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ABSTRACT OF DISSERTATION

Hui Ren

The Graduate School

University of Kentucky

2012

REGULATION OF HEPATIC GENE EXPRESSION DURING LIVER DEVELOPMENT AND DISEASE

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in the Department of
Microbiology, Immunology and Molecular Genetics
College of Medicine at the University of Kentucky

By
Hui Ren

Director Dr. Brett T Spear, Professor of
Microbiology, Immunology and Molecular Genetics

Lexington, Kentucky

2012

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ABSTRACT OF DISSERTATION

REGULATION OF HEPATIC GENE EXPRESSION DURING LIVER DEVELOPMENT AND DISEASE

My first project was to investigate the role of Hepatocyte Nuclear Factor 1 (HNF1) and Nuclear Factor I (NFI) on alpha-fetoprotein (AFP) promoter activity during liver development. AFP is highly expressed in the fetal liver, silenced at birth, and remains at very low levels in the adult liver. A G→A substitution located at -119 of the human AFP promoter is associated with hereditary persistence of AFP (HPAFP) expression in the adult liver (Hum Molec Genet, 1993, 2:379). The -120 region harbors overlapping binding sites for HNF1 and NFI. While it has been shown that the G→A substitution increases HNF1 binding, the role of NFI in AFP regulation has not been investigated. This overlapping HNF1/NFI site is conserved in other mammals, including mice. In this study, I used a combination of biochemical, tissue culture, and animal studies to explore further the role of this HNF1/NFI site in AFP regulation. Transient co-transfections in Hep3B hepatoma cells indicate that HNF1 activates while NFI represses the mouse AFP promoter. EMSAs indicate that HNF1 and NF1 compete for binding to this site. Transgenes regulated by the wild-type AFP promoter are expressed at low levels in the adult liver. Transgenes with a GG→AA mutation (similar to the G-A human mutation) are more active in the adult liver. My data indicate that HNF1 and NFI compete for binding to the -120 region of the AFP promoter and this competition is involved in postnatal AFP repression.

My second project was to study the control of Elongation of very long chain fatty acids like 3 (Elovl3) in the liver by Zinc fingers and homeoboxes 2 (Zhx2). The Zhx2 gene was originally characterized in our lab based on its ability to control the developmental repression of several hepatic genes, including AFP (PNAS, 102:401). Zhx2 is a member of a small family of proteins found only in vertebrates that also includes Zhx1 and Zhx3. These proteins all contain two zinc fingers and four homeodomains, suggesting that they function as regulators of gene expression. My study shows that Zhx2 regulates Elovl3 expression in female liver. Mouse strain-specific differences in adult liver Elovl3 mRNA levels and transgenic mouse data indicate that Zhx2 activates Elovl3 expression in the female adult liver. I also demonstrate that Elovl3 is repressed in the regenerating liver and that the level of Elovl3 repression is controlled by alpha-fetoprotein regulator 2 (Afr2). In addition, I show that Elovl3 expression is reduced in liver tumors, fibrotic livers and fatty livers, raising the possibility that Elovl3 can serve as a marker for HCC and liver damage.

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June 29, 2012

REGULATION OF HEPATIC GENE EXPRESSION DURING LIVER DEVELOPMENT AND DISEASE

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June 29, 2012

DISSERTATION

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DISSERTATION

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TABLE OF CONTENTS

List of tables i

List of figures ii

Chapter One: Introduction 1

Liver architecture and cell composition 1

Liver development and liver disease 2

NAFLD 3

Fibrosis and cirrhosis 5

HCC 6

Alpha-Fetoprotein (AFP) 7

Transcriptional regulation of AFP expression 8

Zinc Fingers and Homeoboxes 2 (Zhx2) 13

Identification 13

Function 15

Elovl3 18

Function 18

Regulation of Elovl expression 20

Chapter Two: Materials and Methods 29

Mice 29

Mouse Genotyping 29

Cloning 30

Transformation 31

Miniprep 31

Maxi Prep 32

Cell Culture.....33

Reporter Gene Assays 33

Preparation of Nuclear extracts and Electrophoretic Mobility Shift Assay (EMSA) 34

RNA extraction and Real-Time PCR 36

Cell Cycle Analysis 37

Statistical analysis 37

Chapter Three: Regulation of Mouse Alpha-fetoprotein Promoter Activity by HNF1 and NFI Binding 41

Introduction.....41

Results 43

The mouse AFP promoter is activated by HNF1 and repressed by NFI 43

HNF1 and NFI compete for binding to -120 region of the mouse AFP promoter.....45

Functional analysis of HNF1 and NFI sites in the mouse AFP promoter 46

In vivo analysis of AFP 47

AFP expression is repressed in the adult liver of opossum 48

Discussion.....49

Chapter Four: The Elongation of Very Long Chain Fatty Acids-like 3 Gene Is A Target of Zhx2 in The Liver 61

Introduction.....61

Results 65

Elovl3 levels are controlled by Zhx2 in adult female livers 65

Elovl3 levels are not controlled by Zhx2 in adult male livers, brown adipose tissue and skin 65

Other Elovl family members are not targets of Zhx2.....66

Elovl3 expression increases during liver development 66

Elovl3 is regulated by Afr2.....67

Elovl3 expression is repressed in fibrotic livers 68

Elovl3 expression is repressed in response to a high fat diet.....68

Elovl3 can alter cell cycle progression.....69

Discussion.....70

Chapter Five: Summary & Future Directions 83

NFI 83

Zhx2 85

Afr2 87

Elovl3 89

References 91

Vita.....100

List of tables

Table 1. List of Plasmids 38

Table 2. Oligos 39

Table 3. A comparison of HNF1/NFI site of AFP promoter from a number of different mammals 53

List of figures

Figure 1. Schematic diagram of the anatomy of the adult mammalian liver 24

Figure 2. Signaling pathways and molecular targeted agents in HCC.....25

Figure 3. Alignment of the mouse and human AFP promoters and locations of major transcription factor binding sites.....26

Figure 4. The mouse Zhx family 27

Figure 5. Regulation of Elovl3 and VLCFA synthesis 28

Figure 6. The mouse AFP promoter is activated by HNF1 and repressed by NFI.....54

Figure 7. EMSA analysis of HNF1 and NFI binding to AFP promoter 55

Figure 8. Functional analysis of HNF1 and NFI sites in the mouse AFP promoter.....56

Figure 9. The AFP(250)^{Mut1}-D^{d} transgene is expressed at higher levels than the AFP(250)- D^{d} transgene in the adult liver 58

Figure 10. Postnatal AFP repression is delayed in the absence of NFIX.....59

Figure 11. AFP is developmentally repressed in opossum liver 60

Figure 12. Elovl3 is regulated by Zhx2 in the adult female liver 74

Figure 13. Regulation of Elovl3 by Zhx2 is not observed in the adult male liver, brown adipose tissure or skin.....76

Figure 14. Other Elovl family members are not regulated by Zhx2. 77

Figure 15. Elovl3 is developmentally activated in the perinatal liver 78

Figure 16. Elovl3 is repressed in the regenerating liver and controlled by Afr2.....79

Figure 17. Elovl3 expression is repressed in fibrotic livers 80

Figure 18. Elovl3 expression is repressed in response to high fat diet induced stress.....81

Figure 19. Elovl3 alters cell cycle progression.....82

CHAPTER 1

Introduction

Liver architecture and cell composition

The adult liver has a unique structure of repeating hexagonal units termed lobules (Fig. 1, left panel) [1]. Lobules are contiguous throughout the liver's three-dimensional architecture. The middle of each lobule contains a central vein, whereas each of the six corners consists of the portal triad [2]. The portal triad consists of three main vessels: the bile duct, hepatic artery, and portal vein (Fig. 1, middle panel). The hepatic artery supplies oxygen-rich blood while the portal vein supplies blood low in oxygen, but rich in nutrients and metabolic byproducts from the gastrointestinal tract. Blood enters the liver and flows along sinusoids towards the central veins, and eventually re-enters the circulation through the vena cava [1]. Bile flows in the opposite direction of blood flow, towards the portal triad where they are transported via bile ducts to the gall bladder for storage before release into the small intestine (Fig. 1, right panel) [3].

The adult liver is comprised of numerous cell types. Hepatocytes account for 60-70% of the normal liver parenchyma. Hepatocytes are highly proliferative in the fetal liver. In the adult liver, hepatocytes are generally quiescent although they are capable of regeneration in response to hepatocyte loss [4]. Hepatocytes contain a high percentage of endoplasmic reticulum as well as over one thousand mitochondria per cell, providing this cell with great synthetic potential [5].

The majority of the liver function can be attributed to the hepatocyte. Hepatocytes are polarized epithelial cells. The apical portion bridges the canalicular lining where bile flow and secretion occurs. The basolateral sides of the hepatocyte are involved in nutrients, toxins, and metabolic byproducts exchange [1]. Sinusiodal endothelial cells (SECs) which make up about 20% of the cellular content of the liver [5]. These cells form the blood hepatocyte barrier and are important for the exchange of materials between blood and the space of Disse. Biliary Epithelial Cells (BECs) are bile duct epithelial cells, which share a common lineage with hepatocytes [1]. Kupffer cells derive from bone marrow and are resident macrophages of the liver. Kupffer cells account for about 50% of the macrophages in adult mammals [6]. Kupffer cells reside primarily in the sinusoids nearer the portal vein. They are phagocytic for bacteria, damaged and aged red blood cells, as well as macromolecules from the circulation [7]. Kupffer cells can also present antigens and produce a variety of cytokines and chemokines [1]. Pit cells are resident natural killer cells of the liver and anchored to the sinusoidal endothelium by pseudopodia [8]. Hepatic stellate cells (HSC) are the major sites of vitamin A storage in adults. Stellate cells are important in chronic injury that leads to liver fibrosis, which will be discussed in the next section [9].

Liver development and liver disease

Cells in the mature liver must function in a coordinated manner for the liver to function properly. Proper liver function is essential for maintaining metabolic homeostasis in mammals. These functions include (a) the production of serum proteins, including clotting factors and transport proteins such as albumin and transferrin; (b) the removal and breakdown of serum

proteins, red blood cells and microbes; (c) the production or removal of glucose during periods of fasting or eating, respectively; (d) the processing of fatty acids and triglycerides; (e) the maintenance of cholesterol homeostasis via synthesis or catabolism; (f) the synthesis and interconversion of non-essential amino acids; (g) the breakdown of toxic endogenous compounds such as ammonia; (h) the production and excretion of bile components; (i) the detoxification of xenobiotic agents; and (j) the storage of numerous substances [1]. The processes require the coordinated regulation of numerous genes, and a number of transcription factors that control these genes have been identified. Transcription factors, including Foxa1/2, Hnf4 and Gata 4/6, are important regulators of early liver development [10-12].

With the liver carrying out so many crucial functions, it is perhaps not surprising that the full spectrum of chronic liver disease is a significant health problem worldwide [13]. HBV and HCV, alcohol, and a variety of metabolic disorders contribute to this problem. The increasing prevalence of obesity and insulin resistance will lead to increasing number of individuals with non-alcoholic fatty liver disease (NAFLD), which is predicted to increase the frequency of steatosis, steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [14]. Thus, while not considered to be a major health concern in the United States at this time, the burden of liver disease in the US healthcare enterprise is expected to grow.

NAFLD. More than 20% of Americans have NAFLD, and this is the leading cause of liver enzyme abnormalities in the United States [13]. The development of NAFLD is determined by the interaction of genetic and environmental factors [15]. NAFLD parallels the frequency of

central adiposity, obesity, insulin resistance, metabolic syndrome and type 2 diabetes [16]. Nonalcoholic steatohepatitis (NASH), a more serious form of NAFLD, can proceed to cirrhosis and hepatocellular carcinoma (HCC) [13]. It is predicted that one third of NAFLD cased progress to NASH, and why this happens is still not fully understood [16]. A 'two-hit' hypothesis has been proposed for the pathogenesis of NASH. The first hit, steatosis, increases the sensitivity of the liver to the induction of inflammation by a second pathogenic hit that promotes oxidative stress and hence steatohepatitis [17]. Certain proinflammatory cytokines, oxidative stress and possibly industrial toxins could all be the second hit to transform simple steatosis into NASH [13]. The present optimal therapy for NASH is modest weight reduction [16]. The complications of NASH, including cirrhosis and HCC, are expected to increase with the growing epidemic of diabetes and obesity [18].

To date, there is no single biochemical marker that can confirm a diagnosis of NAFLD or distinguish between steatosis, NASH, and cirrhosis. Common biomarkers include elevated serum alanine aminotransferase (ALT) and Gamma-glutamyltransferase (GGT) levels. Novel potential biomarkers include: TNF-α and C-reactive protein (CRP), which are associated with inflammation; type IV collagen 7S domain and HA, which indicate the level of fibrosis; Thioredoxin (TRX), which suggests oxidative stresses; caspase-generated cytokeratin-18 (CK-18) fragments, which are indicative of hepatocyte apoptosis. Improved noninvasive diagnostic imaging technologies and improved scoring systems have also been developed to replace the invasive standard liver biopsy [19].

Fibrosis and cirrhosis. Chronic liver diseases causing hepatic fibrosis and cirrhosis are among the most common digestive diseases in the United States [20]. Cirrhosis can be defined as the end stage consequence of fibrosis. Fibrosis and cirrhosis are the consequences of a sustained wound healing response to chronic liver injury from viral, autoimmune, drug induced, cholestatic and metabolic diseases [21]. Hepatic fibrosis and cirrhosis are a main cause of morbidity and mortality. HCV infection, in particular, affects more than 170 million individuals and causes 300,000 deaths annually worldwide due to cirrhosis and hepatocellular carcinoma [22]. Up to 40% of patients with cirrhosis are asymptomatic and may remain so for more than a decade [21]. The marked variability in progression of fibrosis has been attributed to age, gender, environmental factors and genetic factors [22].

Myofibroblasts (MF) are the most important cells in the production of the extracellular matrix. Hepatic stellate cells (HSC) are the predominant MF-producing liver cells. While normally quiescent in the healthy liver, activation of HSCs in response to repeated liver injuring is associated with extracellular matrix (ECM) remodeling. During this period, the basement membrane-like ECM is gradually replaced by collagen-rich fibers and the production of fibrous bands. Non-MF cells, including hepatocytes and Kupffer cells, also participate actively in the process of fibrogenesis by producing reactive oxygen species (ROS) and recruiting of other inflammatory cells to the site of injury, respectively [23].

Procollagen type I carboxy terminal peptide (PICP), procollagen type III amino-terminal peptide (PIIINP), metalloproteinases (MMPs), and tissue inhibitors of matrix metalloproteinases

(TIMPs) are fragments of the liver matrix components produced by HSCs during the process of ECM remodeling. As such, they are commonly used as direct biomarkers of fibrosis. Their usefulness in this capacity is limited by the fact that these biomarkers are not liver-specific and their serum levels are influenced by clearance rates [23].

HCC. HCC is the most common primary malignancy of the liver [24]. It is the fifth most common cancer and is the third most common cause of cancer death globally [25]. Although the incidence of HCC in Asia is starting to plateau or decrease, it is increasing in the United States and Europe [26]. This disease carries a devastating prognosis, in that most cases remain undiagnosed until the disease has advanced to a metastatic stage, and the one-year cause-specific survival rates are less than 50% [25]. The risk factors for HCC are well known and include hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol and hepatic metabolic syndrome [14]. Numerous studies have identified the major oncogenic pathways that are known to be disregulated in HCC as well as several drugs used to block these pathways (Fig. 2).

The Wnt signaling pathway is the most frequently mutated pathway in HCC. A key component of this pathway is β-catenin. Under normal (unstimulated) conditions, β-catenin is localized to the cytoplasm and associated with a complex that includes AXIN1, APC, and glycogen synthase kinase 3β (GSK3β). This complex phosphorylates β-catenin at serine/threonine residues which results in its proteosomal degradation. The binding of Wnt proteins to specific Frizzled receptors on the surface of target cells results in the activation of Disheveled that, in turn, leads to GSK3β inactivation. Unphosphorylated β-catenin translocates

into the nucleus where it binds DNA-associated TCF/LEF transcription factors to activate target genes, including those that are important in cancer development. In 10-40% of HCC tumors, β-catenin is mutated, and the phosphorylation site encoded by exon 3 is a hot spot for mutation. In 5-15% of HCC, AXIN1 is mutated; this leads to AXIN1 inactivation and the release of β-catenin by the AXIN1/APC/ GS3Kβ complex. In 13-50% of HCC, the promoter of E-cadherin is hypermethylated which leads to decreased E-cadherin expression. E-cadherin, a calcium-dependent cell adhesion molecule, is physically linked to β-catenin at the cytoplasmic membrane [27]. The MAPK (Ras/Raf/MEK/ERK), and PI3K/Akt/mTOR pathways and several growth factor pathways are also targets of mutations in HCC [28].

Several common serum markers, including alpha-fetoprotein (AFP), des-γ-carboxy prothrombin (DCP), squamous cell carcinoma antigen-immunoglobulin M complexes (SCCA-IgM Cs), have been shown to be elevated in HCC and therefore used for the early diagnosis and prognosis of HCC. However, none of the three biomarkers (AFP, DCP, SCCA-IgM Cs) is optimal. It is recommended that the three biomarkers should be measured simultaneously and in combination with imaging techniques to increase the sensitivity, specificity, diagnostic accuracy in monitoring HCC. [29]

Alpha-Fetoprotein (AFP)

AFP belongs to the gene family that includes albumin (Alb), alpha-albumin (AFM), vitamin D binding protein (DBP) and AFP-related gene (Arg) [30, 31]. Alb is expressed at high

levels in the fetal liver [32]. After birth, Alb continues to be expressed in the adult liver. DBP is activated during midgestation and AFM is activated at birth. Both genes continue to be expressed in the adult liver [33]. AFP is expressed at high levels in the yolk sac and fetal liver, and at low levels in the fetal intestine [34]. At birth, transcription of AFP is rapidly repressed to barely detectable levels [35]. AFP is often reactivated in cancers [36] and during liver regeneration [37]. The unique aspect of AFP expression makes it an excellent model to study liver gene regulation and development repression in mammals.

AFP is a single polypeptide chain of 609 or 605 amino acids in human and mouse, respectively [38]. AFP is a serum protein that transports a variety of molecules, and, due to its high concentration, a regulator of blood osmolarity [39]. As mentioned previously, serum AFP levels are widely used as diagnostic marker for HCC [40]. In addition, maternal serum AFP levels can be monitored during pregnancy to serve as indicators of fetal neural tube defects [41] as well as Down Syndrome [42]. AFP knockout mice appear normal except that female mice are infertile, which suggests an important role for AFP in the female reproductive system [34].

Transcriptional regulation of AFP expression

Studies using tissue culture transfections and transgenic mice have shown that a 7.7 kb region directly upstream of the AFP gene contains all the elements required for normal AFP expression in a tissue specific and developmentally regulated manner [43]. This region contains

three distinct enhancers at -6.5 kb (E3), -5.0 kb (E2) and -2.5 kb (E1), a repressor centered at -0.9 kb and a 250 bp promoter upstream of the AFP transcription start site [44, 45].

The 250 bp AFP promoter has been extensively studied and found to bind a number of liver-enriched and ubiquitous transcription factors (Fig. 3). A region centered at -165 has binding sites for FTF (Fetoprotein Transcription Factor), an orphan nuclear receptor of the FTZ-F1 family [46], and Nkx 2.8, a divergent homeodomain factor [47]. Chromatin immunoprecipitation (ChIP) has demonstrating binding of Nkx2.8 to the AFP promoter. In cultured cells, antisense inhibition of Nkx2.8 reduces expression of both the endogenous human AFP gene and transfected reporters containing the rat AFP promoter [47].

The Foxa (formerly HNF3) family of factors (Foxa1, Foxa2 and Foxa3) contain a winged helix DNA-binding domain that is homologous to the corresponding region of the *Drosophila* forkhead protein which contains a helix-loop-helix motif and two “wings” interacting with DNA [48]. The Spear lab previously showed that Foxa1 and Foxa2 repress AFP promoter activity in HepG2 cells. A mutation that is centered at −165 is able to abolish the repression, although EMSAs indicate that Foxa proteins do not bind DNA from the −205 to −150. This led to the suggestion that Foxa represses AFP promoter activity through indirect mechanisms that modulate the binding or activity of a factor that interacts with the −165 region of the AFP promoter [49].

A site that contains overlapping Hepatocyte Nuclear Factor 1 (HNF1) and Nuclear Factor I (NFI) binding site is centered at -120 [50, 51]. A second HNF1 binding site is centered at -60 [50]. Binding sites for HNF1 are also found in the promoters of Alb, AFM, and DBP, suggesting an essential role for HNF1 in liver-specific control of this gene family [33, 51-53]. HNF1β can act in a dominant manner to inhibit HNF1α dependent transactivation of the AFM and DBP promoter [33]. A G → A mutation at -119 of the human AFP promoter that increased HNF1 binding was identified in four independent families with hereditary persistence of AFP (HPAFP) expression in adults, whereas a C→A change at –55 has been identified in a different HPAFP pedigree and also increased HNF1 binding to this region [54-58]. A Major part of my dissertation research (Chapter 3) is to investigate further HNF1 and NFI mediated regulation of AFP promoter activity.

The CAAT/Enhancer Binding Protein (C/EBP) family of transcription factors include the leucine zipper proteins C/EBPα, C/EBPβ and C/EBPγ. These three protein, which can form homodimers or heterodimers with each other, bind to the AFP promoter. C/EBPα has been shown to activate the AFP promoter in transient transfections, and several C/EBP binding sites have been identified (Fig. 2). Although the mRNA levels of C/EBPα, C/EBPβ and C/EBPγ all increase during liver development, only C/EBPβ and C/EBPγ are expressed at high levels in yolk sac and fetal liver, where C/EBPα is poorly expressed, suggesting that C/EBPβ and C/EBPγ are early regulators of the AFP gene in the liver [59]. Binding sites for C/EBP are found in the promoters of Alb and DBP, suggesting an essential role for C/EBP in liver-specific control of this gene family [59-61].

More recently, it was found that mice with a liver-specific deletion of Zbtb20 had increased AFP in the adult liver, suggesting that this protein is required for normal postnatal AFP repression [62]. Zbtb20, which belongs to the BTB/POZ zinc finger family, binds to the AFP promoter fragment between -108 and -53. Two Zbtb20 isoforms exist due to the alternative translation initiation, with both containing an intact N-terminal BTB domain and a C-terminal zinc finger domain. Consistent with its role as a repressor of AFP, Zbtb20 inhibits AFP promoter-driven transcriptional activity. Zbtb20 and AFP gene expression are inversely correlated in the liver.

A region centered around 850 bp upstream of the AFP promoter was found to function as a repressor region [63]. Deletion of this element leads to continued expression of AFP transgenes in the adult liver and gut. This negative element acts as a repressor in a position-dependent manner [63]. The region contains binding sites for Foxa proteins and p53, with Foxa activating and p53 repressing AFP transcription. Foxa and p53 bind to the repressor region in a mutually exclusive manner. Studies in fibroblast cells lacking Foxa show that AFP repression by p53 occurs by two mechanisms, physical exclusion of Foxa binding and active transcription interference [64]. *In vitro* transcription experiments showed that chromatin assembly establishes a barrier to block inappropriate expression of AFP in non-hepatic tissues and that tissue-specific factors, such as Foxa, can alleviate the chromatin-mediated repression [65]. These studies suggest the possibility that during tumorigenesis, p53-mediated repression of AFP may be lost, allowing Foxa-mediated AFP activation; this is in contrast to postnatal AFP repression, when

increased hepatic p53 levels lead to AFP repression. Consistent with this, AFP repression is slightly delayed in p53-deficient mice [65]. Studies from the Spear lab shows that a transgene with the 250 bp AFP promoter region linked to AFP enhancer element E2 is expressed in the fetal liver and is postnatally repressed [66]. Therefore, the repressor region contributes to, but is not essential for, postnatal AFP repression.

Several studies have also elucidated aspects of AFP reactivation in regenerating liver. The repressor region also appears to be involved in AFP reactivation during liver regeneration. This is based on studies which showed that AFP transgenes with a deletion of the region between -1,010 and -838 bp were not reactivated in regenerating liver, whereas transgenes containing this region were reactivated in regenerating liver similarly to the endogenous AFP gene [67]. Furthermore, AFP induction is significantly lower in C57BL/6 mice than in other mouse strains [68]. This strain-specific difference in AFP induction is due to a gene called Alpha-fetoprotein regulator 2 (Afr2) [69]. The $Afr2^{b}$ allele in C57BL/6 mice and the $Afr2^{a}$ allele found in other mouse strains are co-dominant [50]. Afr2 has been mapped to mouse Chromosome 2, however, the Afr2 gene has not been identified [70]. In addition to AFP, H19 and Glypican 3 (Gpc3) are the only other known targets of Afr2 [71]. Interestingly, these three genes are frequently reactivated in HCC, suggesting that Afr2 may be involved in liver cancer progression. A second major part of my dissertation is the identification of Elongation of very long chain fatty acids 3 (Elovl3) as another target of Afr2.

The three AFP enhancers, E1, E2 and E3, are each around 300 bp in length [72]. Using transgenic mice, Hammer, *et al.* showed that each enhancer can direct expression in the appropriate tissues, the visceral endoderm of the yolk sac, the fetal liver, and the gastrointestinal tract with different influences [73]. E1 has been shown to binds to C/EBP [50], whereas E2 has not been characterized at the molecular level. In contrast to these two enhancers, E3 has been well studied and found to contain binding sites for members of nuclear receptor family, Foxa/HNF6 and C/EBP, all of which are at the 5' end of E3 [74]. More recently, our lab identified a TCF site at the 3' end of E3; this site is required for responsiveness to β-catenin (Clinkinbeard *et al*. in press). Using transgenic mice in which each AFP enhancer was individually linked to the heterologous human b-globin promoter, our lab previously showed that E1 and E2 are active in all hepatocytes in the adult liver, with highest activity in cells surrounding the central vein; in contrast E3 was active exclusively in hepatocytes surrounding the central vein [75]. Peyton *et al.* showed that lack of E3 activity in all hepatocytes except those encircling the central veins is due to active repression in non-pericentral hepatocytes [76]. Enhancer knockout experiments showed that these enhancers are also required for AFP and Alb activation early in liver development [77].

Zinc Fingers and Homeoboxes 2 (Zhx2)

Identification. Zhx2 was originally defined by mouse strain-specific differences in serum AFP levels by Rhouslatti and colleagues [78]. They found low AFP levels in most strains, as expected since AFP is repressed at birth, with the single exception being BALB/cJ, which had

~20-fold higher serum AFP levels. Further analysis revealed that this trait was due to a single gene called *regulator of AFP* (*Raf*) and later renamed *Alpha-fetoprotein regulator 1* (*Afr1*). The $Afr1^b$ allele in BALB/cJ mice was recessive to the wild-type $Afr1^a$ allele found in other mouse strains. Studies by Tilghman, *et al*. found differences in steady state adult liver AFP mRNA levels between BALB/cJ and other mouse strains [68], while Blankenhorn and colleagues found that Afr1 maps to chromosome 15, whereas AFP is on chromosome 5 [79]. Tilghman, *et al*. also identified another target of Afr1, which they called H19 [69]. By positional cloning, the Spear lab identified Zhx2 as the gene responsible for the Afr1 trait [80]. The mutated Zhx2 allele in BALB/cJ mice contains a mouse endogenous retroviral (MERV) element in its first intron, resulting in the production of an aberrant transcript that can no longer encode a functional protein [81]. Although BALB/cJ mice have lower levels of Zhx2 levels and higher hepatic AFP and H19 expression than other strains, they do express low levels of Zhx2; thus, the naturally occurring mutation in BALB/cJ mice is a hypomorphic allele rather than a complete null allele. Based on data from our lab and other labs, we have proposed that Zhx2 is a repressor of AFP and H19 expression that silences these genes at birth in the liver; the reduction in Zhx2 levels in BALB/cJ mice leads to persistent AFP and H19 expression in the adult liver [82].

Zhx2 belongs to a small gene family that also includes Zhx1 and Zhx3 (Fig. 4). All three Zhx proteins are predicted to contain two C2H2-type zinc fingers and four or five homeodomains [82]. In addition, all Zhx proteins can form homodimers and heterodimers with each other and with NF-YA [83-85]. *In vitro* assays suggest that Zhx2 is a transcriptional repressor that is localized in the nuclei [84]. Data from our lab and our collaborator Dr. Peterson indicate that

Zhx2 also acts at the posttranscriptional level to regulate steady-state AFP and H19 mRNA levels (L. A. Morford, B. T. Spear, and M. L. Peterson, unpubl. obs.). *Zhx* genes have been identified in vertebrates, including amphibians, birds, fish, and mammals, but have not been found in non-vertebrates [82].

Function. To date, little is known about the biological function of Zhx2 or any of the other Zhx proteins. Since AFP is also frequently reactivated in HCC, there is interest in whether Zhx2 is also disregulated in liver cancer. In a screen to identify sequences that are differentially methylated between the Hepatocellular carcinoma (HCC) genomes and adjacent nontumorous liver tissues, Lv, *et al.* found that the CpG island in the Zhx2 promoter is hypermethylated in HCC [86]. Further studies demonstrated, by bisulfate sequencing, that Zhx2 5'-CpG island is hypermethylated in some HCC and HepG2 cell lines, but not in 6 normal liver tissue samples. In addition, they showed that the hypermethylation of Zhx2 promoter is associated with low Zhx2 mRNA levels in HCC samples. In contrast, Hu, *et al.* showed that Zhx2 is detected only in HCC tissues by immunohistochemistry. They further showed that Zhx2 expression is associated with clinical stage of the disease. In stage III-IV, which are more advanced, the rate of Zhx2 expression was approximately twice as high as in stage I-II. In addition, they found that Zhx2 expression in primary lesions with metastasis is significantly higher than without metastasis, suggesting that Zhx2 is associated with metastasis in HCC [87]. Yue, *et al.* showed that Zhx2 overexpression reduces proliferation of HCC cells and growth of HepG2 tumor xenografts in nude mice. They also demonstrated that nuclear localization of Zhx2 is reduced in human HCC samples, which is correlated with reduced survival times of patients, high levels of tumor

microvascularization, and hepatocyte proliferation [88]. More studies will be needed to resolve these conflicting data.

Although Zhx2 was originally identified as a regulator of hepatic genes, Zhx2 is not a liver-specific factor but is ubiquitously expressed [89]. Zhx2 has been identified in several screens for genes that are disregulated during disease and development, including erythrocyte development [90], B-cell development [91], multiple myeloma progression [92] and kidney disease [93]. Andrade, *et al.* differentiated peripheral blood mononuclear cells by the addition of erythropoietin and obtained gene expression patterns from cells undergoing erythroid differentiation relative to undifferentiated cells. They found that Zhx2 is downregulated in differentiated erythroid cells, suggesting that Zhx2 may participate in globin regulation and may be important in the normal physiology of erythrocytes [90]. Hystad, *et al.* characterized several stages of normal human B cell development in adult bone marrow by gene expression profiling of hematopoietic stem cells, early B, pro-B, pre-B, and immature B cells, by microarrays. They found that Zhx2 is up-regulated in the transition of hematopoietic stem cells to early B cells and early B cells to pro-B cells and continued to be high during the next differentiation steps, with a similar expression pattern to the essential transcription factors EBF, TCF3, PAX5, and LEF1. [91] Multiple myeloma is a malignant neoplasm of plasma cells [94]. Armellini, *et al*. showed high Zhx2 expression is associated with better response and longer survival after high-dose therapy in multiple myeloma patients and that low Zhx2 expression is associated with high-risk multiple myeloma disease [92]. Their study suggests a potential role for Zhx2 as a biomarker for multiple myeloma [92]. Finally, Liu, *et al*. showed that Zhx1, Zhx2, and Zhx3 are regulators of

podocyte gene expression in glomerular disease. These proteins are predominantly located in the nonnuclear compartment in the normal podocyte. In puromycin-induced nephrosis, Zhx3 expression is transiently downregulated. This was associated with loss of heterodimerization with Zhx1 and Zhx2 proteins, and entry of Zhx3 into the nucleus coinciding with the development of proteinuria [93].

A quantitative trait locus (QTL) on chromosome 15 was reported by the lab of Aldons Lusis to contribute to hyperlipidemia based on a cross between the inbred mouse strains MRL/MpJ and BALB/cJ [95]. A congenic strain (CON15) was generated by placing a subregion of chromosome 15 from MRL/MpJ onto the BALB/cJ genetic background. The congenic mice, but not BALB/cJ mice, exhibited a dramatic (≈30-fold) increase in the atherogenic lesions similarly to MRL/MpJ when placed on a high fat diet. In addition, both triglycerides (TG) and cholesterol levels were significantly higher in the CON15 mice compared to levels in BALB/cJ mice, on either a regular chow diet or a western (high fat) diet. This chromosome 15 QTL was designated *Hyplip2* [96]. Further studies showed that *Hyplip2* caused hypertriglyceridemia by decreased clearance of TG. *Hyplip2* did not appear to affect intestinal TG absorption and VLDL-TG production, but impaired TG clearance from the plasma [97]. However, these studies did not identify the product of the *Hyplip2* QTL or elucidate how *Hyplip2* contributed to atherosclerosis. A collaboration between our lab and Dr. Lusis' lab demonstrated that *Zhx2* resides in the region of mouse chromosome 15 where *Hyplip2* was mapped, and showed that *Zhx2* is differentially expressed in MRL/MpJ and BALB/cJ mouse strains. BALB/cJ mice (which contain a mutated *Zhx2* gene, as mentioned previously) that express a liver-specific Zhx2 transgene exhibit

significantly elevated plasma cholesterol and TG levels, much higher than non-transgenic BALB/cJ mice and identical to the CON15 mice. This data provides direct evidence that *Zhx2* is responsible for the observed hyperlipidemia in MRL/MpJ mice and suggests that Zhx2 is an important regulator of lipid metabolism in the liver [98]. Microarray analysis performed by the Lusis lab identified genes that might be potential targets of Zhx2 [99]. One of the identified genes was elongation of very-long-chain fatty acids-like 3 (Elovl3).

Elovl3

Function. A significant amount of the fatty acids in tissues are further elongated into very-long-chain fatty acids (VLCFAs) by membrane-bound enzymes predominantly located in the endoplasmic reticulum. Members of the elongation of very-long-chain fatty acids gene family (Elovl1-Elovl7) encode for enzymes in the elongation cycle in mammals [100]. These enzymes display differential substrate specificity, tissue distribution, and regulation, making them important regulators of cellular lipid composition as well as specific cellular functions [101]. Elovl1, Elovl3, Elovl6 and Elovl7 prefer saturated and monounsaturated fatty acids as substrate and Elovl2, Elovl4 and Elovl5 are selective for polyunsaturated fatty acids [102]. The fatty acid elongase Elovl3 is involved in the synthesis of C20–C24 saturated and monounsaturated very-long-chain fatty acids (VLCFAs) in triglyceride-rich glands such as the sebaceous and meibomian glands, brown and white adipose tissue, and liver [103].

Westerberg, *et al*. reported in 2004 that Elovl3 has an important role in the development and maintenance of hair and skin function [104]. They showed that Elovl3 expression in the skin was restricted to the sebaceous glands and the epithelial cells of the hair follicles. They generated Elovl3-ablated mice by homologous recombination and found that these mice displayed a sparse hair coat and a hyperplastic pilosebaceous system. Eicosenoic acid (20:1) was exceptionally high in the hair lipid content of Elovl3-ablated mice, however, fatty acids longer than 20 carbon atoms were almost undetectable. As a result, Elovl3-ablated mice exhibited a severe defect in water repulsion and increased trans-epidermal water loss. In summary, their study showed that Elovl3 participates in the formation of certain VLCFA and triglycerides in cells of the hair follicles and sebaceous glands, and that the absence of Elovl3 can have detrimental effects on hair and skin function [104].

Tvrdik, *et al*. reported in 1997 that Elovl3 expression was elevated in brown fat more than 200-fold when mice were exposed to a 3-day cold stress [105]. Prolonged cold exposure of mice for 1 month gradually decreased *Elovl3* expression, but the level still remained 100-fold above the control level. In two other conditions of brown fat recruitment, during perinatal development and after intake of a calorie-rich diet, similar increases in Elovl3 were observed. The induction of Elovl3 expression in the cold could also be mimicked by continuous administration of norepinephrine via osmotic minipumps in mice kept at 28 °C. Based on this data, they proposed that the Elovl3 protein is involved in a pathway connected with brown fat hyperplasia [105]. Following up the studies by Tvrdik, *et al.*, Westerberg, *et al*. reported in 2006 that upon cold stress, Elovl3-ablated mice were unable to hyperrecruit brown adipose tissue and

subsequently maintained body temperature by muscle shivering [106]. In brown adipose tissue of Elovl3-ablated mice, during the initial cold stress, there was a transient decrease in the capacity to elongate saturated fatty acyl-CoAs into very long chain fatty acids. Furthermore, warm-acclimated Elovl3-ablated mice had reduced lipid accumulation and reduced metabolic capacity within the brown fat cells. The authors concluded that Elovl3 is an important regulator of VLCFA and triglyceride formation in brown adipose tissue during the early phase of the tissue recruitment [106].

Zadravec, *et al*. reported that Elovl3 ablation led to reduced lipogenic gene expression and triglyceride content in the liver [103]. As a result, serum VLDL triglyceride levels were significantly reduced. In addition, they showed that Elovl3-ablated mice have increased energy expenditure, reduced serum leptin levels, increased expression of orexigenic peptides in the hypothalamus, and unchanged food intake. As a consequence, Elovl3-ablated mice were lean and resistant to diet-induced obesity. This study indicated that VLCFA produced by Elovl3 are indispensable for appropriate synthesis of liver triglycerides, fatty acid uptake, and storage in adipose tissue [103].

Regulation of Elovl expression. Based on their biological importance, it is important to understand the regulation of enzymes that control VLCFA levels. Wang, *et al*. showed that peroxisome proliferator-activated receptor α (PPARα) agonist WY14,643 increases Elovl1, Elovl5, Elovl6 mRNA abundance in the rat liver [107]. This group also found that Elov5 mRNA is low in fetal liver and increases significantly after birth and that the PPARα-regulated gene

CYP4A has similar developmental pattern as Elovl5. In contrast, Elovl6 mRNA is present in the fetal liver, absent immediately after birth, and reappears in the adult liver, which paralleled the protein level of hepatic sterol regulatory element binding protein 1c (SREBP1c) in the nucleus. The authors also showed the profile of Elovl expression in several rat tissues. Elovl1 was expressed in lung, brain, kidney, and heart but was absent from liver, brown adipose tissue, and skin. Elovl2 was expressed in the liver, lung, brain, and kidney. Elovl3 was only detected in skin. Elovl5 and Elovl6 expression is detected in all tissues examined [107].

In another paper, Wang, *et al*. showed that Elovl1, Elovl2, Elovl5, and Elovl6 are the only four elongases expressed in rat liver [108]. The relative mRNA abundance of elongase expression in rat, mouse, and human liver is Elovl5 > Elovl1 = Elovl2 = Elovl6. They also demonstrated that PPARα was required for WY14643-mediated induction of Elovl5 and Elovl6 by studies with PPARα-deficient mice. LXR agonist TO-901317 induces nuclear SREBP1 nuclear abundance, but has no effect on SREBP2 in rat primary hepatocytes. LXR agonist also modestly induces Elovl6, but has no significant effect on Elovl1, Elovl2, or Elovl5 expression in rat primary hepatocytes. Overexpressed SREBP1c induces transcripts encoding Elovl2, Elovl6 rat in primary hepatocytes [108].

While the two studies mentioned above used rats to study Elovl gene regulation, a mouse study by Panda, *et al*. showed that steady-state Elovl3 mRNA levels follow a robust circadian profile in the liver, which is perturbed in the CLOCK mutant mice [109]. Anzulovich, *et al*. confirmed the rhythmic Elovl3 transcript level in the liver [110]. They showed that the Elovl3

promoter (-956 to +165) was not activated by BMAL/ CLOCK in transfected cells, in spite of the presence of a perfect E-box in this promoter region. Instead, they found that RevErbα represses whereas SREBP1 activates Elovl3 promoter (-956 to +165) activity in transfected cells. They also demonstrated that the feeding schedule modulates the rhythm of proteolytic activation of SREBP1 and the circadian Elovl3 transcript level in the liver. Anzulovich, *et al*. also found that Elovl3 levels exhibit sexual dimorphism, with substantially higher expression in the male liver than in the female liver [111]. Brolinson, *et al*. showed that hepatic Elovl3 expression is induced upon injection of the synthetic glucocorticoid dexamethasone, suggesting that hepatic Elovl3 expression controlled by circulating steroid hormones such as glucocorticoids and androgens. They also found that Elovl3 levels were elevated in peroxisomal transporter ABCD2 ablated mice and reduced in ABCD2 overexpressing mice. Since VLCFAs are selectively degraded by peroxisomal β-oxidation, their finding suggests a cross talk between very long chain fatty acid synthesis and peroxisomal fatty acid oxidation [110].

Jakobsson, *et al*. demonstrated reduced Elovl3 expression in BAT of cold-exposed PPARα ablated mice [112]. They also found that a mixture of norepinephrine, dexamethasone, and the PPARα ligand Wy-14643, which rendered the adipocytes a high oxidative state, induced Elovl3 expression in cultured brown adipocytes. In addition, they found that stimulation of Elovl3 expression was independent of LXR and SREBP1 activation in cultured brown adipocytes. They also showed that LXR agonist TO-901317 repressed Elovl3 expression [112]. Jörgensen, *et al.* showed that norepinephrine and the PPARα ligand rosiglitazone synergistically induce Elovl3 gene expression in primary cultures of brown adipocytes [100]. They found that

norepinephrine and rosiglitazone increase Elovl3 mRNA level by both inducing transcription and increasing mRNA stability, and that the whole process required novel protein synthesis [113]. To summarize data from adipose tissue and liver, Elovl3 expression is induced by factors stimulating fatty acid oxidation, such as NE, while it is inhibited by LXR. Stimulation with PPARα and glucocorticoids also promoted Elovl3 expression (Fig. 5) [100].

My dissertation study shows that hepatic Elovl3 expression is under the control of Zhx2. In addition, Elovl3 expression is reduced in fatty livers, fibrotic livers, regenerating livers and liver tumors, raising the possibility that Elovl3 may be involved in progression of liver damage and HCC and possibly serve as a biomarker for these events.

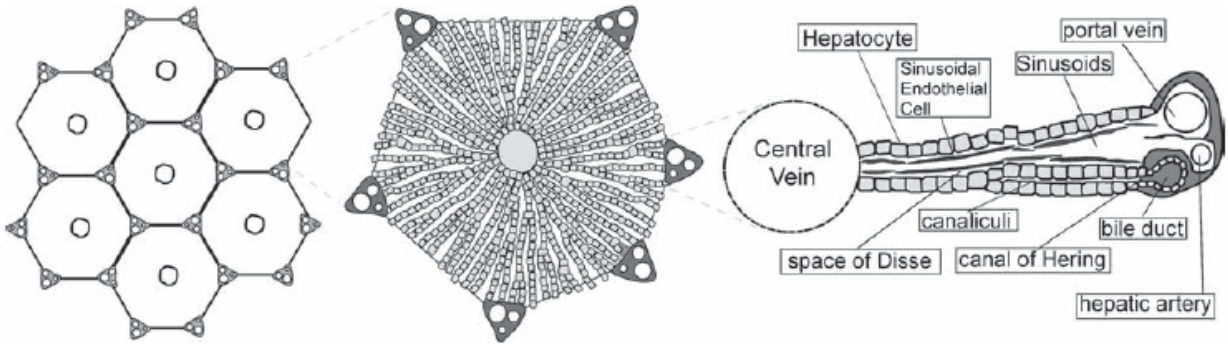

Figure 1. Schematic diagram of the anatomy of the adult mammalian liver

Left: The lobule structure of the adult liver show the repeating hexagonal lobules that are found in the adult mammalian liver. Middle: Each lobule is centered around the central vein. Each corner of the hexagon contains a portal triad which is composed of a portal vein, hepatic artery, and bile duct. Plates of hepatocytes extend outward from the central vein. Right: The portocentral axis of the liver lobule. Blood enters the liver through the portal vein and hepatic artery (periportal region) and flows along sinusoids towards the central vein (pericentral region). Transfer of materials between the blood and hepatocytes occurs in the space of Disse. Canaliculi transport bile from hepatocytes to the bile duct [1]. Copyright 2006 Cell. Mol. Life Sci.

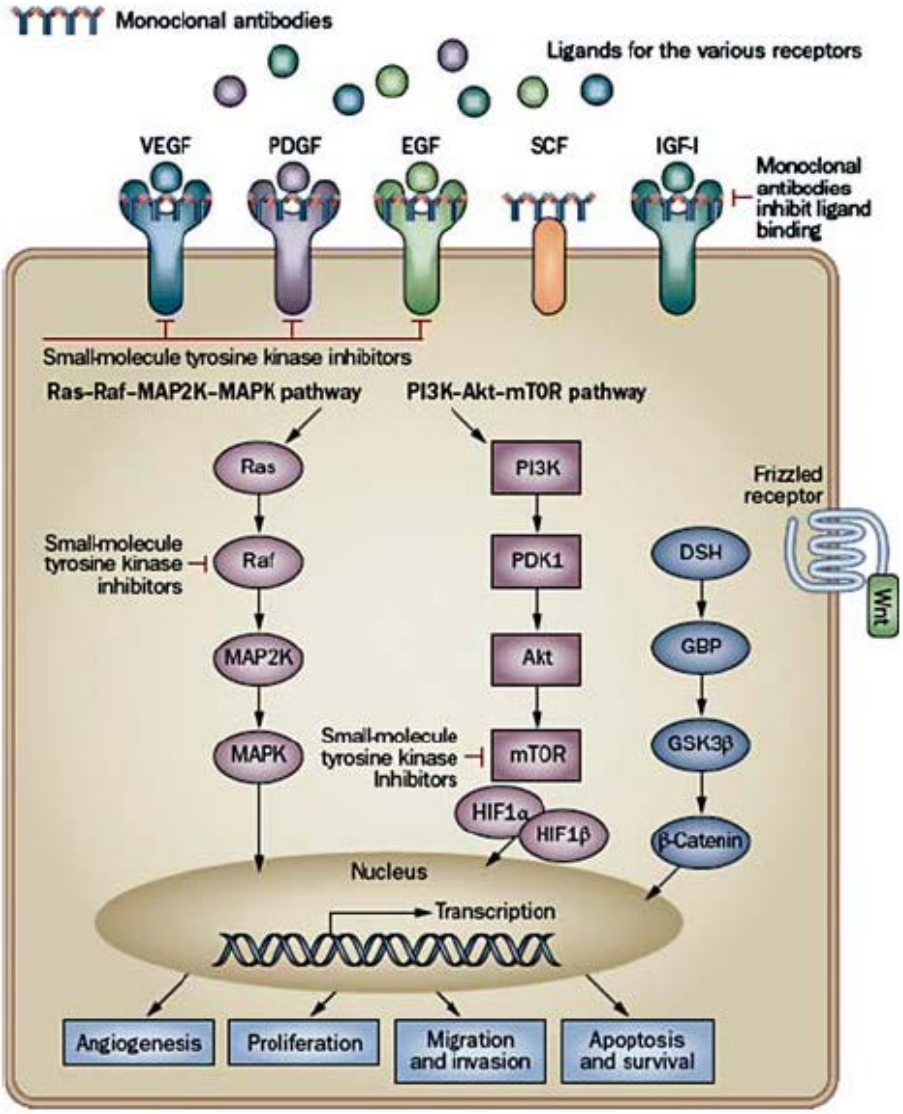

Figure 2. Signaling pathways and molecular targeted agents in HCC

Based on these pathways, anti-HCC drugs include monoclonal antibodies (VEGFR: bevacizumab, EGFR: cetuximab), tyrosine kinase inhibitors (VEGFR: sorafenib, brivanib, linifanib, axitinib, EGFR: erlotinib, lapatinib), serine/threonine kinase inhibitors (Raf: sorafenib, mTOR: rapamycin and everolimus, PIK:KL-755) [28]. Copyright 2011 Digestive Diseases

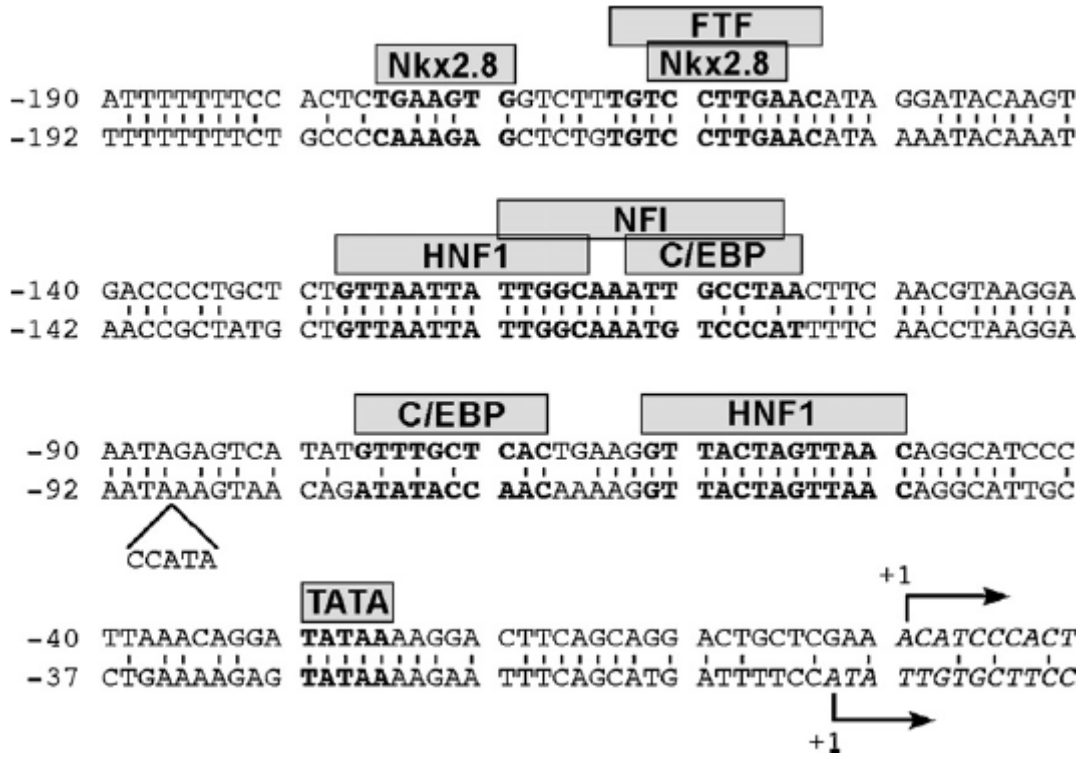

Figure 3. Alignment of the mouse and human AFP promoters and locations of major transcription factor binding sites

The upper rows represent the mouse sequence, the lower rows represent human sequence; vertical lines indicate conserved nucleotides. The arrows represent the sites of transcription initiation (designated +1), nucleotides in italics correspond to the 5' end of exon 1. Locations of factor binding sites are shown. While most of these sites are conserved between mouse and human, the C/EBP sites, distal Nkx2.8 site (−176 to −170), as well as the 3' end of the NFI site, are less conserved [114]. Copyright 2011 Semin Cancer Biol

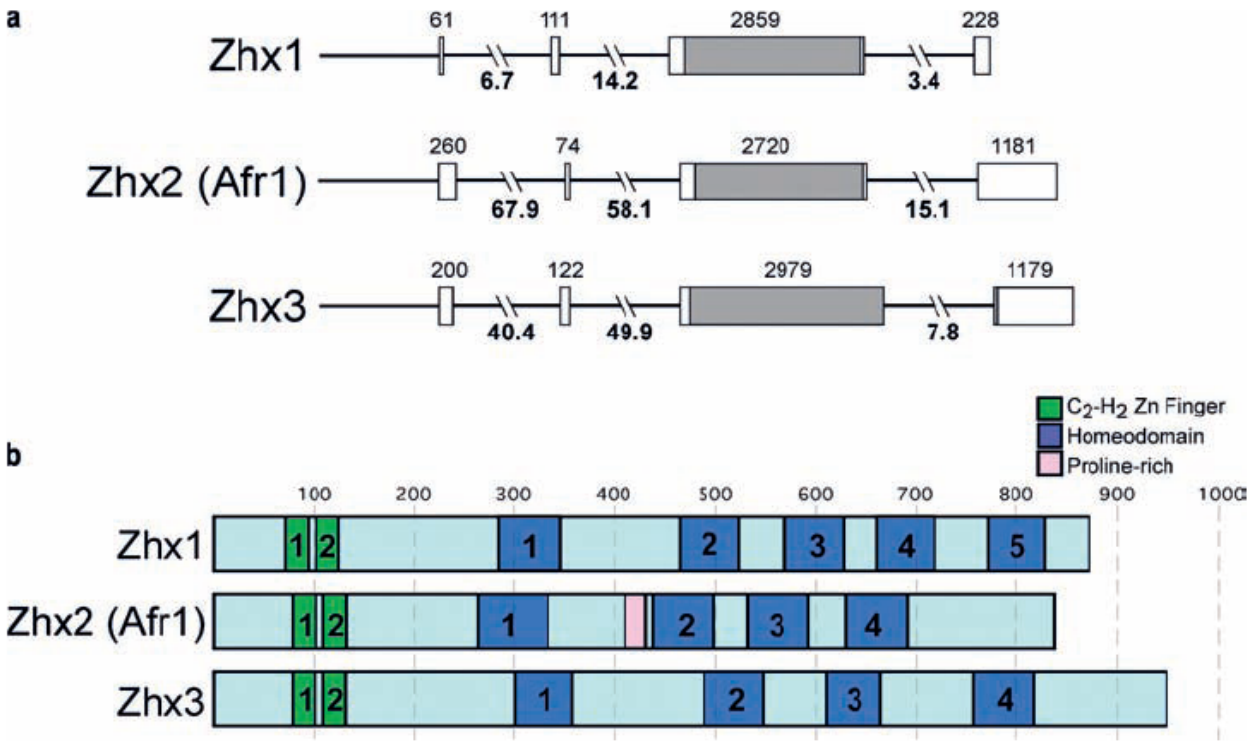

Figure 4. The mouse Zhx family

(a) The three mouse Zhx genes show a similar structure. Each gene contains two small 5′ non-coding exons (boxes, numbers above boxes indicate size of exons in bp), followed by an unusually large internal exon 3. The entire coding region of Zhx1 and Zhx2 is contained in this exon; 8 bp of coding sequence is found in the terminal exon of Zhx3. Numbers below the lines indicate the size of the introns (in kb). (b) The three Zhx3 proteins contain conserved C2-H2 zinc fingers in their amino end (green boxes) and four (or five, in the case of Zhx1) conserved homeodomains (dark blue boxes). Zhx2 contains a predicted proline-rich region (pink box) adjacent to homeodomain 2. Numbers above the dashed lines represent amino acids [1]. Copyright 2006 Cell. Mol. Life Sci.

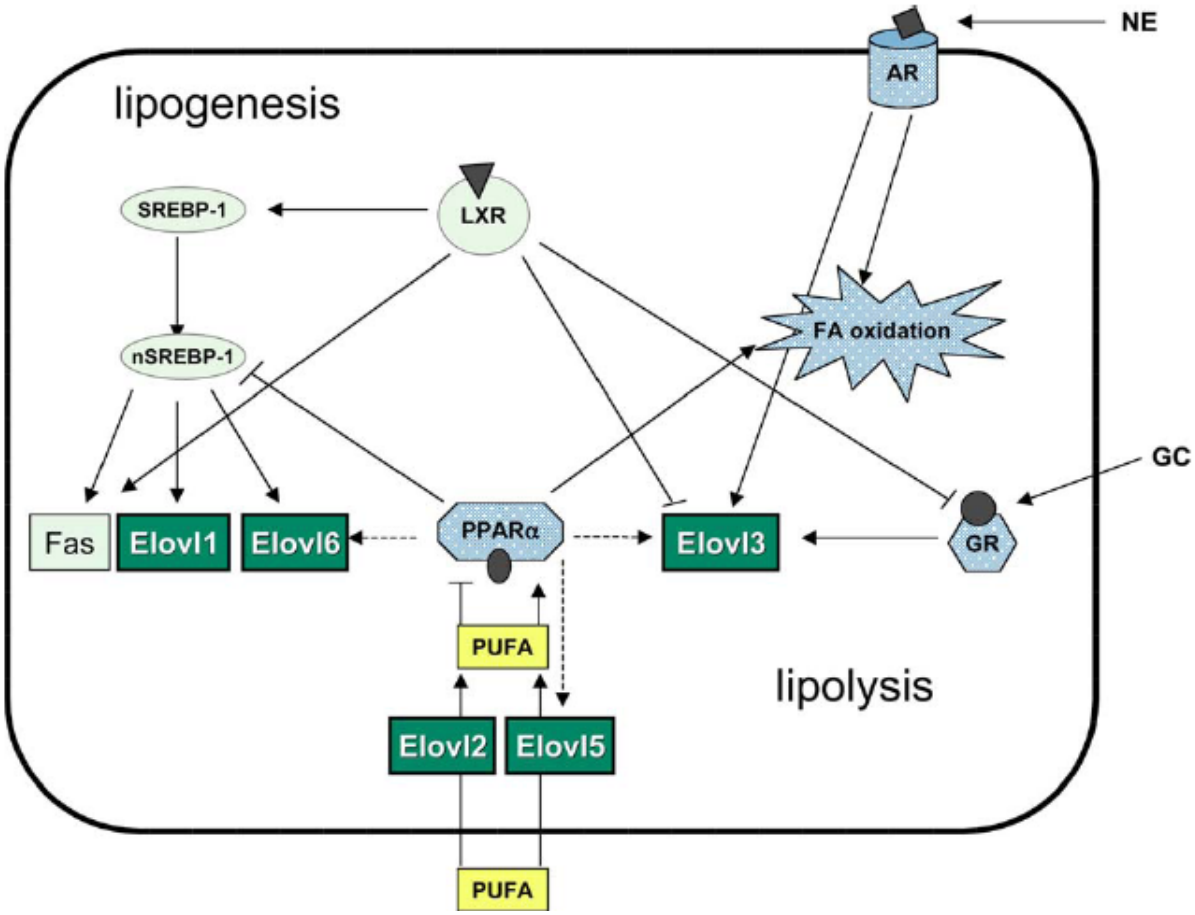

Figure 5. Regulation of Elovl3 and VLCFA synthesis

A schematic picture based on data on regulation of Elovl3 expression in adipose tissue and liver. Elovl3 expression is induced by factors stimulating fatty acid oxidation, such as NE, while it is inhibited by LXR. Stimulation of PPARα has also been shown to promote the expression of Elovl3 in a more long-term perspective. Glucocorticoids (GC) induce Elovl3 expression [100]. Copyright 2006 Progress in Lipid Research

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CHAPTER 2

Materials and Methods

Mice. All transgenic mice were generated by the University of Kentucky Transgenic Facility and were housed in the Division of Laboratory Animal Research (DLAR) facility in accordance with an Institutional Animal Care and Use Committee (IACUC) approved protocol. All mice had access to food and water *ad libitum* and were kept on a 12hr-12hr light-dark cycle. Mice containing the E2-AFP(250)-D^d transgene [66] or E2-AFP$(250)^{Mut1}$-D^d transgene were maintained by breeding with Bl6/C3H mice (Jackson Lab). Mice containing the TTR-Zhx2-Flag transgene were bred with BALB/cJ mice (The Jackson Lab). $NFIX^{-/-}$, $NFIX^{+/-}$and $NFIX^{+/+}$mice were generated in Dr. Richard Gronostajski's lab (University at Buffalo, the State University of New York).

Mouse Genotyping. At approximately postntatal day 14 (p14) mouse pups were ear tagged for identification and 5 mm tail snips were taken. To extract the DNA [115], tail pieces were incubated overnight at 52^oC in 0.5ml tail lysis buffer (100mM Tris-HCl pH 8.5, 5mM EDTA, 200mM NaCl, and 2.5 units proteinase K (Sigma). The following day, tubes were centrifuged at 14,000RPM for 12 min. The DNA-containing supernatant was decanted into an equal volume of isopropanol. Tubes were inverted to precipitate the DNA. After another centrifugation for 1 min the liquid was decanted and the pelleted DNA was washed 1X with 95% ethanol and dried for 5 min. The resulting DNA was resuspended in 250 μL water and mixed by

vortexing. For genotyping, 2.5 μL of DNA was added to 12.5 μL ThermoStart Master Mix (ThermoScientific), 5 μL water and 5 μL of 100 nM primer mix. The 0.2 mL tubes were placed in the thermocycler (Gene Amp PCR system 9700 by Applied Biosystems) for 25 cycles. The Y-linked *Uty* gene was used to determine the gender of mice [116]. All primers used for genotyping are listed in Table I. All samples underwent the same PCR protocol: 95°C for 15 min, cycles with 95°C 30 sec, 55°C for 30 sec, 72°C for 20 sec and then a final 72°C for 5 min. When complete, 5 μL of 6X loading dye (Fermentas) was added to each sample and mixed by pipetting. Ten μL of sample was loaded into the wells of a 1.5% w/v agarose (SeaKem) gel along with a 100 bp ladder (Fermentas). Electrophoresis separation was done at 140V for 45 min using 1X TBE. Bands were visualized with UV light.

Cloning. Oligonucleotides were purchased from Integrated DNA Technologies. The mouse AFP promoter fragments were excised from AFP(250)-lacZ vector [49] and re-cloned into the promoterless luciferase vector pGL3-Basic (Promega). Using the full-length wild-type 250 promoter as a template, mutations were introduced into the HNF1/NFI region at -120 by the megaprimer method to generate Mut1, Mut2 and Mut3 using primers listed in Table I (Sarkar and Sommer, 1990). Mutated constructs were confirmed by DNA sequencing.

Full-length expression vectors for mouse HNF1α and HNF1β were generated by PCR amplification of mouse liver cDNA[33]. The 5' oligonucleotide contained a Kozak consensus, whereas the 3' oligonucleotide was flanked by a BamHI site. Amplicons were cloned into pGEM-T Easy, sequenced, excised using EcoRI and BamHI, and cloned into the pcDNA3.1

FLAG expression vector (Invitrogen), which provides a C-terminal FLAG epitope tag~~s~~. Full-length expression vectors for mouse NFIB, NFIC and NFIX were provided by Dr. Richard Gronostajski (University at Buffalo, the State University of New York) [117]. A Full-length expression vectors for mouse Elovl3 were generated by PCR amplification of mouse liver cDNA. Amplicons were cloned into pGEM-T Easy, sequenced, excised using EcoRI and BamHI, and cloned into the pcDNA3.1 expression vector (Invitrogen). Ligation of the gel purified insert and dephosphorylated vector was performed using the Promega pGEM-T Easy ligase with the 2X ligation buffer.

Transformation. Plasmids were transformed into JM109 *E coli* bacteria by incubating 10 μL of plasmid DNA with 100 μL of competent JM109 cells on ice for 30 min. Cells were heat shocked for 45 seconds at 45°C and then incubated on ice for 2 minutes. 900 μL S.O.C. media was added and the cells were shaken at 37°C for one hour. Cells were then pelleted in a 1.5 ml Eppendorf tube at 14,000 RPM during a one minute centrifugation. S.O.C. was decanted and the cells resuspended in the remaining liquid. Cells were then plated on LB plates containing ampicillin (Sigma) and incubated overnight at 37°C.

Miniprep. Individual colonies were picked and placed in 1.5mL of LB media containing ampicillin (50 μg/mL final concentration). Cells were grown overnight in a shaker at 37°C. Cells were pelleted in a 1.5 ml Eppendorf tube at 14,000 RPM in a one minute centrifugation and the LB media was aspirated. Cells were resuspended in 100 μL of Plasmid Prep Solution 1 (50 mM sucrose, 25 mM Tris pH8, 10 mM EDTA) through vortexing. Freshly

prepared plasmid prep solution 2 (0.2N NaOH, 1%SDS; 200 μL) was added to the cell mixture. Following mixing by inversion, 150 μL of 3M potassium acetate (pH) was added to each sample. Following mixing by inversion, 150 μL Chloroform:Isoamyl Alcohol (24:1) was added and each sample was mixed. Samples were centrifuged for three minutes at 14,000 RPM. The interface was removed with a toothpick and the upper (organic) layer was removed, followed by the addition of 900 μL of 100% ethanol. Following mixing by inversion, the precipitated DNA was then pelleted by centrifugation for 3 min at 14,000 RPM. All liquid was aspirated and the pellet was resuspended in 100 μL of water. After vortexing to dissolve pellet, 100 μL of 3M Ammonium Acetate (Sigma) was added and samples were mixed by vortexing. Five hundred micro liter of 100% ethanol was added and samples were mixed by vortexing and spun for 3 min at 14,000 RPM. All liquid was removed and the pellet washed in 95% ethanol. After drying on the bench for 5 minutes the pellet was resuspended in 50 μL of water prior to restriction enzyme digestion. 3 μL of miniprep DNA was digested with 2 μL 10X enzyme buffer, 0.5 μL RNAse, 1.0μL of enzyme, and water in a total volume of 20 μL.

Maxi Prep. For large-scale plasmid preps, transformed cells were grown in 450 mL LB with ampicillin (50 μg/mL final concentration). After overnight growth at 37°C, the bacteria was pelleted by centrifugation in a Jouan CR412 centrifuge at 4000 RPM for 20min at 4°C. The liquid was decanted and cells were resuspended in 10 mL of plasmid prep solution 1. With gentle vortexing, 20 mL of freshly prepared plasmid prep solution 2 was added, followed by the addition of 10mL of 3M KoAc. After mixing, samples were centrifuged for 10min at 3500 RPM at 4 ^{o}C. The supernatant was clarified by passing through cotton gauze and precipitated by the

addition of an equal volume of isopropanol. After mixing, samples were centrifuged for 10 min at 3500 RPM at 4 ^{o}C. Pelleted DNA was resuspended in 3 mL of TE, followed by the addition of 4.59 g of cesium chloride (CsCl). After the CsCl dissolved, 225 μL of ethidium bromide was added. Samples were placed in a sealable Beckman centrifuge tube, sealed and centrifuged overnight in a Vti 65.2 rotor at 55,000 RPM under vacuum. Plasmid bands were isolated and transferred to a new centrifuge tube with TE-CsCl. After a 6 hour centrifugation in a Vti 65.2 rotor at 55,000 RPM under a vacuum, plasmids were isolated and ethidium bromide was extracted using isoamyl alcohol. Samples were then dialyzed twice against 0.5M EDTA and 1M Tris-EDTA. After dialysis, the solution was transferred to a 15 mL conical tube and DNA was precipitated by the addition of 1/10 volume of 5M NaCl and 2X volume of ethanol. DNA concentrations were measured using a spectrophotometer and diluted to 1 μg/μL.

Cell Culture. Cryopreserved Hep3B, HepG2 and HEK293 cells were removed from liquid nitrogen and placed in the appropriate media in T75 Flasks. Hep3B and HEK293 cells were grown in Dulbecco's minimal eagle's media (DMEM, Cellgro) supplemented with 10% fetal bovine serum (FBS, Cellgro), 1% L-glutamine and 1% Pen-Strep (Gibco). HepG2 cells were grown in media containing 1:1 mix of DMEM: Ham's F-12(Gibco) supplemented with 10% FBS, 1% L-glutamine, 1% penstrep and 0.1% insulin (Sigma). All cells were cultured in a Napco incubator at 37°C and 5% CO_2.

Reporter Gene Assays. HepG2 and Hep3B cells were seeded onto 12 well plates. The following day, cells were transfected using the calcium phosphate protocol [118]. For each well,

cells were transfected with 500 ng of luciferase reporter gene, 1 μg of transcription factor expression vector, and 12.5 ng of renilla luciferase vector in a total volume of 100 μL. For titration of NFIC, 0.04 μg, 0.11 μg, 0.33 μg and 1 μg of NFIC expression vector were used; 0.1 μg of HNF1α expression vector were used (Fig. 5B). Each transfection was performed in duplicate. Six hours after the addition of DNA, cells were washed 1X in phosphate buffered saline (PBS) and refed with fresh media.

After 48 hours, cells were washed 1X with PBS followed by the addition of 200 μL Glo Lysis Buffer (Promega). After 10 min incubation at room temperature, samples were transferred to 1.5 ml eppindorf tubes and pelleted by centrifugation at 13,000 RPM for 30 sec. Supernatants were transferred to separate tubes and used immediately or stored at -80°C. Aliquots (25 μL of Hep3B or 20 μL of HepG2) of supernatants were placed in duplicate into 96-well luciferase plates (CoStar). Analysis was performed using an Applied Biosystems TR717 Microplate luminometer that injected 25 μL of both dual luciferase substrates (Promega) into the designated wells. Luciferase values were normalized to renilla values to control for variations in transfection efficiency.

Preparation of Nuclear extracts and Electrophoretic Mobility Shift Assay (EMSA). HEK293 cells were seeded onto 10 cm plates. The following day cells were transfected using the calcium phosphate protocol [118] containing 15 μg of HNF1β and NFIC expression vectors. Six hours later the media was changed and after 48 hours 1 ml of PBS was added and the cells were scrapped from the plate and transferred to a 1.5 ml eppindorf tube. Cells were pelleted for 5 min

at 2,000RPM and the supernatant was removed. Cell fractions were collected from the cells using the NE-PER nuclear and cytoplasmic extraction kit following manufacturer's protocol (Thermo-scientific). Protein concentrations from nuclear fractions were analyzed from each sample in duplicate using the BCA protein concentration assay kit (Peirce) at 562 nm in the spectrophotometer. Extracts were used immediately or stored at -80°C.

Oligonucleotides (obtained from IDT, listed in Table 2) were resuspended in water to a final concentration of 1 μg/μL. Oligos were annealed using 10 μg of each in 10 mM NaCl (with a total volume of 100 μL) and by heating to 75°C for 3 minutes and slowly cooling to room temperature. The products were checked on a 12% acrylamide gel using a 29:1 bis:acrylamide mixture to insure the oligonucleotides had annealed. Ten μL of annealed products were radiolabeled with ^{32}P using T4 kinase (Lucigen) according to manufacturer's protocol. After quenching the reaction by adding EDTA to a final concentration of 50 mM, the sample was passed through a centri-spin20 column (Princeton Separations) to purify the radiolabeled oligonucleotide away from free ^{32}P. Radioactive counts of 1 μL of sample in 2 mL of scintillation fluid (Research Products Int) were measured in scintillation counter. Labeled probes were diluted with water to 15,000 cpm/μL and used immediately or stored at -20°C for up to several weeks. Binding reactions with oligonucleotide, dI:dC and extracts were carried out as described [119]. For cold competitions, unlabeled probe and extracts were incubated on ice for 10 min before addition of the radiolableled probe, followed by an additional 30 min incubation at room temperature. Samples were then loaded on a 5% acrylamide gel using a 75:1 bis:acryl mixture and run at 230V for about 1.5 hours in TBE buffer at room temperature. After drying,

the gel was placed onto a phosphorus screen overnight in a cassette in the dark. Screens were analyzed with Storm 860 phosphoimager (ThermoScientific) with ImageQuant software.

RNA extraction and Real-Time PCR. Approximately 100 mg of liver was placed into 1 mL of Trizol (Invitrogen) in a 2063 tube and homogenized at highest speed for 30 sec (Ultra-Turrax T25 Basic from IKA Works). The homogenate was transferred to 1.5 mL microfuge tube. Two hundred μL of chloroform was added to each sample which was then vigorously mixed for 15 sec. Samples were centrifuged at 10,000 RPM for 15 min at 4°C. The top (clear) aqueous layer was carefully removed and transferred into a new Eppindorf tube which was then filled with 500 μL of isopropanol. After incubation at room temperature for 10 min the samples were centrifuged for 10 min at 10,000 RPM at 4°C. Liquid was decanted and the RNA pellet washed with 1 mL 95% ethanol. Samples were spun again for 5 min and the wash fluid removed. Pellets were air-dried at the bench for 10 min, resuspended in 100 μL water, and placed in a 52°C waterbath for 10 min. After a second round of Trizol extraction, the RNA was quantified at 260 nm using a Biomate 3 spectrophotometer (Thermo Scientific) by placing 2 μL of RNA in 500 μL of water. Opossum RNA samples were provided by Dr. Steve Munroe from Marquette University [120]. One microgram of RNA was processed into cDNA using the qscript kit (Quanta Biosciences) according to the manufacturer's instructions and run in the reverse transcriptase protocol in an iCycler (BioRad). Each sample was then diluted 1:5 with water. Quantitative PCR was carried out using 2 μL of diluted cDNA with 1X Sybr Green (Quanta Biosciences), 100 nM primer mix and water in a total volume of 25 μL. Samples were placed in duplicate in a 96 well plate and covered with film and analyzed with Bio-rad iCycler. All CT

(MyIQ) levels were normalized using the ΔΔCT method with values normalized to ribosomal gene L30 [121].

Cell Cycle Analysis. HEK 293 cells were plated in 10 cm dishes. Cells were transfected with either PcDNA3.1 or Elovl3 expression plasmids using the calcium phosphate protocol [118]. After 5 hours, cells were serum starved by incubation in serum-free media for 24 hours[122]. Cells were then refed with standard media containing 10% FBS for 12 hours. Cells were then trypsinized, centrifuged, and washed with cold 5 mL PBS. The cell pellets were then resuspended in 0.5 mL of cold PBS. With gentle vortexing, 5 mL of 70% ethanol was slowly added. After vortexing, cells were centrifuged and PBS/Ethanol was discarded. Cells were washed again with 5 ml of cold PBS and centrifuged. After discarding supernatant, cells were resuspended in PBS. RNase (50 mg RNAase, 50 μL 1M Tris, 15 μL 5M NaCl, 5ml H2O) was added to the final concentration of 1mg/ml. Propidium iodide (Roche; from a stock solution of 0.5 mg/mL) was added to the final concentration of 0.25 mg/mL. Triton-X (Amersham Biosciences; from a stock solution of 100%) was added to the final concentration of 0.3% and cells were broght to a final volume of 1 mL. After incubation in the dark at 4°C for 45 minutes, cells were passed through mesh to remove clumped cells. Cells were analyzed for cell cycle by FACS at the University of Kentucky Flow Cytometry Service Facility.

Statistical analysis. All values within a group were averaged and plotted as mean +/- standard deviation. p-values were calculated between two groups using student's t-test. A p-value less than 0.05 was considered significant.

Table 1. List of Plasmids

Plasmid Name	**Description**
pGL3-Basic	Promega SV40 promoterless luciferase vector
Renilla	Promega CMV driven luciferase vector
AFP(250)-lacZ	Wildtype AFP 250bp promoter in lacZ vector
WT-pGL3	Wildtype AFP 250bp promoter in pGL3 vector
Mut1-pGL3	Mutated AFP 250bp promoter in pGL3 vector
Mut2-pGL3	Mutated AFP 250bp promoter in pGL3 vector
Mut3-pGL3	Mutated AFP 250bp promoter in pGL3 vector
pcDNA 3.1	Empty expression vector
HNF1α	HNF1α expression plasmid
HNF1β	HNF1β expression plasmid
NFIB	NFIB expression plasmid obtained from Dr. Richard Gronostajski
NFIC	NFIC expression plasmid obtained from Dr. Richard Gronostajski
NFIX	NFIX expression plasmid obtained from Dr. Richard Gronostajski
Elovl3	Elovl3 expression plasmid

Table 2. Oligos

Genotyping

AFP(250)-Dd transgene	F	5'CCCCTGCTCTGTTAATTATTGGCAAATTGCCTAACT
	R	5' CACTCACCAGCGCGGGTCTGAGTC
AFP(250)^{Mut1}-Dd transgene	F	5'CCCCTGCTCTGTTAATTATTAACAAATTGCCTAACT
	R	5' CACTCACCAGCGCGGGTCTGAGTC
Uty	F	5’ GGCTAGAGGCGAGGGCGAAG
	R	5’ TGGCGCCATCTTTGCATCGG

Real-time

Tg	F	5' ACTGCCTGCGGGGTCGACAGAT
	R	5’ TGTACCGGGGCTCCCCGAAG
oAFP	F	5’ CCACTTGTTGCCAAGCTGAAAATGC
	R	5’ AGACCGTTCTCCAAACTTCCTCAGA
oAlb	F	5' CAGAGATGCTGTGAGGGAAT
	R	5' TGCCTTGACAGCTCTCTCT
oβactin	F	5’ TTGCTGACAGGATGCAGAAG
	R	5’ GAGCCTCCAATCCAGACAGA
Elovl1	F	5’ GTGGCCCAGCCCTACCTTTGG
	R	5’ TGGTAGTTGCAGCTGGGCATGA
Elovl2	F	5’ TCACCACGCGTCCATGTTCAACA
	R	5’ AAGCTGTTCAGGGTGGGTCCAA
Elovl3	F	5’ CCTCTGGTCCTTCCTGGCA
	R	5’ CGGCGTCATCCGTGTAGATGGC
Elovl5	F	5’ ACTGGGTTCCCTGCGGCCAT
	R	5’ TTCCACCAGAGGTAGGGACGCA
Elovl6	F	5’ TCCTGTTTTCTGCGCTGTACGCT
	R	5’ GCACCAGTTCGAAGAGCACCGA
Zhx2	F	5’ AGGCCGGCCAAGCCTAGACA
	R	5’ TGAGGTGGCCCACAGCCACT
AFP	F	5' CCGGAAGCCACCGAGGAGGA
	R	5’ TGGGACAGAGGCCGGAGCAG
L30	F	5’ ATGGTGGCCGCAAAGAAGACGAA
	R	5’ CCTCAAAGCTGGACAGTTGTTGGCA

Oligos continued on next page

Probes

WT	F	5’CCCCTGCTCTGTTAATTATTGGCAAATTGCCTA ACT
	R	5’ AGTTAGGCAA TTTGCCAATA ATTAACAGAG CAGGGG
Mut1	F	5’CCCCTGCTCTGTTAATTATTAACAAATTGCCTA ACT
	R	5’ AGTTAGGCAA TTTGTTAATA ATTAACAGAG CAGGGG
Mut2	F	5’CCCCTGCTCTGGCGATTATTGGCAAATTGCCTA ACT
	R	5’ AGTTAGGCAA TTTGCCAATA ATCGCCAGAG CAGGGG
Mut3	F	5'CCCCTGCTCTGTTAATTATTGGCAAATTGCCAA ACT
	R	5’ AGTTTGGCAA TTTGCCAATA ATTAACAGAG CAGGGG

Cloning

HNF1α	F	5’ GGATCCCTGGGAAGAGGAGGC
	R	5’ GGATCCCTGGGAAGAGGAGGC
HNF1β	F	5’ GCCACCATGGTGTCCAAGCTCACGT
	R	5’ GGATCCCCAGGCTTGCAGTGGACA
Elovl3	F	5' GCCACCATGGACACATCCATGAATTTCTCAC
	R	5’ GGATCCTTGGCTCTTGGATGCAACTTTG
Mut1	Flanking oligos	5’ GATCTGGATCCGGGGAAATAATCT
		5’ ACCAGCGCGGGTCTGAGTCGGACC
	Oligo with mutation	5’ GGCAATTTGTTAATAATTAACAGAGCAGG
Mut2	Flanking oligos	5’ GATCTGGATCCGGGGAAATAATCT
		5’ ACCAGCGCGGGTCTGAGTCGGACC
	Oligo with mutation	5’AGTTAGGCAATTTGCCAATAATCGCCAGAGCA GGGG
Mut3	Flanking oligos	5’ GATCTGGATCCGGGGAAATAATCT
		5’ ACCAGCGCGGGTCTGAGTCGGACC
	Oligo with mutation	5’ CGTTGAAGTTTGGCAATTTGCCAATAATT

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CHAPTER 3

Regulation of Mouse Alpha-fetoprotein Promoter Activity by HNF1 and NFI Binding

Introduction

AFP belongs to a small family of five serum transport proteins that also includes albumin (Alb), alpha-albumin (AFM), vitamin D binding protein (DBP), and AFP-related gene (Arg) [30, 31]. AFP has been used extensively as a model of gene regulation during liver development [123]. AFP is expressed at low levels in the developing foregut endoderm but is highly induced in hepatoblasts that form the liver bud [124]. AFP continues to be highly expressed in the fetal liver but is dramatically repressed at birth resulting in a 10,000-reduction in steady state AFP levels during the perinatal period [68]. AFP levels remain low in the adult liver, but this silencing is reversible since the gene can be activated during liver regeneration and in liver cancer [125].

The *cis*-elements that control AFP transcription have been well characterized in cultured cells and transgenic mice. These elements include three distinct upstream enhancers (E1, E2 and E3), each 200-400 bp in length, and a promoter within the first ~250 bp upstream of exon 1 [72, 126, 127]. The 250 bp AFP promoter contains binding sites for numerous factors. Binding sites for Nkx2.8 and FTF are centered at -165 [46, 47]. A region at -120 contains overlapping binding sites for Hepatocyte Nuclear Factor 1 (HNF1) and Nuclear Factor I (NFI) [50, 51]. A second

HNF1 binding site is centered at -60 [50]. C/EBPα has been shown to activate AFP in transient transfections, and several C/EBP binding sites have been identified [59]. Clinical studies have also provided insight into AFP regulation. In particular, Hereditary Persistence of AFP (HPAFP), in which AFP continues to be expressed at elevated levels in the adult liver, has demonstrated the important of HNF1 in AFP regulation. A G → A mutation at -119 of human promoter that increased HNF1 binding was identified in four independent families with hereditary persistence of AFP (HPAFP) expression in adults, whereas a C→A change at –55 has been identified in a different pedigree and also increased HNF1 binding to this region [54-58].

HNF1 was originally characterized as an activator of α1-antitrypsin, albumin and β-fibrinogen, and has since been shown to control a large number of genes in the liver and in other organs, including the kidney [128, 129]. Two HNF1 isoforms, HNF1α and HNF1β, have been characterized. These structurally related proteins contain a homeodomain, POU domain, and dimerization motif [130, 131]. During embryonic development, HNF1β is induced upon the onset of hepatic differentiation, whereas HNF1α is activated later and continues to be expressed in terminally differentiated hepatocytes [132]. In general HNF1α is a more potent activator than HNF1β; this has been shown for several genes including DBP and AFM [33, 53]. HNF1 proteins bind as homodimers or heterodimers to the palindromic GTTAATnATTAAC sequence, although HNF1 binding to half-sites has been reported [129]. NFI proteins were originally identified as regulators of adenovirus replication and subsequently shown to influence transcription [133, 134]. The NFI family is comprised of four ubiquitously expressed members, NFIA, NFIB, NFIC and NFIX; alternative processing increases the number of potential NFI

isoforms [135-137]. These proteins can act as activators or repressors of gene expression in a context-dependent manner [134]. NFI proteins bind as dimers to the consensus TTGGCn_{5-6}GCCAA but, similarly to HNF1, can also bind to half-sites to control target promoters [134].

While HPAFP studies demonstrated that the natural mutations in the AFP promoter resulted in enhanced HNF1 binding, the role of NFI was not fully investigated. Here, we characterize the overlapping HNF1/NFI site of the mouse AFP promoter. Our studies confirm that HNF1 is a positive regulator of AFP promoter activity whereas we show that several NFI isoforms repress the AFP promoter. Mutations that improve HNF1 binding increase promoter activity in cultured cells and transgenic mice, whereas increased NFI binding reduces promoter activity in liver cell lines. Consistent with this data, loss of NFIX in the developing mouse liver results in delayed postnatal AFP repression. We also show that the opossum AFP promoter contains a variation that corresponds to the -119 region of the human gene; in contrast to the human mutation, the variation in opossum is not predicted to increase HNF1 binding. AFP repression appears normal in opossum. Taken together, this data indicates that the balance between HNF1 and NFI binding controls AFP promoter activity and is important for normal AFP repression after birth.

Results

The mouse AFP promoter is activated by HNF1 and repressed by NFI. The -120 region of the AFP promoter contains binding sites for HNF1 and NFI, with overlap between the

last five nucleotides of the HNF1 and the first five nucleotides of the NFI site. A comparison of this region from a number of different mammals indicates that the HNF1 binding motif is highly conserved (Table 3). In fact, the first 9 nucleotides of the HNF1 site are conserved between all 30 species and are a perfect match with the HNF1 consensus. Nucleotides 10-12, which overlap with the NFI site, are less conserved. There is greater variation in the NFI motif, with the distal NFI dyad (the region that overlaps with the HNF1 site) being more conserved that the proximal NFI dyad. The region of HNF1/NFI overlap most often contains a GG dinucleotide (-118 and -117 of the human gene); this is closer to the NFI consensus than that of HNF1.

Transient transfections were performed to explore further HNF1 and NFI control of AFP promoter activity. Hep3B is a hepatoma cell line. Hep3B cells were used since they contain low levels of HNF1 activity. HNF1α and HNF1β expression vectors were co-transfected with the Luciferase reporter fused to the 250 bp AFP promoter [AFP(250)-Luc]; Renilla luciferase was included to control for variations in transfection efficiency. Both HNF1 isoforms could transactivate the AFP promoter, although HNF1α was a more potent activator than HNF1β (Fig. 6A). Co-transfections were also performed with NFI expression vectors. NFIC and NFIX repressed AFP promoter activity, whereas NFIB had no effect (Fig. 6A). To test the activating and repressive activities, HNF1α and increasing amounts of NFIC were transfected together with AFP(250)-Luc. The transactivating activity of HNF1α was inhibited by NFIC in a dose-dependent manner, suggesting that the activities of these two factors are in competition (Fig. 6B). Taken together, these data confirm that the mouse AFP promoter is transactivated by both HNF1 family members and repressed by several NFI family members.

HNF1 and NFI compete for binding to -120 region of the mouse AFP promoter. Since the HNF1 and NFI sites centered at -120 are overlapping, we asked whether these proteins compete for binding to this region of the AFP promoter; since these proteins have been shown to bind to a half-site of its consensus palindromic motif, it is possible that both proteins could bind simultaneously. EMSAs were performed using the -120 region of the AFP promoter as a radiolabeled probe. Since HNF1 and NFI proteins are not found in HEK293 cells, nuclear extracts were prepared from these cells that were transiently transfected with HNF1β and NFIC expression vectors. EMSAs were then performed with constant amounts of NFIC extracts and increasing amounts of HNF1β extracts (Fig. 7, lanes 1-10). The band corresponding to the NFIC complex did not change as increasing amounts of HNF1β were added. However, as the band corresponding to the HNF1β complex increased in intensity, the amount of free probe diminished. A similar result is observed with increasing amounts of NFIC extracts are added to a constant amount if HNF1β extract; the NFIC does not diminish the HNF1β band but the amount of free probe is reduced (Fig. 7, lanes 11-17). A new complex is not observed when HNF1β and NFIC extracts are added together. One possibility is that HNF1β and NFIC cannot bind to the overlapping sites in the AFP promoter at the same time. It is also possible that the complex with HNF1β and NFIC migrates to the same location as either the HNF1β or NFIC complex; however, this possibility is low. Another possibility is that the complex with HNF1β and NFIC stays in the well and cannot migrate into the gel. Given the overlapping spacing of HNF1 and NFI sites, we favor the possibility that the binding of HNF1β and NFIC is mutually exclusive. These data also suggest that both HNF1β and NFIC binding to their cognate sites on the AFP

promoter with high avidity, since increasing levels of NFIC or HNF1β do not reduce the binding of HNF1β or NFIC, respectively, but rather bind to the excess free probe that is available.

Functional analysis of HNF1 and NFI sites in the mouse AFP promoter. To explore further the importance of HNF1/NFI binding to this region, we generated a series of mutations (Fig. 8A). The first mutation (Mut1) changes the GG → AA at -118/-117; this mutation is predicted to increase HNF1 binding and decrease NFI binding and is similar to the natural single nucleotide G → A mutation at -119 of the human AFP gene that leads to HPAFP. We also generated a mutation in the 5' HNF1 half site (Mut2). This mutation is predicted to reduce HNF1 binding but should not alter NFI binding. We attempted to generate a mutation that would diminish NFI binding without altering HNF1 binding; several mutations were generated in the 3' NFI half site (since mutations in the 5' NFI half site would also affect HNF1 binding) but none of these reduced NFI binding in EMSAs (data not shown). Therefore, we made a T → A change at –107 (Mut3), which is predicted to improve NFI binding but should not alter HNF1 binding to this region.

These series of mutations were first tested as cold competitors in EMSAs, using the wild-type AFP -120 region as a radiolabeled probe, to determine their affect on HNF1 and NFI binding. When HNF1β extracts were used, the wild-type competitor could compete for binding in a dose-dependent manner (Fig. 8B). Mut1, which is predicted to improve HNF1 binding, was a more effective competitor than the wild-type fragment. In contrast, Mut2 could not compete for binding even at the highest concentration. Mut3, in which HNF1 binding should not be

altered, was similar to the wild-type fragment as a cold competitor. A similar experiment was performed with NFIC extracts (Fig. 8C). The wild-type probe could compete for binding in a dose-dependent manner. Mut1 was a less effective competitor, consistent with the change that is predicted to decrease NFI binding. Mut2, which should not have altered NFI binding, competed for binding similarly to the wild-type fragment. However, Mut3 was the most effective competitor, consistent with the change that is predicted to improve NFI binding.

The three mutations were introduced into the AFP(250)-Luc plasmid to test their transcriptional effect. This series was transiently transfected into HepG2 cells, since the AFP promoter has robust activity in these cells (Fig. 8D). The Mut1 mutation increased promoter activity ~3-fold, consistent with the human promoter mutation that increases HNF1 binding and leads to HPAFP. The Mut2 mutation, which dramatically reduces HNF1 binding, reduced promoter activity ~6-fold. The Mut3 mutation, which increases NFI binding but had no effect on HNF1 binding in vitro, also reduced promoter activity ~6-fold. Taken together, this data indicates that the balance between HNF1 and NFI binding determines the overall strength of the AFP promoter.

In vivo analysis of AFP. To extend this EMSA and tissue culture data, we performed several experiments in mice. First, we generated transgenic mice with Mut1-containing transgenes. The Mut1 mutation (GG → AA at positions -118, -117) is similar to the human AFP mutation (G → A at position -119) that leads to HPAFP. We previously showed that a transgene with AFP enhancer E2 and the 250 bp AFP promoter fused to the histocompatibility class I H-

$2D^d$ reporter gene [E2-AFP(250)-D^d] was developmentally repressed in mice [66]. This construct was generated with the Mut1 change to generate E2-AFP(250^{mut1})-D^d. This transgene was expressed in the adult liver at levels that were significantly higher the wild-type E2-AFP(250)-D^d transgene (Fig. 9).

Our in vitro data indicated that NFIX is a potent repressor of AFP promoter activity. If NFI proteins are important for developmental AFP regulation, the loss of NFIX should result in increased AFP expression. To test this, we collaborated with Dr. Gronostaski's lab (University at Buffalo, the State University of New York). They monitored hepatic AFP expression at several different timepoints in NFIX-deficient mice (Fig. 10). At postnatal day 7 (p7), AFP levels was essentially the same in wild-type and $NFIX^{-/-}$ livers. However, at p16, postnatal AFP repression was delayed in the absence of NFIX. The AFP gene was eventually repressed in the $NFIX^{-/-}$ mice to the same extent as in wild-type mice, but the delay in postnatal shutoff suggests that NFIX is required for proper repression.

AFP expression is repressed in the adult liver of opossum. In most species the AFP gene contains GG residues in the HNF1-NFI overlapping region; these two residues are more similar to the NFI consensus than the HNF1 site. Variations that change one or both of these G residues to A, which increases and decreases HNF1 and NFI binding, respectively, results in HPAFP in humans and mice. A natural variation in this position is found in the opossum AFP promoter; this variation changes a G to a T residue. While this change is predicted to reduce NFI binding, it should not increase HNF1 binding. To test whether this variation influenced AFP

expression, hepatic AFP and albumin mRNA levels in Opossum were monitored at several developmental time-points (Fig. 11). This indicated that AFP and albumin were both expressed in 2 week and 4 week livers (these time-points in marsupials would correspond to the fetal period in mice). However AFP was repressed 2 and 4 months after birth while albumin continued to be expressed. These data indicate that the opossum AFP gene is developmentally repressed similarly to the mouse and human AFP genes.

Discussion

The AFP gene provides a model system to study developmental gene silencing, since steady state AFP mRNA levels are reduced 10,000-fold in the perinatal liver [35]. Early insight into postnatal AFP repression first came from a human family which exhibited HPAFP [54]. Molecular analysis revealed the causative mutation as a G→A transition in the overlapping HNF1/NFI site centered at -120 of the AFP promoter. The same mutation was subsequently found in several independent families with HPAFP. This transition results in increased HNF1 binding, which has been the focus of several studies [55-57]. Although not previously analyzed, this mutation is also predicted to decrease NFI binding. Data shown here indicates that changes in NFI binding can also influence AFP promoter activity. Using transgenic mice, we demonstrate that mutation in the mouse AFP promoter similar to the natural human mutation results in continued AFP expression in the adult liver. We also show that mutations that increase NFI binding but do not alter HNF1 binding diminish AFP promoter activity. Several NFI isoforms can also repress AFP promoter activity in cultured cells, and postnatal AFP repression

is delayed in NFIX knock-out mice. Our data indicates that NFI and HNF1 cannot bind the AFP promoter at the same time. Taken together, this data indicates that the balance of HNF1 and NFI binding is important for developmental control of AFP promoter activity. Our data supports the model that HNF1 activates the AFP promoter early in hepatogenesis and that increased levels of several NFI isoforms later in liver development (NFIX and/or NFIC) leads to a displacement of HNF1 by NFI; this change reduces AFP promoter activity and decreases AFP mRNA levels. This model is consistent with expression patterns of HNF1 and NFI, since HNF1α is activated early in hepatogenesis whereas NFI is activated later during liver development [117, 132].

The five members of the albumin gene family (Alb, AFP, AFM, Arg and DBP) are expressed primarily in the liver [30, 31]. These evolutionarily related genes arose from a series of duplications and are found on chromosome 5 in mice, with all but DBP being adjacent to each other [31, 32]. AFP and Alb are highly activated as soon as the liver bud forms and continue to be expressed in the fetal liver, whereas AFM, Arg, and DBP are activated later during liver development [31, 138-140]. Alb, AFP, AFM and DBP are activated by HNF1 through the action of one (Alb) or two HNF1 sites; Arg, which is expressed at very low levels in the mouse liver, does not appear to be regulated by HNF1 ([31] and data not shown). Our sequence analysis did not identify strong NFI binding sites in the AFM, Arg or DBP promoters (data not shown), and there is no evidence that NFI controls these genes. An NFI site is present in the -120 region of the Alb promoter, and DNAse footprinting indicates that NFI can bind this region. In contrast to AFP, transient co-transfections indicate that NFI modestly activates, rather than represses, the

Alb promoter [141]. Since the Alb NFI site is 60 bp upstream of the single Alb promoter HNF1 site (-52 to -64), it is unlikely that NFI binding could block HNF1 binding to the Alb promoter.

While the repression of AFP-driven reporter genes by NFIC and NFIX and the delayed AFP repression in NFIX deficient livers indicate that NFI is required for normal AFP silencing, postnatal AFP repression is a complex process that involves multiple factors [62, 64, 80]. A natural mutation in the Zinc fingers and homeoboxes 2 (Zhx2) gene in the BALB/cJ strain of mice leads to incomplete postnatal AFP repression [80]. Zhx2 represses AFP through the AFP promoter, although the specific site required for Zhx2 responsiveness has not been identified. Also, the targeted deletion of Zbtb20 in hepatocytes also results in continued AFP expression in the adult liver. EMSAs indicate that Zbtb20 binds to the AFP promoter region between -108 and -53 [62]. Forced expression of Zhx2 and Zbtb20 represses AFP-driven reporter genes, consistent with the repressive activities of these proteins on AFP expression (data not shown and [62]). The binding of p53 to the repressor region (located roughly 1 kb upstream of the AFP promoter) also appears to be involved in postnatal AFP repression [64]. We have also found that AFP enhancer element E3 is a potent negative element in non-pericentral hepatocytes in the adult liver, although the factors involved in this negative regulation have not been identified [76]. Since NFI, Zhx2, and Zbtb20 all act through the AFP promoter region, it is of interest to consider whether these factors interact with each other or work cooperatively to repress AFP promoter activity at birth. All three of these factors are expressed at higher levels in the adult than in the fetal liver, although NFI isoforms are induced earlier in liver development than Zhx2 or Zbtb20.

NFI proteins have been implicated in the control of cell growth in both humans and model systems [134]. Overexpression of NFI proteins renders chick embryo fibroblasts cells resistant to transformation by a number of nuclear oncogenes, including fos, jun and qin [142]. While the mechanism of resistance is not known, the finding that the cells are not resistant to transformation by several oncogenes that function in the cytoplasm suggests some specificity to the suppression of oncogenic susceptibility. Overexpression of NFIX prevents the growth arrest of mink lung epithelial cells by TGF-β, further implicating NFI proteins in the TGF-β signal transduction pathway and cell proliferation [143]. Since AFP expression is reactivated during HCC and liver regeneration, and NFI was first discovered as a protein required for viral DNA replication [144], it will be interesting to investigate the role of NFI proteins in liver proliferation.

		HNF1	NFI
HNF1 consensus	GTTAATnATTAAC		
NFI consensus	TTGGC (n_{5-6}) GCCAA		
Human	GTTAATtATTGGCaaatgTCCCA	10/12	8/10
Human**	GTTAATtATTGACaaatgTCCCA	11/12	7/10
Chimp	GTTAATtATTGGCaaatgTCCCA	10/12	8/10
Orangutan	GTTAATtATTGGCaaatgTCCCA	10/12	8/10
Gibbon	GTTAATtATTGGCaaatgTCCCA	10/12	8/10
Rhesus	GTTAATtATTGGCaaatgTCCCA	10/12	8/10
Lemur	GTTAATtATTGGCaaattgTCCCA	10/12	8/10
Tarsir	GTTAATtATTGGCaaattgTCCAA	10/12	9/10
Elephant	GTTAATtATTGGCaaatcaTCCCA	10/12	8/10
Rabbit	GTTAATtATTTACacatcaTCCCA	11/12	6/10
Pika	GTTAATtATTGACacatcaTCCCA	11/12	7/10
Shrew	GTTAATtATTGGCaaattgTCCCA	10/12	8/10
Horse	GTTAATtATTGGCaagttgCTCCA	10/12	7/10
Dog	GTTAATtATTGACaagttgTCCCA	11/12	7/10
Cow	GTTAATtATTAGCaagtcaTCCTA	11/12	7/10
Sheep	GTTAATgATTAGCaagtcaTCCTA	11/12	7/10
Dolphin	GTTAATtATTAGCaagtcaTCCCA	11/12	7/10
Pig	GTTAATtATTAGCaagttGTCCC	11/12	7/10
Alpaca	GTTAATtATTAGCaagttGTACA	11/12	6/10
Shrew	GTTAATtATTGGCaagtcaTCCCA	10/12	8/10
Hedgehog	GTTAATtATTAGCaagtcaTCCCA	11/12	7/10
Squirrel	GTTAATtATTGGCaaaatgTCCCA	10/12	8/10
Armadillo	GTTAATtATTGGCaaattgGTCCA	10/12	8/10
Hamster	GTTAATtATTGGCaaattGCCTC	10/12	8/10
Rat	GTTAATtATTGGCaaattGCCTA	10/12	9/10
Mouse	GTTAATtATTGGCaaattGCCTA	10/12	9/10
Opossum	GTTAATtATTGTCatattgTCCAA	10/12	8/10

Table 3. A comparison of HNF1/NFI site of AFP promoter from a number of different mammals Blue shows HNF1 binding nucleotides that are the same as HNF1 consensus binding sequence (the number of nucleotides are shown in the right) ; yellow shows NFI binding nucleotides that are the same as NFI consensus binding sequence (the number of nucleotides are shown in the right); green shows the overlapping nucleotides . Human **shows the human AFP promoter sequence in individuals with HPAFP.

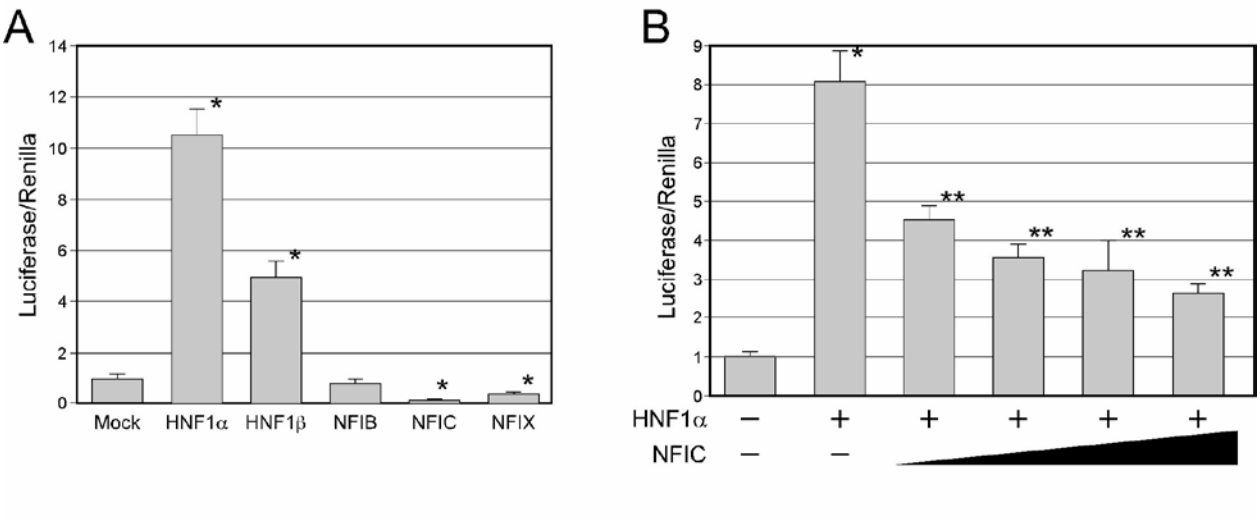

Figure 6. The mouse AFP promoter is activated by HNF1 and repressed by NFI

(A) Relative luciferase reporter gene activity in Hep3B cells, compared to Renilla control. Cells were transiently transfected with the AFP-luciferase constructs and expression vectors for HNF1α, HNF1β, NFIB, NFIC, NFIX or empty vector control; the Renilla luciferase expression vector pRL-CMV was included to normalize for variations in transfection efficiency. * indicates statistical significant in comparison with empty vector control; * $p<0.05$ (B) Relative luciferase reporter gene activity in Hep3B cells. Cells were transiently transfected with the AFP-luciferase constructs and expression vectors for HNF1α and NFIC; the Renilla luciferase expression vector pRL-CMV was included to normalize for variations in transfection efficiency. * indicates statistical significant in comparison with empty vector control; ** indicates statistical significant in comparison with HNF1α transfection; * and **$p<0.05$

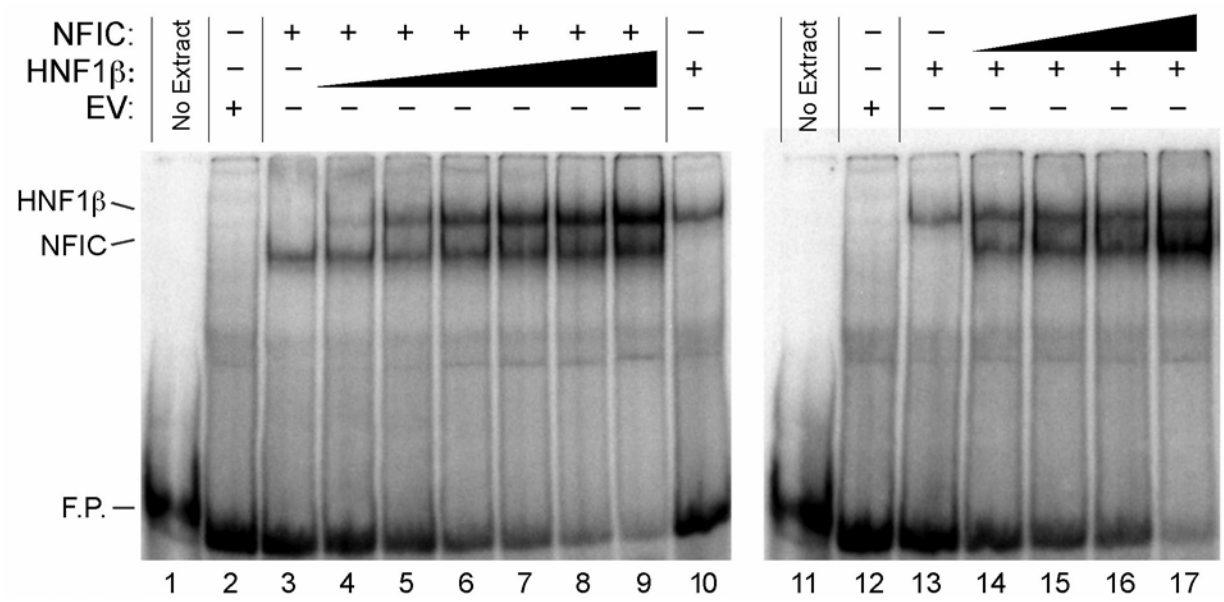

Figure 7. EMSA analysis of HNF1 and NFI binding to AFP promoter

Lanes 1-10 show that NFIC competes with HNF1β for binding to the free probe. EMSAs were performed with same amount of NFIC nuclear extracts (0.9 ug) and increasing amounts of HNF1β nuclear extracts (lanes 4-10: 0.5 ug, 1.5 ug, 2.5 ug, 3.5 ug, 4.5 ug, 5.5 ug, 2.5 ug). Lanes 11-17 show that HNF1β competes with NFIC for binding to the free probe. EMSAs were performed with same amount of HNF1β nuclear extracts (2.5 ug) and increasing amounts of NFIC nuclear extracts (lanes 14-17: 0.5 ug, 1 ug, 1.5 ug, 2 ug). Nuclear extracts were prepared from HEK293 cells that were transfected with empty vector (pcDNA) or HNF1β or NFIC. Extracts were incubated with radiolabeled probes of -120 region of AFP promoter (36 bp).

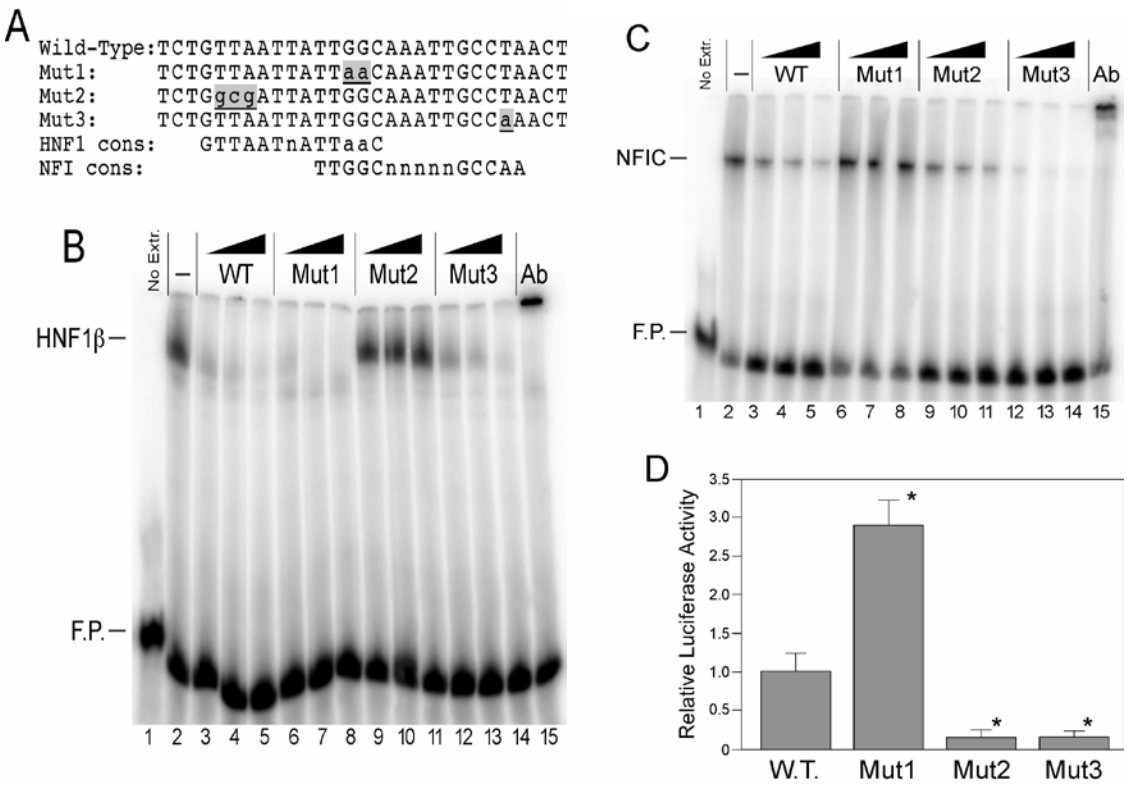

Figure 8. Functional analysis of HNF1 and NFI sites in the mouse AFP promoter

(A) Alignment of the wildtype, Mut1, Mut2 and Mut3 mouse AFP promoters. Regions shown are from -140 to -105. HNF1 and NFI consensus sequence are shown at the bottom. (B, C) Nuclear extracts were prepared from HEK293 cells that were transfected with HNF1β (B) or NFIC (C). Extracts were incubated with radiolabeled probes of -120 region of AFP promoter. EMSAs were performed with no competitor or with cold competitors, including wild-type, Mut1, Mut2 and Mut3. In (B), the concentration of cold competitors was 0.75, 1.5 and 3 fold of the radiolabeled probe. In (C), the concentration of cold competitors was 3.75, 7.5 and 15 fold of the radiolabeled probe. Supershift experiments were performed with antibodies against HNF1β or NFIC as shown. (D) Relative luciferase reporter gene activity in HepG2 cells. Cells were transiently transfected with wildtype AFP-Luc or mutated AFP-Luc; the Renilla luciferase

expression vector pRL-CMV was included to normalize for variations in transfection efficiency.

*indicates statistical significant in comparison with W.T; * $p<0.05$

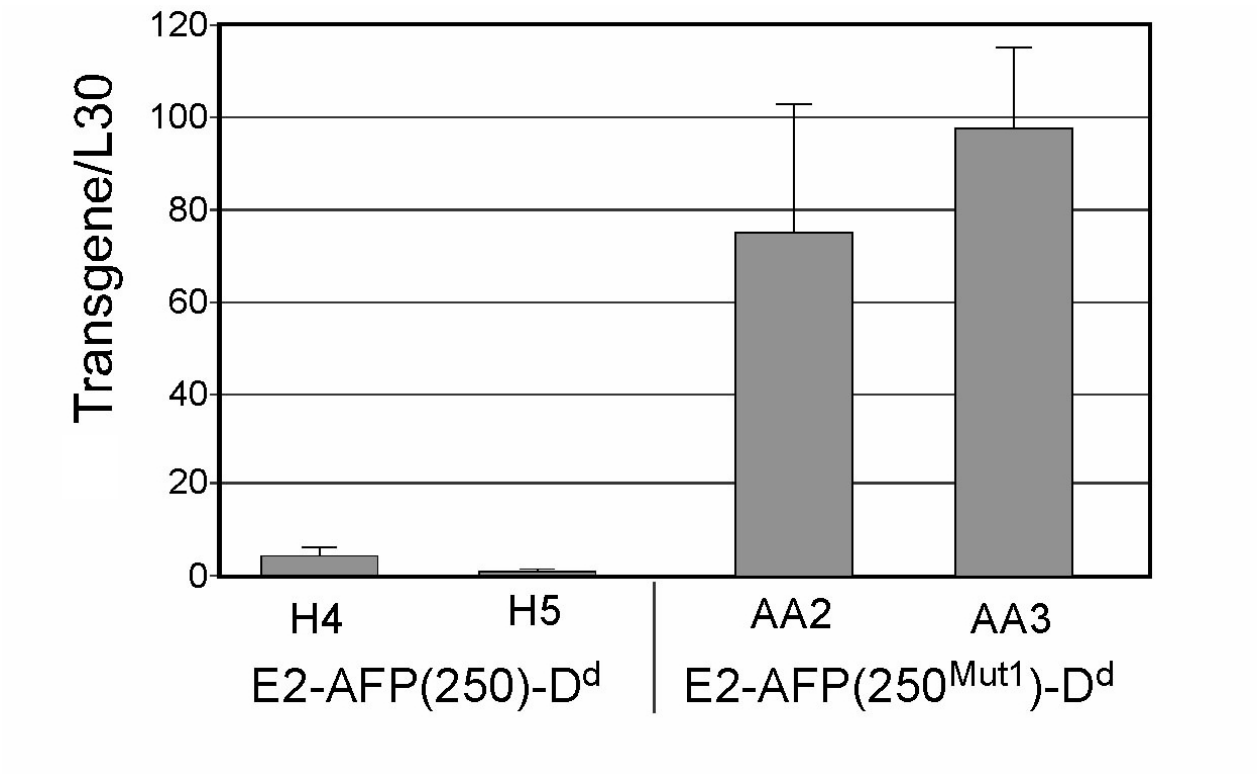

Figure 9. The AFP(250)^{Mut1}-D^{d} transgene is expressed at higher levels than the AFP(250)- D^{d} transgene in the adult liver

Livers were removed from AFP(250)-D^{d} and AFP(250)^{Mut1}- D^{d} transgenic mice at 4 weeks age. H4, H5, and AA2 group contain 3 mice; AA3 group contain 4 mice. RNA was prepared and analyzed using real-time RT-PCR. Transgene levels were normalized to L30. Transgene expression from H5 line is arbitrarily set to be 1.

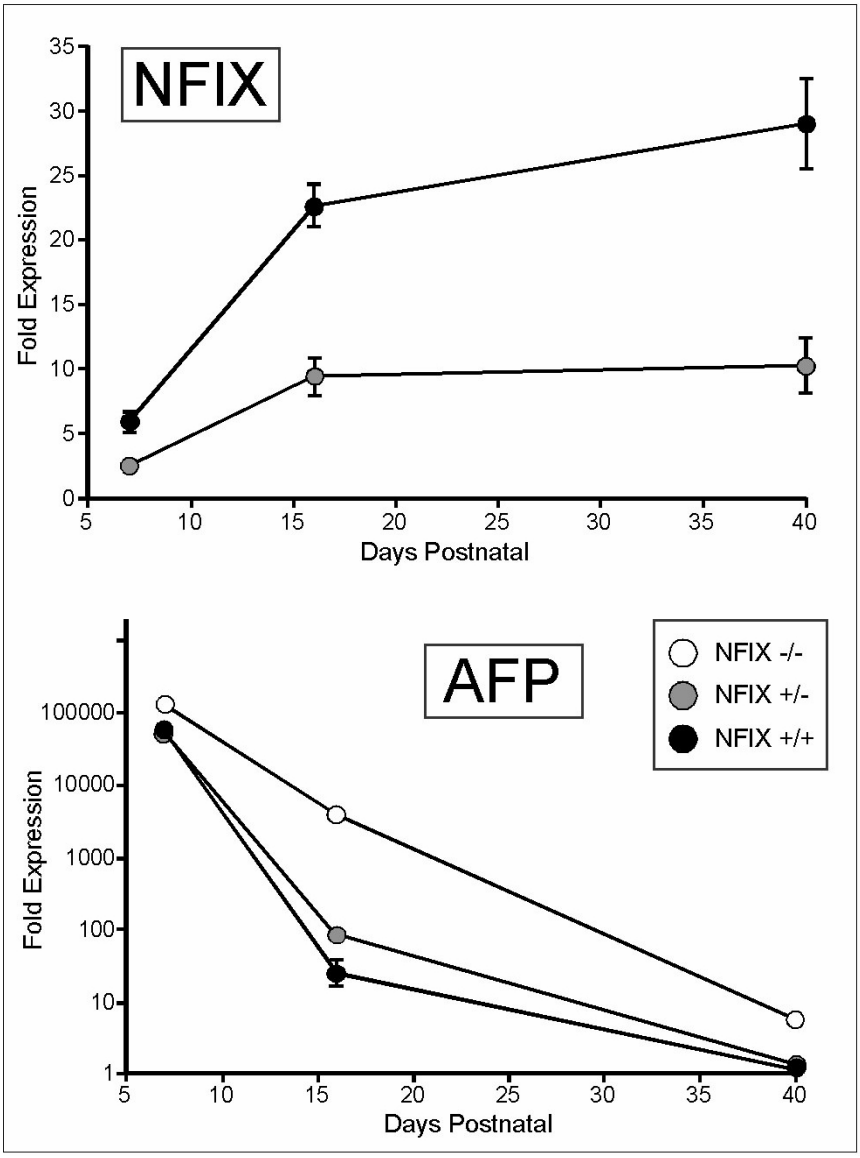

Figure 10. Postnatal AFP repression is delayed in the absence of NFIX

Livers were removed from mice at postnatal day 7, day 16 and day 40 from NFIX$^{-/-}$, NFIX$^{+/-}$and NFIX$^{+/+}$mice. RNA was prepared and analyzed with real-time RT-PCR. NFIX was not detectable in NFIX$^{-/-}$ mice. The analysis was performed by Dr. Richard Gronostajski's lab (University at Buffalo, the State University of New York).

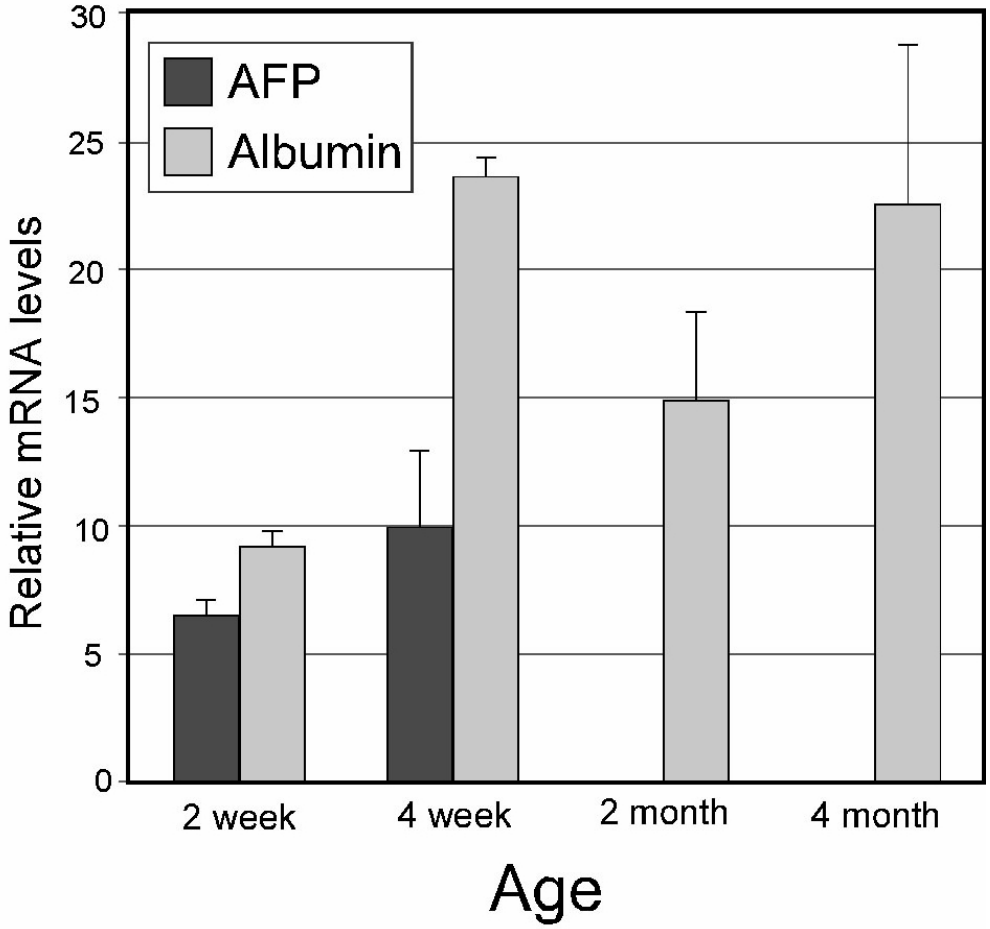

Figure 11. AFP is developmentally repressed in opossum liver

Livers were removed from opossum at 2 weeks, 4 weeks, 2 months and 4 months. RNA was prepared and analyzed with real-time RT-PCR. AFP and Albumin mRNA levels were normalized to β-actin which remained unchanged during this perinatal period in the liver samples. AFP and Albumin mRNA expression of 1 week body is arbitrarily set to be 1. Opossum RNA samples were provided by Dr. Steve Munroe from Marquette University.

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CHAPTER 4

The Elongation of Very Long Chain Fatty Acids-like 3 Gene Is A Target of Zhx2 in The Liver

Introduction

The Zhx2 gene was originally identified in our lab based on its ability to control the developmental repression of several genes in the liver, including AFP, H19 and Gpc3, all of which are normally expressed at high levels in the fetal liver and very low levels in the adult liver. The data from our lab is consistent with a model in which Zhx2 represses AFP, H19 and Gpc3 expression during the perinatal period. A natural hypomorphic mutation in the BALB/cJ dramatically reduces Zhx2 expression and results in persistent AFP, H19 and Gpc3 expression in the adult liver [71, 80].

Zhx2 is a member of a small family of genes that also includes Zhx1 and Zhx3 [82]. These genes share a similar structure in that the first two exons are non-coding, the third exon is unusually large for an internal exon and encodes the entire protein (or all but 2 amino acids for Zhx3), and a moderately-sized terminal exon. The Zhx1 and Zhx2 genes are tightly linked on mouse chromosome 15 (human Chromosome 8) whereas Zhx3 is located on mouse chromosome 2 (human chromosome 20). It is likely that these three genes arose from the duplication of a common ancestral gene. Zhx genes have been identified in a number of different vertebrates,

including amphibians, birds, fish, and mammals, but have not been found in non-vertebrates [82]. The Zhx proteins all contain two C2H2-type zinc fingers and four or five homeodomains, suggesting that they function as regulators of gene expression (Fig. 3) [82]. In addition, all Zhx proteins can form homodimers and heterodimers with each other and with NF-YA [83-85]. *In vitro* assays suggest that Zhx2 is a transcriptional repressor that is localized in the nuclei [84]. Data from our lab in collaboration with Martha Peterson's lab, indicate that Zhx2 also acts at the posttranscriptional level to regulate steady-state AFP and H19 mRNA levels (L. A. Morford, B. T. Spear, and M. L. Peterson, unpubl. obs.).

The known targets of Zhx2, including AFP, H19 and Gpc3, are all frequently reactivated in hepatocellular carcinoma (HCC) [71]. Thus, there is considerable interest in whether Zhx2 is also disregulated in liver cancer and involved in re-expression of its target genes. One study suggested that Zhx2 expression was silenced in HCC samples, and that this silencing was correlated with hypermethylation of the Zhx2 promoter [86]. However, a second study found that Zhx2 protein levels were increased in HCC samples compared to adjacent non-tumor cells [87]. A recent study with our collaborator in China, Chunhong Ma (Shandong University), suggested that nuclear localization of Zhx2 might be reduced in HCC [88]. It should also be noted that Zhx2 is not a liver-specific factor but is ubiquitously expressed [89]. Zhx2 has been identified in several screens for genes mis-regulated during disease and development, including erythrocyte development [90], B-cell development [91], multiple myeloma progression [92] and kidney disease [93, 145].

In contrast to other mouse strains, BALB/cJ mice, which have a natural mutation in the Zhx2 gene, exhibit reduced serum lipid levels (including fatty acids) and atherosclerotic plaques when placed on a high fat diet [99]. The quantitative trait locus (QTL) associated with this trait was found on Chromosome 15 and called *Hyperlipidemia 2 (Hyplip2)* [96]. In a collaboration between our lab and the lab of Jake Lusis (UCLA), we found that the Hyplip2 phenotype is due to the Zhx2 mutation in BALB/cJ mice [98]. A recent GWAS study identified a strong association between Zhx2 and carotid intima media thickness, providing further evidence that Zhx2 is a genetic risk factor for cardiovascular disease (CVD) [146].

A significant amount of the fatty acids are further elongated into very-long-chain fatty acids (VLCFAs) by membrane-bound enzymes predominantly located in the endoplasmic reticulum. Members of the elongation of very-long-chain fatty acids gene family (Elovl1-Elovl7) encode for enzymes that catalyze this elongation cycle in mammals [100]. These elongases display differential substrate specificity, tissue distribution, and regulation, making them important regulators of cellular lipid composition as well as specific cellular functions [101]. The fatty acid elongase Elovl3 is involved in the synthesis of C20–C24 saturated and monounsaturated very-long-chain fatty acids (VLCFAs) in triglyceride-rich glands such as the sebaceous and meibomian glands, brown and white adipose tissue, and liver [103].

To date, little is known about the regulation of Elovl3 expression in the liver (Fig. 4). It has been shown that steady-state Elovl3 mRNA levels follow a robust circadian profile in the liver, and that this regulation is perturbed in the CLOCK mutant mice [109]. However, Elovl3

gene promoter was not activated by BMAL/CLOCK in transfected cells, despite the presence of a perfect E-box. However, it has been found that RevErbα represses Elovl3 promoter (-956 to +165) activity and the sterol regulatory element binding protein-1 (SREBP1) transcription factors activate Elovl3 promoter activity in transfected cells. [111] It has also been reported that hepatic Elovl3 expression is induced in mice injected with the synthetic glucocorticoid dexamethasone, suggesting that Elovl3 expression in mouse liver is under control by circulating steroid hormones such as glucocorticoids [110]. In fact, Elovl3 is a sex-specific gene, with high and low expression in the male and female liver, respectively [111]. Studies in mice also demonstrated that hepatic Elovl3 expression increases in peroxisomal transporter ABCD2 ablated mice and decreases in ABCD2 overexpressing mice [110]. Since VLCFAs are selectively degraded by peroxisomal β-oxidation, the finding suggests a tight cross talk between very long chain fatty acid synthesis and peroxisomal fatty acid oxidation [110].

We hypothesize that Zhx2 contributes to cardiovascular disease (CVD), at least in part, by regulating hepatic genes involved in lipid and cholesterol homeostasis. Microarray data provided to us by the Lusis lab indicated that Elovl3 was a candidate target of Zhx2 [96]. In this chapter, I further explore the regulation of Elovl3 by Zhx2, and investigate this regulation relative to other known Zhx2 targets. This analysis revealed that the Zhx2 is a positive regulator of Elovl3 gene. The regulation of Elovl3 by Zhx2 is observed in the livers of female mice, where Elovl3 levels are low, but not in male mice, where Elovl3 levels are much higher. This regulation is specific for Elovl3; none of the other Elovl elongases is regulated by Zhx2. These data raise the possibility that the effects of Zhx2 in CVD may be due to its regulation of Elovl3. I also

demonstrate that Elovl3 is repressed in the regenerating liver and that the level of Elovl3 repression is controlled by alpha-fetoprotein regulator 2 (Afr2). In addition, I show that Elovl3 expression is reduced in fibrotic livers and fatty livers, suggesting that Elovl3 can serve as a marker for liver damage.

Results

Elovl3 levels are controlled by Zhx2 in adult female livers. Microarray data from the Lusis lab indicated that Elovl3 levels were reduced in the livers of BALB/cJ mice compared to mice that have the wild-type Zhx2 gene. To validate this data, adult liver Elovl3 mRNA levels were compared between female BALB/cJ and BALB/c mice. As expected, the Zhx2 mRNA levels were substantially higher in BALB/c livers than in BALB/cJ livers (Fig.12A). Difference in the steady-state Elovl3 mRNA levels between BALB/c and BALB/cJ livers indicates that this gene is a target of Zhx2 (Fig. 12B). This pattern was somewhat surprising in that higher Elovl3 levels were observed in mice with the wild-type Zhx2 gene; other known targets of Zhx2 are lower in the presence of Zhx2 [71, 80]. This data suggests that Elovl3 is positively regulated by Zhx2.

Elovl3 levels are not controlled by Zhx2 in adult male livers, brown adipose tissue and skin. Anzulovich, *et al*. showed that Elovl3 levels exhibit sexual dimorphism, with substantially higher expression in the male liver than in the female liver [111]. To confirm this data, adult liver Elovl3 mRNA levels were examined in male BALB/cJ and BALB/c mice. The

Elovl3 mRNA levels were more than 100-fold higher in male livers than in female livers (Fig.12B and Fig. 13A). Difference in the steady-state Elovl3 mRNA levels between male BALB/c and BALB/cJ livers was not observed (Fig. 13A). We also examined Elovl3 mRNA expression in brown adipose tissue (Fig 13B) and skin (Fig 13C), and did not detect differences between BALB/c and BALB/cJ. This data suggests that Elovl3 is not regulated by Zhx2 in male livers, brown adipose tissue and skin.

Other Elovl family members are not targets of Zhx2. Elovl3 belongs to a family of elongases that include 6 other members. We examined whether other Elovl family members are also targets of Zhx2. To accomplish this, mRNA levels were compared between female BALB/cJ and BALB/c mice. RNA was prepared from adult livers and analyzed by real-time RT-PCR. No differences in Elovl1, Elovl2, Elovl5 and Elovl6 mRNA levels between Zhx2- and Zhx2+ mice were found (Fig 14). Expression of Elovl4 and Elovl7 were extremely low in the liver, which is consistent with rat studies, and could not be measured [107].

Elovl3 expression increases during liver development. The control of Elovl3 by Zhx2 led us to consider other aspects of Elol3 regulation. Since other Zhx2 targets are silenced after birth, postnatal Elovl3 levels were examined by real-time RT-PCR using RNA from what B6C3F1 (wild-type Zhx2) female mouse livers obtained at embryonic day 18 (e18) and postnatal day 1 (d1), d7, d28, d56. This indicated that hepatic Elovl3 steady-state mRNA levels increased roughly 5-fold between e18 and p56 (Fig. 15B). Although the extent of Zhx2 induction during

this perinatal period was more dramatic than that of Elovl3 (Fig. 15A), the two genes showed similar temporal patterns of induction in the liver after birth.

Elovl3 is regulated by Afr2. Although AFP, H19 and Gpc3 are silenced in the liver after birth and remain repressed in the normal adult liver, this silencing is reversible because both genes are transiently reactivated during liver regeneration. Strain-specific differences in the degree of AFP reactivation identified a second postnatal regulator of these genes called Alpha-fetoprotein regulator 2 (Afr2). Mice with the $Afr2^a$ allele, found in most strains of mice, including C3H/HeJ, exhibit higher AFP mRNA levels during liver regeneration than mice containing the rare $Afr2^b$ allele (C57BL/6). While some studies indicate that the Afr^b allele is dominant over $Afr1^a$, other studies suggest that these two alleles are co-dominant [50]. Since every target of Zhx2 is regulated by Afr2, we tested whether Elovl3 would also be regulated by Afr2. Liver regeneration was initiated in C3H/HeJ ($Afr2^a$) and C57BL/6 ($Afr2^b$) mice by a single intraperitoneal injection of the hepatotoxin CCl_4 in mineral oil (control mice received an injection of mineral oil alone). The livers were removed after 72 hours, a time in which AFP mRNA levels are highest during the regenerative period. Liver RNA was prepared, and both AFP and Elovl3 levels were analyzed using real-time RT-PCR (Fig. 14). As expected, AFP was highly induced by CCl_4 in C3H/HeJ mice and this induction was dramatically reduced in C57BL/6 mice (Fig. 16A). In contrast, Elovl3 was repressed during regeneration, and the CCl_4 repression was greater in C3H/HeJ mice than in C57BL/6J mice (Fig. 16B). Thus, Elovl3 is also a target of Afr2-mediated regulation in the regenerating adult liver but with a pattern that is opposite of other Afr2 targets.

Elovl3 expression is repressed in fibrotic livers. Whereas liver damage can occur transiently after a single treatment with the hepatotoxin CCl_4, long-term treatment results in liver fibrosis. To test whether Elovl3 expression was altered during liver fibrosis, 6-week old BALB/cJ male mice were treated with CCl_4 twice weekly intraperitoneally (2.5 uL per gram body weight as a 1:4 mixture with mineral oil) for 6 weeks. As controls, age-matched mice were treated with mineral oil alone. At the end the six weeks, mice were sacrificed. Part of the liver was sectioned for H&E staining to confirm the development of liver fibrosis. Mice treated with CCl_4 (Fig 17B), but not mineral oil (Fig 17A), developed fibrosis. Part of the liver was used for RNA preparation. AFP and the Elovl3 mRNA levels were analyzed with real-time RT-PCR. These data revealed that AFP was highly induced in fibrotic livers (Fig. 17C). In contrast, Elovl3 was repressed in fibrotic livers (Fig. 17D).

Elovl3 expression is repressed in response to a high fat diet. We previously showed that the loss of Zhx2 in the livers of BALB/cJ mice could be complemented, as judged by AFP and H19 mRNA levels, by the overexpression of a Zhx2 transgene [80]. In these mice, a Zhx2 cDNA was driven by a TTR enhancer/promoter expression cassette (TTR-Zhx2); this TTR cassette results in expression of Zhx2 in the liver that is similar to levels found in wild-type mice (L. Turcios, data not shown). To confirm that Zhx2 regulates Elovl3, we tested whether the Elovl3 levels were responsive to the TTR-Zhx2 transgene (Fig.18). To accomplish this, TTR-Zhx2 transgenic (on a B6C3F1 background) mice were crossed to BALB/cJ mice; the resulting TTR-Zhx2 positive F1 mice were backcrossed to BALB/cJ. The resulting F2 offspring were

genotyped for the endogenous Zhx2 allele ($Zhx2^{Afr1/Afr1}$or $Zhx2^{Afr1/+}$) and the TTR-Zhx2 transgene. As expected, the presence of the Zhx2 transgene repressed AFP expression (Fig. 18A, low fat diet). In agreement with the previous data (Fig. 12B), the Elovl3 mRNA levels were higher in mice with Zhx2 transgene (Fig. 18B, low fat diet), demonstrating that transgene-derived Zhx2 could lead to increased Elovl3 mRNA levels.

BALB/cJ mice and BALB/cJ mice with Zhx2 transgene (BALB/cJ+Zhx2) were also placed on a high fat diet for 8 weeks to study the influence of high fat diet on Elovl3 expression. At the end of the 8-week period, livers samples were obtained. The mice on the high fat diet had fatty livers (E.Clinkenbeard and B. T. Spear, manuscript in preparation). It was found that hepatic AFP levels were activated in BALB/cJ mice, but not BALB/cJ mice with Zhx2 transgene on a high fat diet, compared to on a low fat diet (Fig. 18A). In contrast, hepatic Elovl3 levels were substantially repressed in BALB/cJ mice with Zhx2 transgene, but not BALB/cJ mice on a high fat diet, compared to on a low fat diet (Fig. 18B). Similar results were seen when the experiment was performed with BALB/c mice and BALB/cJ mice (data not shown). These data suggest that Elovl3 expression is repressed in response to high fat diet induced hepatic stress in the presence of Zhx2.

Elovl3 can alter cell cycle progression. The Zhx2 targets AFP, H19 and Gpc3 expression are frequently reactivated during liver cancer development. Since Elovl3 is also a target of Zhx2, we were interested in Elovl3 expression in liver tumors. Microarray data from Dr. Mark Hoenerhoff's lab showed that Elovl3 expression was reduced in spontaneous HCC,

compared to normal non-tumor livers, with the highest fold reduction among all genes identified [25]. The dramatic reduction in Elovl3 levels in tumors led us to consider whether Elovl3 might contribute to cell proliferation. To test this, we performed cell cycle analysis in HEK293 cells that were transfected with Elovl3 expression vectors. Briefly, cells were transfected with an Elovl3 expression vector or with an empty vector (pcDNA3.1, EV) along with GFP. Cells were synchronized by serum starvation for 24 hours, followed by the addition of 10% FBS. Twelve hours later, cells were harvested, stained with propidium iodide (PI), and analyzed by FACS (Fig. 19). Cells were gated for GFP expression (to restrict analysis to transfected cells) and monitored for DNA content by PI analysis. These data indicated that the Elovl3 overexpression led to an increase in the percentage of cells in S phase and decrease in the percentage of cells in the G2 phase of the cell cycle when compared to EV-transfected controls. This data suggests that Elovl3 can alter cell cycle progression.

Discussion

The regulation of Elovl3 in the liver is not well understood. The data presented here identified Zhx2 as a factor that controls hepatic Elovl3 mRNA levels. This was shown by the low Elovl3 expression in the adult BALB/cJ liver, high Elovl3 expression in the adult BALB/c liver, and by the ability of a Zhx2 transgene to activate Elovl3 in a BALB/cJ background. It is intriguing that the three known targets of Zhx2 repression, AFP, H19, and Gpc3 are commonly reactivated in HCC, and the fourth target, Elovl3 is repressed in HCC. Although our study did not test whether Zhx2 is involved in the loss of Elovl3 expression in HCC, it has been shown in

one study that nuclear localization of Zhx2 is reduced in human HCC [88]. It is possible that the loss of nuclear Zhx2 in HCC would reduce Elovl3 expression in tumors. There are two additional studies regarding Zhx2 in HCC: one study suggested that Zhx2 was silenced in liver cancer, whereas another study saw increased Zhx2 in HCC [147, 148]. More studies will be needed to resolve these conflicting data.

When maintained on a high fat diet, BALB/cJ mice (which contain a mutated Zhx2 gene) that express a liver-specific Zhx2 transgene exhibit significantly elevated plasma cholesterol and TG levels than non-transgenic BALB/cJ mice. This data shows that Zhx2 is an important regulator of lipid metabolism in the liver [98]. Elovl3 is involved in the synthesis of C20–C24 saturated and monounsaturated very-long-chain fatty acids (VLCFAs). It has been shown that ablation of Elovl3 significantly reduced the VLDL triglyceride level in serum [103]. In BALB/cJ mice, reduced Zhx2 levels leads to low Elovl3 levels, which is associated with reduced serum VLDL triglyceride level. Therefore, the effects of Zhx2 in CVD may be due, at least in part, through its regulation of hepatic Elovl3 level.

The presence of homeodomains and zinc fingers in Zhx2 suggests that it functions as a DNA-binding protein, although the consensus DNA sequences bound by any Zhx proteins have not been identified [82]. Previous data from our laboratory showed that the 250 bp AFP promoter was sufficient to confer Zhx2 control to a linked transgene, indicating that the cis-acting site required for AFP regulation resides in this 250 bp region [66]. However, the specific motif required for Zhx2 control has not been identified. Furthermore, it has been shown that Zhx2 also

acts at the posttranscriptional level to regulate steady-state AFP and H19 mRNA levels [149]. It will be of interest to determine whether Elovl3 is also regulated through posttranscriptional mechanism.

The difference in AFP and H19 mRNA levels between BALB/cJ and other mouse strains is seen only in the adult liver; furthermore, the AFP and H19 mRNA levels in the gut and muscle are the same in these different mouse strains [68, 69, 150]. The Gpc3 mRNA levels are the same in the hearts, lungs, spleens, and kidneys of adult BALB/cJ and C3H/HeJ mice [71]. The adult Elovl3 mRNA levels are also the same in the skin, brown adipose tissue, white adipose tissue of BALB/cJ and BALB/c mice. The liver specificity of the BALB/cJ phenotype is curious, since Zhx2 is ubiquitously expressed. The mechanisms account for the liver-specific Zhx2 phenotype is not clear. It is possible that Zhx2 interacts with other liver-specific factors to regulate target genes. Alternatively, other Zhx proteins can compensate for the reduction of Zhx2 in organs other than the liver [1].

We also show that Elovl3 is repressed in the regenerating liver after CCl_4 intoxication and that this reduction is controlled by the regulator Afr2. This result again demonstrates an interesting inverse correlation between Elovl3 and AFP/H19/Gpc3 regulation. Although the product of Afr2 has not yet been identified [70], this is another factor that regulates Elovl3 expression. It will be interesting to examine Elovl3 expression using the partial hepatectomy models of liver regeneration. Because there is potential for Elovl3 to be used as a biomarker for fatty liver, fibrotic liver and liver tumors, it will be important to evaluate whether

Elovl3 levels decrease during liver injury in humans. It is curious that all the known targets of Zhx2 are also regulated by Afr2. The mechanism for this correlation is not clear. It is possible that Zhx2 and Afr2 have the same direct target which regulates AFP/H19/Gpc3 and Elovl3.

In summary, we have identified Zhx2 as a regulator of Elovl3 in the adult liver. Further studies will be needed to determine whether Zhx2 is involved in Elovl3 repression in HCC. We also have shown that Elovl3 is repressed in the regenerating liver after a CCl_4 treatment and that this induction is controlled by Afr2. Elovl3 is the fourth known target of Zhx2, but the first that appears to be regulated in a positive manner. Microarray analysis using $Zhx2^{+/+}$ and $Zhx2^{-/-}$ livers, ChlP-CHIP experiments will help to identify additional targets and elucidate further the mechanism of Zhx2 in the control of hepatic gene expression during liver development and in liver disease.

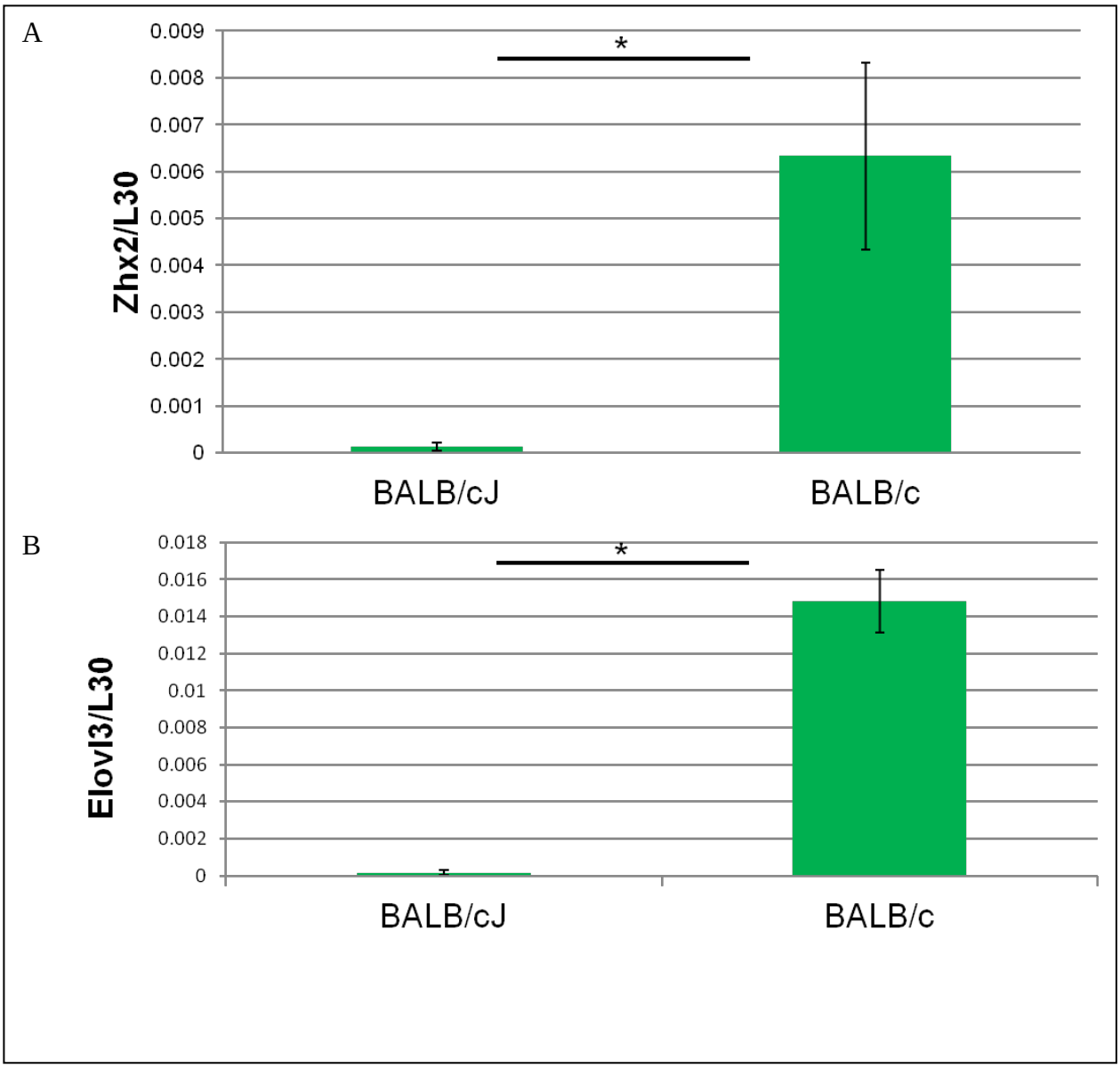
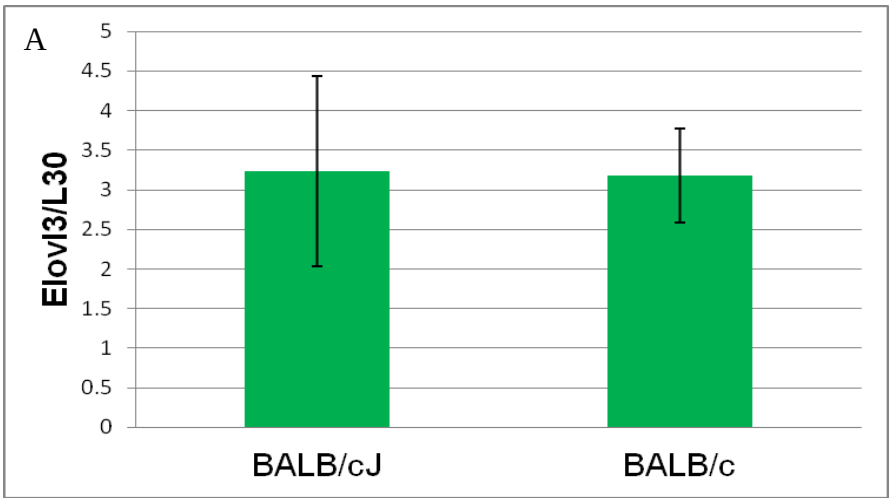


Figure 12. Elovl3 is regulated by Zhx2 in the adult female liver

Livers were removed from female BALB/cJ (n=4) and BALB/c (n=5) mice. Mice were fasted overnight before sacrifice. The livers were removed at the same time of the day. RNA was prepared and analyzed with real-time RT-PCR. (A) Zhx2, (B) Elovl3 mRNA levels were normalized to L30. * $p<0.05$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Elovl3/L30
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BALB/cJ
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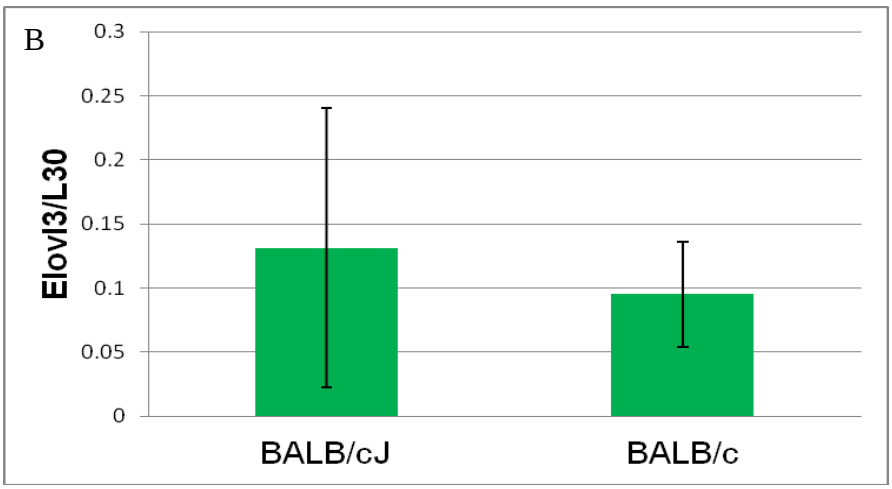
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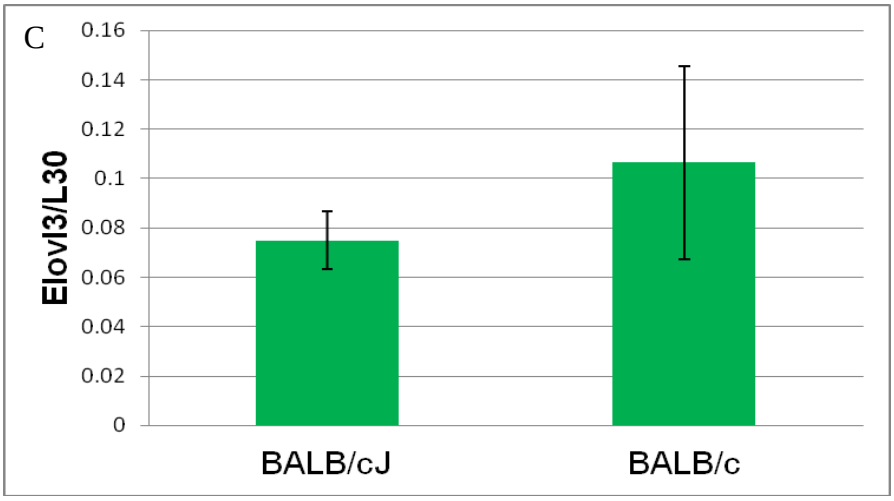
C
Elovl3/L30
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BALB/cJ
BALB/c

Figure 13. Regulation of Elovl3 by Zhx2 is not observed in the adult male liver, brown adipose tissure or skin

Livers (A), brown adipose tissue (B) and skin (C) were removed from male BALB/cJ (n=3) and BALB/c (n=3) mice. Mice were fasted overnight before sacrifice. The livers were removed at the same time of the day. RNA was prepared and analyzed with real-time RT-PCR. Elovl3 mRNA levels were normalized to L30.

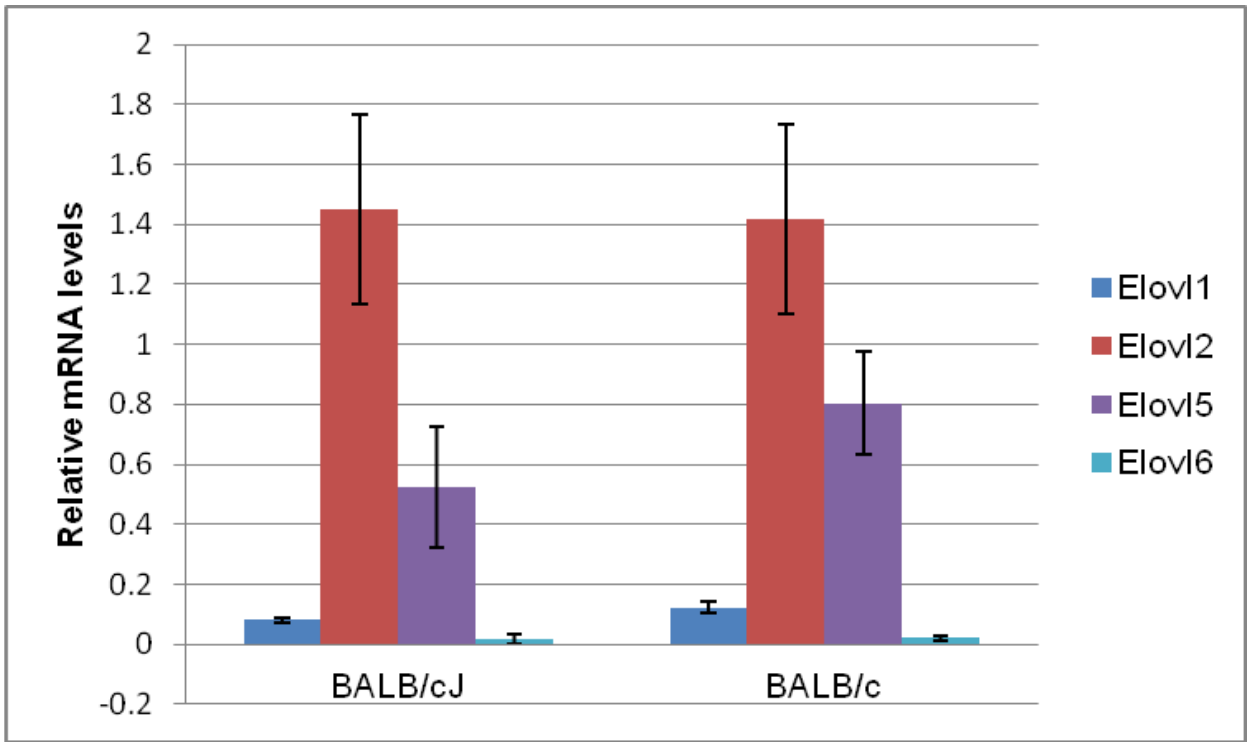

Figure 14. Other Elovl family members are not regulated by Zhx2.

Livers were removed from female BALB/cJ (n=4) and BALB/c (n=5) mice. Mice were fasted overnight before sacrifice. The livers were removed at the same time of the day. RNA was prepared and analyzed with real-time RT-PCR. Elovl1, Elovl2, Elovl5, Elovl6 mRNA levels were normalized to L30.

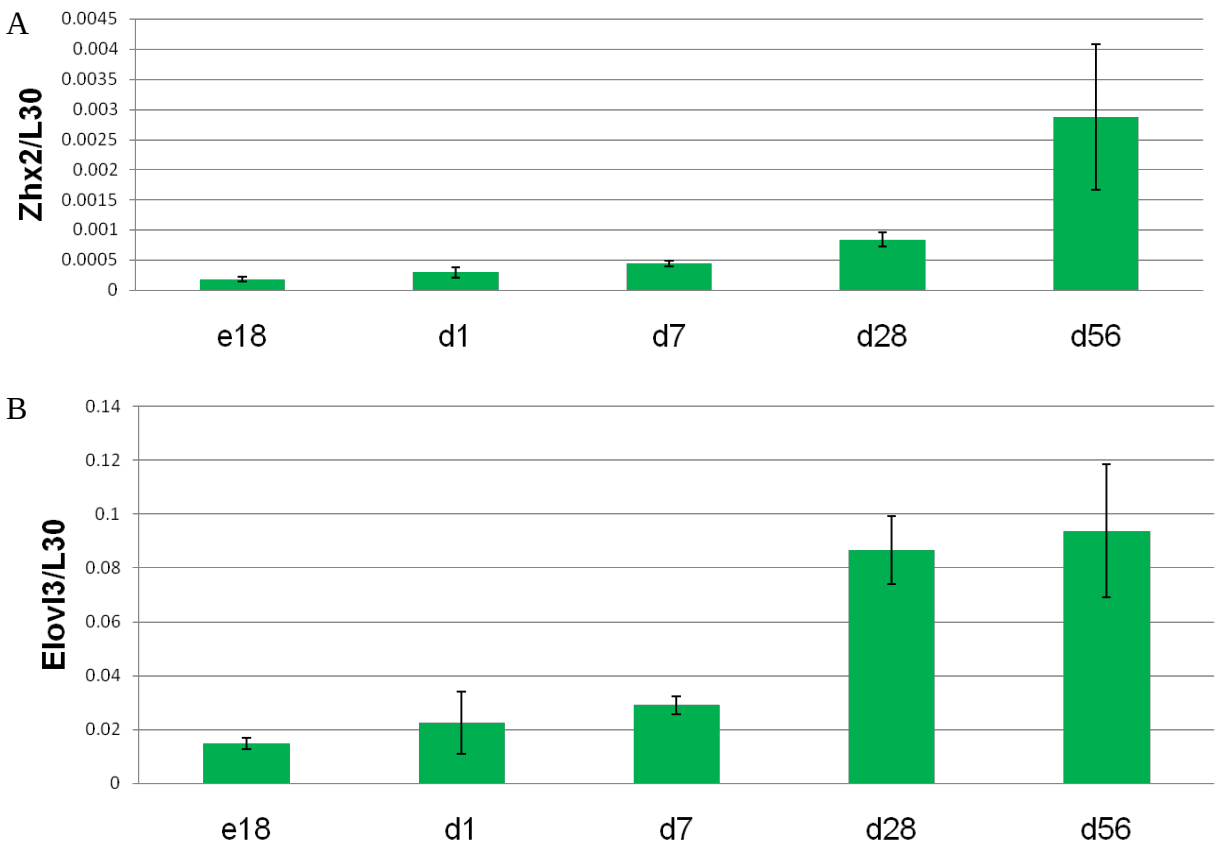

Figure 15. Elovl3 is developmentally activated in the perinatal liver

Livers were removed from female mice at e18, postnatal d1, d7, d28 and d56. The livers were removed at the same time of the day. Each group contained 3 mice. RNA was prepared and analyzed with real-time RT-PCR. (A)Zhx2, (B) Elovl3 mRNA levels were normalized to L30 which remained unchanged during this perinatal period in the liver samples.

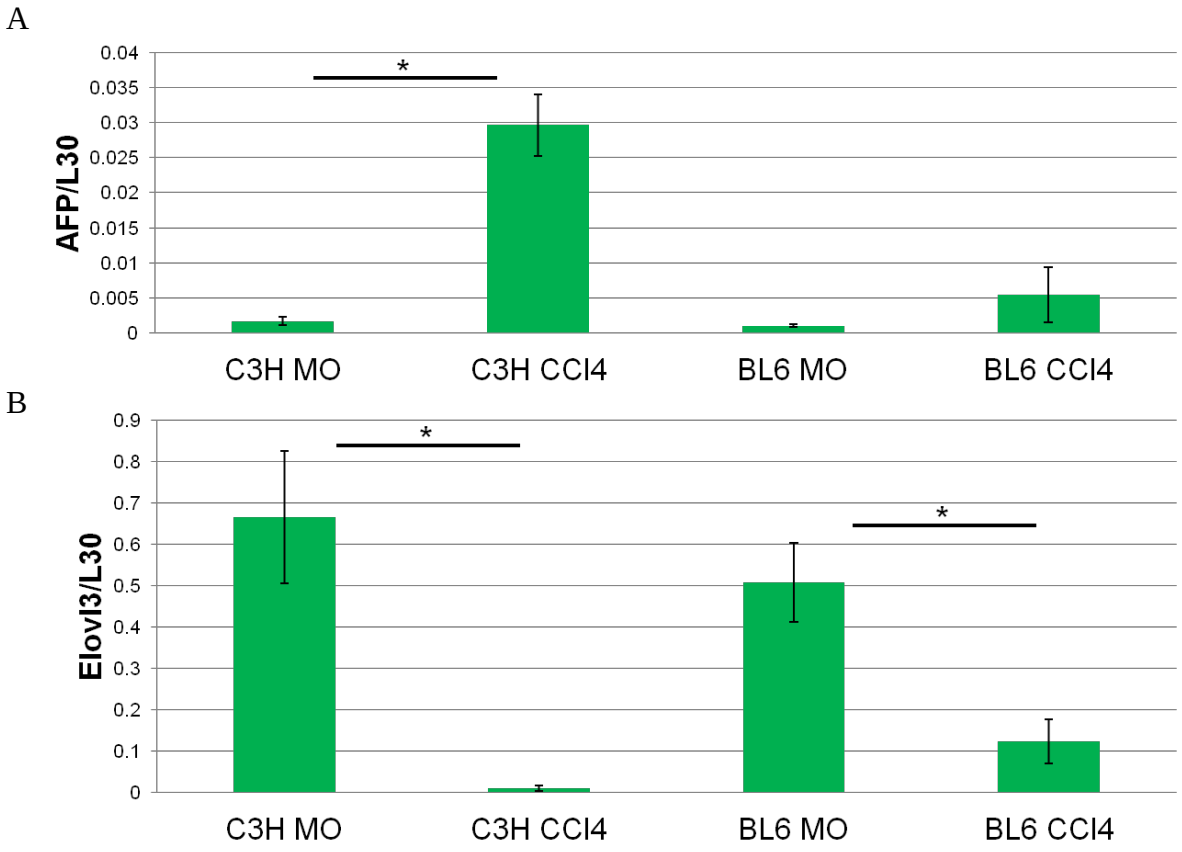

Figure 16. Elovl3 is repressed in the regenerating liver and controlled by Afr2

Age-matched adult male C57BL/6J ($Afr2^b$) and C3H/H3J ($Afr2^a$) mice were given a single intraperitoneal injection of 0.05 mL of mineral oil (MO) or 0.05 mL of 10% CCl_4 in MO. Each group contained 5 mice. After 3 days, the livers were removed, and RNA was prepared and analyzed by Real-time RT-PCR using primers for AFP, Elovl3 and L30. * $p<0.05$

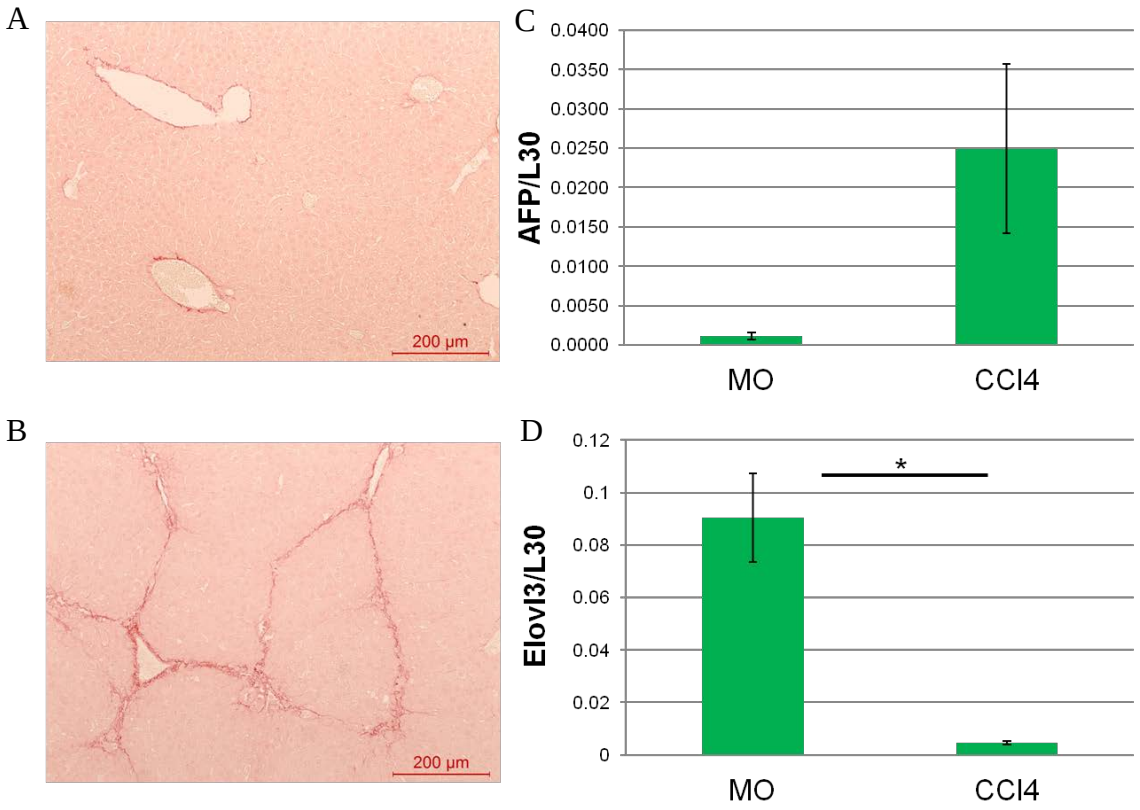

Figure 17. Elovl3 expression is repressed in fibrotic livers

6-week old BALB/cJ male mice were treated with CCl_4 twice weekly intraperitoneally (2.5 uL per gram body weight as a 1:4 mixture with mineral oil) for 6 weeks (n=3). As controls, age-matched mice were treated with mineral oil (n=4). At the end of 6 weeks, livers were removed. Mineral oil (A) CCl_4 (B) treated livers were sectioned for H&E staining, to examine the development of liver fibrosis. RNA was prepared from the livers and analyzed with real-time RT-PCR. AFP (C) and Elovl3 (D) mRNA levels were normalized to L30. Experiments in Fig. 17 were performed by Xin Lu. * $p<0.05$

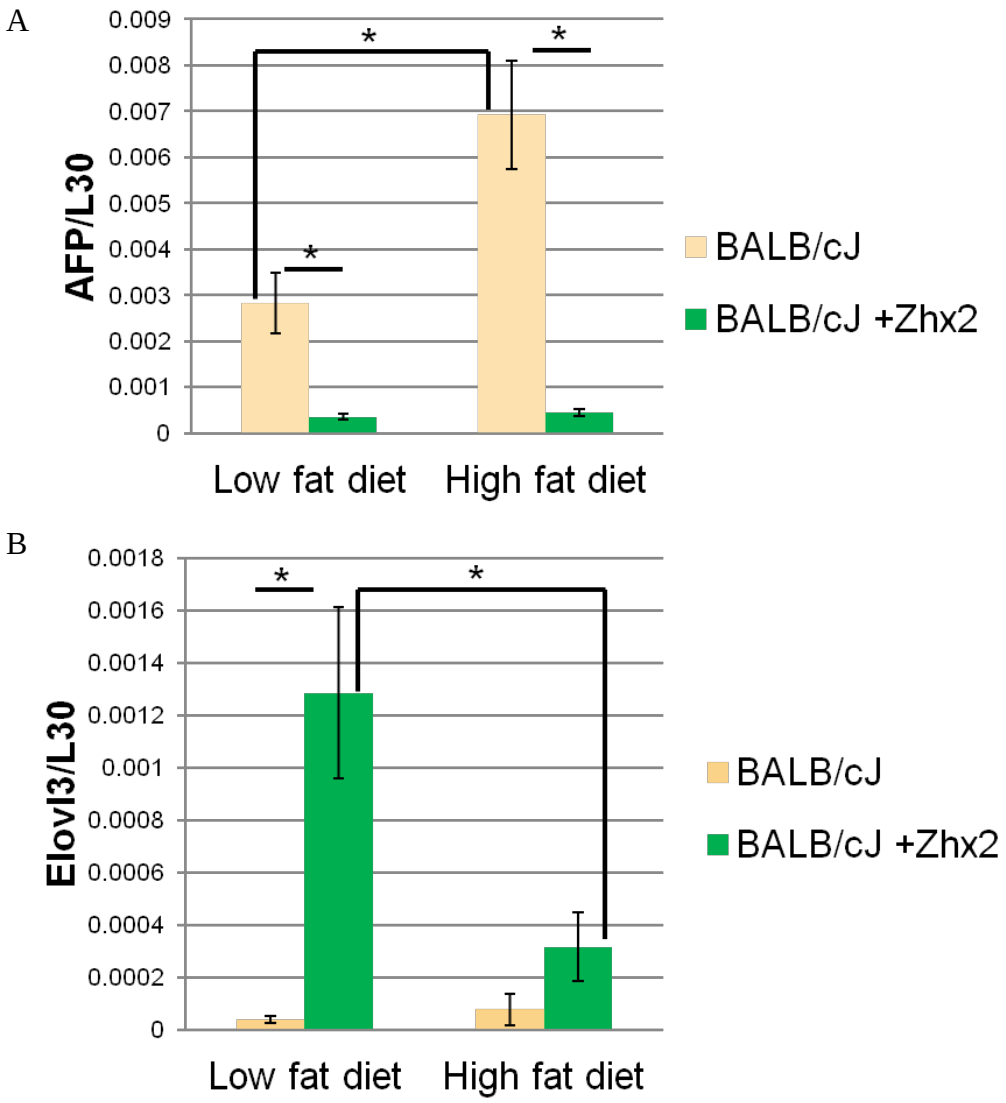

Figure 18. Elovl3 expression is repressed in response to high fat diet induced stress

Livers were removed from female BALB/cJ mice and BALB/cJ mice with TTR-Zhx2 transgene that were maintained on a normal chow diet or high fat diet for 8 weeks. Mice were fasted overnight before sacrifice. The livers were removed at the same time of the day. Each group contained 5 mice. RNA was prepared and analyzed with real-time RT-PCR. AFP and Elovl3 mRNA levels were normalized to L30. Experiments in Fig. 18 were performed by Erica Clinkenbeard. * $p<0.05$

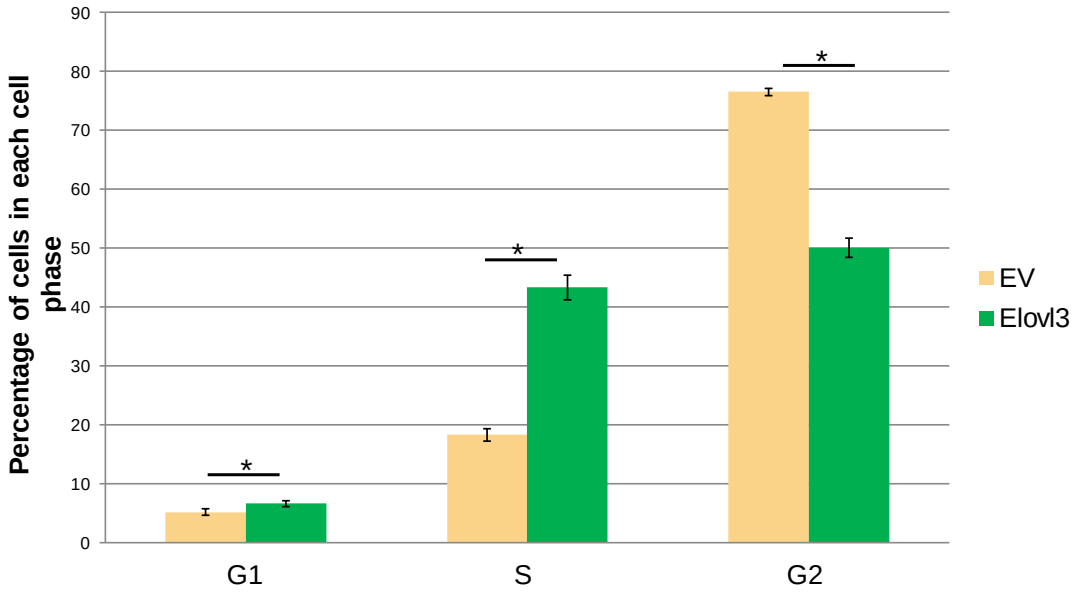

Figure 19. Elovl3 alters cell cycle progression

HEK 293 cells were transfected with either PcDNA3.1 or Elovl3 expression plasmids. Then cells were synchronized by serum starvation. Forty-eight hours after transfection, cells were harvested, stained by propidium iodide, and analyzed for cell cycle by FACS. Each bar represents data from three independent transfections. * $p<0.05$

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CHAPTER 5

Summary & Future Directions

Because of many of its unique properties, the liver is one of the best model systems for studying gene regulation. From developmental gene regulation, liver regeneration and diseases, the liver provides many different opportunities to study mechanisms of transcriptional regulation. AFP is expressed in the fetal liver, silenced at birth and reactivated in regenerating livers and HCC, thereby serving as an ideal gene to study to gain knowledge of transcriptional regulation. The cis-elements that control AFP transcription (three enhancers and promoter) have been well characterized in culture cells and transgenic mice. The 250 bp promoter contains binding sites for numerous factors, including HNF1, NFI, C/EBP, Nkx2.8, FTF and Zbtb20 [46, 49, 51, 59, 62, 141, 151-154]. Zhx2, another important regulator of AFP, was recently implicated in CVD [98]. While my work has provided further insights into transcriptional regulation of AFP on the 250 bp promoter and hepatic enzyme controlled by Zhx2 contributing to CVD, future studies will build upon these observations to better understand these mechanisms of regulation.

NFI. AFP is the most abundantly expressed gene in the fetal liver. It is repressed 10,000-fold at birth. A region that centered at -120 of AFP promoter contains overlapping binding site for HNF1 and NFI [50, 51]. A second HNF1 binding site is centered at -60 [50]. Binding sites for HNF1 are also found in the promoters of Alb, AFM and DBP, suggesting an essential role for HNF1 in liver-specific control of this gene family [33, 51-53]. However,

repression of gene expression by NFI is unique for AFP. Alb, AFM and DBP continue to express in the adult liver while AFP expression is shutoff at birth, indicating that NFI plays an important role in regulation. My studies presented here showed that NFIC and NFIX can repress AFP promoter activity in cultured cells. Furthermore, my data indicate that NFI and HNF1 cannot bind the AFP promoter at the same time. Therefore, NFI represses AFP expression by two mechanisms, active transcription repression and physical exclusion of HNF1 binding.

I also show that mutations that increase NFI binding but do not alter HNF1 binding diminish AFP promoter activity in cultured cells; mutations that decrease NFI binding and increase HNF1 binding enhance AFP promoter activity in cultured cells and in the liver. In addition, postnatal AFP repression is delayed in NFIX knock-out mice. The NFI family is comprised of four members, NFIA, NFIB, NFIC and NFIX. It is possible that the AFP repression is only delayed in NFIX knock-out mice, and not completely abolished, because other members compensate for the function of NFIX. Analysis of AFP expression profile during development in NFIC and NFIX double knock-out mice will further elucidate the role NFI plays in AFP repression. Based on my tissue culture data, I would predict that AFP repression will be further relieved in NFIC and NFIX double knock-out mice, compared to NFIX knock-out mice.

NFI, Zbtb20, and P53 are known transcriptional repressors of AFP that directly bind to AFP regulatory region. Zbtb20 ablation in liver led to dramatic derepression of the AFP gene in the liver throughout adult life [62]. AFP repression is slightly delayed in p53-deficient mice [65]. Zhx2 is a repressor of AFP that does not bind AFP promoter directly, as judged by EMSAs (data

not shown). BALB/cJ mice, which have mutated Zhx2 gene, were found to have 5- to 20-fold higher adult serum AFP levels compared with all other mouse strains. Since NFI, Zbtb20 and Zhx2 all act through the AFP promoter region[62, 66], future studies should test whether these factors interact with each other or work cooperatively to repress AFP promoter activity at birth. Co-immunoprecipitation experiment in cultured cells and the adult liver can be performed to test the interaction of those proteins.

AFP repression is reversible since the gene is activated during liver regeneration and HCC. Maybe repression and reactivation are mechanistically linked. It is of interest to consider whether binding of NFI, Zbtb20 and P53 to the promoter is lost during AFP reactivation. EMSAs and ChIP experiments can be done to examine the binding of the proteins to the AFP promoter. It is possible that during AFP reactivation, the balance of repressors and activators shifts again: HNF1 replaces NFI; Foxa replaces P53; Zbtb20 may also be displaced from the promoter despite the fact that it is expressed abundantly in the adult liver.

Zhx2. Zhx2 is a repressor of AFP, H19 and Gpc3 expression that silences these genes at birth in the liver; the reduction of Zhx2 levels in mice leads to persistent AFP, H19 and Gpc3 expression in the adult liver [71, 80]. Our lab and the Lusis lab have shown that Zhx2 regulates cholesterol and TG metabolism in mice and is a genetic risk factor for CVD [98]. My study presented here identified another target of Zhx2, Elovl3. It has been reported that ablation of Elovl3 leads to significantly reduced serum VLDL triglyceride [103]. BALB/cJ mice, which have a natural mutation in Zhx2 gene, have low serum triglyceride and are less susceptible to

atherosclerosis [96], compared to other stains of mice. Because Zhx2 positively regulate Elovl3, BALB/cJ mice have low Elovl3 level. It is possible that low serum triglyceride level in BALB/cJ mice is due to low Elovl3 level. Therefore, the effects of Zhx2 in CVD are likely achieved, at least partly, through its regulation of hepatic Elovl3 levels.

To further understand the mechanism how Zhx2 contributes to CVD, identifying more Zhx2 targets in the liver is important. We have microarray data comparing hepatic gene expression between $Zhx2^{+}$ and $Zhx2^{Afr1}$ mice. This data has identified a number of potential Zhx2 targets, Elovl3 is one of which I have validated. Our lab is in the process of breeding liver-specific $Zhx2^{-/-}$ mice. It is of interest to determine hepatic expression of Zhx2 as well as Zhx2 target genes AFP, H19, Gpc3 and Elovl3. It is important to compare the levels of these targets with age-matched BALB/cJ mice. This comparison will determine whether the complete absence of Zhx2 leads to a more dramatic effect on target gene expression, or is similar to what is seen in BALB/cJ mice which have the hypomorphic Zhx2 allele. If the complete absence of Zhx2 results in a more dramatic effect on target gene expression, it would be valuable to perform microarray analysis with liver RNAs from $Zhx2^{-/-}$ mice and $Zhx2^{+/+}$ littermates. Identifying additional Zhx2 targets will not only help us to understand its role in CVD, but also in HCC, since its known targets are all disregulated in HCC. It will also be important to identify common regulatory motifs that may be involved in Zhx2 binding or posttranscriptional control. Comparison of AFP, H19, Gpc3 and Elovl3 promoter region did not lead to a common regulatory motif (data not shown). Chip-CHIP experiments will identify cis-acting control regions to which Zhx2 binds.

Although Zhx2 and its target genes are expressed in multiple tissues, the regulation occurs only in the liver, as judged by comparison of target gene expression between BALB/cJ and BALB/c mice. Zhx2 is expressed at lower levels in the liver compared to other tissues. It might be that the loss of Zhx2 in the liver exerts a stronger effect because the basal level is low. Another interesting aspect is that AFP, H19, Gpc3 and Elovl3 levels are also very low in the adult liver. Take Elovl3 for example, Elovl3 is regulated by Zhx2 in the female liver but not male liver. Elovl3 is expressed at much higher levels in the adult male liver compared to adult female liver. Gpc3 also appears not to be regulated in tissues where it is more abundantly expressed [71]. Whole body Zhx2 knock-out mice will help to elucidate the mechanism. If AFP, H19, Gpc3 and Elovl3 are still only regulated in the liver, as judged by comparison between $Zhx2^{-/-}$ and $Zhx2^{+/+}$ mice, it might be that liver specific factors are involved in the regulation; or that Zhx1 and Zhx3 cannot compensate for Zhx2 function in the liver.

Afr2. Afr2 is the regulator of AFP during liver regeneration. Afr2 has been mapped to mouse Chromosome 2, however, the Afr2 gene has not been identified [70]. AFP, H19, Gpc3 and Elovl3 are the only known targets of Afr2. It is quite puzzling and interesting that all the known targets of Zhx2 are also regulated by Afr2. Zhx2 regulates gene expression during liver development, and is important for proper shutoff of AFP, H19 and Gpc3 as well as for proper induction of Elovl3. The 250 bp AFP promoter is sufficient to confer Zhx2 regulation[66]. Afr2 regulates target gene expression during liver regeneration. The region between -1,010 and -838 bp upstream of AFP promoter is required for Afr2 regulation [67]. Zhx2 does not regulate the expression of target genes during liver regeneration, neither does Afr2 regulate the expression of

target genes during liver development. It seems that Zhx2 and Afr2 respond to different upstream signals, however, have common downstream targets, which are direct regulators of AFP, H19, Gpc3 and Elovl3.

Our lab has performed microarray experiment with RNAs from C3H/HeJ and C57BL/6 mice. These mice were either given a single intraperitoneal injection of the hepatotoxin CCl_4 in mineral oil or mineral oil alone. The livers were removed 72 hours after the injection and RNA were extracted. Analysis of the microarray data and identifying signaling pathways will be crucial to understand the mechanism by which Afr2 regulates gene expression. Since we also have microarray data comparing hepatic gene expression between $Zhx2^{+}$ and $Zhx2^{Afr1}$ mice, finding common pathways might help to resolve the puzzle of same targets.

Although Afr2 has been mapped to a small interval on mouse chromosome 2 [70], Afr2 gene has not been identified. Mapping data from our lab contradicts published data regarding the position of Afr2. Because we do not know the nature or function of the Afr2-encoded protein, positional cloning is likely to be the best strategy to identify the Afr2 gene.

Zhx2 and Afr2 regulate AFP expression during liver development and liver regeneration, respectively. It is quite likely that there are genes regulating AFP expression during liver tumorgenesis. p53 might be one of them. p53 negatively regulates AFP promoter activity [64]. In addition, p53 negatively regulates H19 promoter activity [155]. It is also been reported

that p53 has inhibitory effect on H19 elevation upon hypoxia [156]. Future studies should examine p53 regulation of AFP, H19, Gpc3 and Elovl3 in HCC.

Elovl3. My study shows that Elovl3 expression is reduced in regenerating livers, fibrotic livers and fatty livers. Microarray data from Dr. Mark Hoenerhoff's lab shows that Elovl3 expression is reduced in spontaneous HCC [25]. Therefore, Elovl3 is a candidate marker for liver damage. Levels of reactive oxygen species (ROS) and inflammatory molecules are often associated with liver damage. Future studies should test whether ROS and inflammatory molecules regulate Elovl3 expression. I have generated a construct containing the 1000 bp Elovl3 promoter linked to the luciferase reporter gene [Elovl3(1000)-Luc]. Response of Elovl3 promoter to ROS and inflammatory molecules could be tested in cultured cells. If specific molecules have been identified, they could be further examined in mice.

Elovl3 is predominantly located in the endoplasmic reticulum. Therefore, another possible cause for reduced Elovl3 expression during liver damage is unfolded protein response (UPR) in ER. To test this, mice could be injected I. P. with tumicamycin, which inhibits protein glycosylation in the ER, or with vehicle alone. After 24 hours, livers will be removed and RNA analyzed. If Elovl3 indeed responds to UPR, tumicamycin injected mice should have lower Elovl3 expression.

The fold reduction of liver Elovl3 expression between BALB/c and BALB/cJ mice (Fig. 12B) is much greater than that between BALB/cJ with hepatic Zhx2 transgene and BALB/cJ

mice (Fig. 18B), leading to the possibility that the regulation of Elovl3 by Zhx2 occurs not only in hepatocytes. It will be interesting to test this regulation in Kupffer cells, given the role Kupffer cells play in inflammation. It is tempting to consider generating macrophage-specific Zhx2 knockout mice. The mice will not only be useful in testing the regulation of Elovl3 by Zhx2 in Kupffer cells, but also in elucidating the role Zhx2 plays in atherosclerosis.

Studies by Zadravec, *et al*. demonstrated that expression levels of lipogenic enzymes including FAS, DGAT2, and fatty acid transporter CD36 were significantly reduced in Elovl3$^{-/-}$ mice. Fsp27, which promotes fatty acid and triglyceride accumulation within lipid droplets, and PPARγ, a transcription factor that regulates Fsp27 expression, also displayed lower expression in Elovl3$^{-/-}$ mice. In addition, they found that female livers have lower levels of fatty acid C22:1n-9, compared to male livers, which is consistent with Elovl3 levels in female and male livers. C22:1n-9 is almost undetectable in the Elovl3$^{-/-}$ mice [103]. It is not clear how Elovl3 and VLCFAs affect lipogenic enzymes in liver. It seems that in the absence of certain VLCFAs species, lipogenesis is disturbed in the liver. My study identified the regulation of Elovl3 by Zhx2. Future studies should examine fatty acid composition in the livers of BALB/cJ and BALB/c mice, as well as Zhx2$^{-/-}$ mice and WT littermates. It is possible that fatty acid composition also contributes to the Zhx2 phenotype in CVD.

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Vita

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EDUCATION

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PUBLICATIONS

Liu, H., **H. Ren**, and B.T. Spear. The Mouse Alpha-Albumin (Afamin) Promoter Is Differentially Regulated by Hepatocyte Nuclear Factor 1alpha and Hepatocyte Nuclear Factor 1beta. DNA Cell Biol, 2011

POSTERS

April, 2011, Experimental Biology Meeting, Washington,D.C. Poster presentation entitled "Zhx2, a novel regulator of hepatic genes during liver development and disease" Hui Ren, Martha Peterson, Brett Spear.

March 22, 2011, Markey Cancer Center Research Day, Lexington, KY. Poster presentation entitled "Zhx2, a novel regulator of hepatic genes during liver development and disease" Hui Ren, Martha Peterson, Brett Spear.

Arpril 27, 2010, Experimental Biology Meeting, Anaheim, CA. Poster presentation entitled "Regulation of Mouse Alpha-fetoprotein Promoter Activity by HNF1 and NFI Binding". Hui Ren, Martha Peterson, Brett T. Spear

April 14, 2010, Markey Cancer Center Research Day, Lexington, KY. Poster presentation entitled "Regulation of Mouse Alpha-fetoprotein Promoter Activity by HNF1 and NFI Binding". Hui Ren, Martha Peterson, Brett T. Spear

PRESENTATIONS

March 25, 2011 – "Zhx2: A novel regulator of hepatic genes during liver development and disease" Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky

February 5, 2010 – "Circadian Clock Feedback Cycle through NAD Biosynthesis" Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky

April 17, 2009 – "Regulation of Mouse Alpha-fetoprotein Gene Expression by an HNF1/NFI Overlapping Site" Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky

October 12, 2007 – "RBP2, a Histone 3 Lysine 4 Demethylase, Regulates Gene Expression" Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky

AWARDS

7/1/2010-6/30/2012–American Heart Association Predoctoral Fellowship
Title of Project: Zhx2, a new gene for hyperlipidemia, post-transcriptionally regulating gene expression in a promoter dependent manner