 5' end of both 3'UTRs.

In conclusion, dysfunction of transcriptional and posttranscriptional regulation of the two promoters and 3'UTRs of *HNF4A*, respectively, mediated by proteins and miRNAs, alters the HNF4A dependent network cascade and likely contributes to the development and/or progression of diabetes and RCC.

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Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Erklärung:
Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Impact of transcriptional and posttranscriptional regulation of HNF4A and its target genes on diabetes and cancer“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Frau Andrea Wirsing befürworte.

Essen, den _____ _____
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