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ZHX2 REGULATION OF LIPID METABOLISM AND THE BALANCE BETWEEN CARDIOVASCULAR AND HEPATIC HEALTH

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ZHX2 REGULATION OF LIPID METABOLISM AND THE BALANCE BETWEEN CARDIOVASCULAR AND HEPATIC HEALTH

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

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2015

ABSTRACT OF DISSERTATION

ZHX2 REGULATION OF LIPID METABOLISM AND THE BALANCE BETWEEN CARDIOVASCULAR AND HEPATIC HEALTH

The growing obesity epidemic in America carries with it numerous health risks, including diabetes, increased serum lipid levels, and excess fat accumulation in the liver. If these conditions persist or become exacerbated, they may lead to the development of cardiovascular disease, the current leading cause of death among Americans, or to nonalcoholic fatty liver disease (NAFLD) which can progress to hepatocellular carcinoma (HCC), one of the deadliest forms of cancer. Better understanding of the genes involved in these diseases can lead to improved identification of at-risk individuals and treatment strategies.

Our lab previously identified zinc fingers and homeoboxes 2 (Zhx2) as a regulator of hepatic gene expression. The BALB/cJ mouse strain has a hypomorphic mutation in the *Zhx2* gene, causing a 95% reduction in Zhx2 protein expression. The near ablation of Zhx2 in BALB/cJ mice confers protection from cardiovascular disease when fed a high fat diet, yet these mice show increased hepatic lipid accumulation and liver damage. Microarray data indicates Zhx2 may be involved in the regulation of numerous genes involved in lipid metabolism. Recent GWAS studies indicate ZHX2 may contribute to the risk of cardiovascular disease and liver damage in humans as well.

In this dissertation, I characterize the role of Zhx2 expression in the liver and how it affects the risk of both cardiovascular disease and liver damage. I generated liver-specific Zhx2 knockout mice and confirmed Zhx2 regulates several novel targets that could contribute to the fatty liver phenotype seen in BALB/cJ mice. Further studies revealed that hepatic Zhx2 expression is necessary for proper sex-specific expression of several Cyptochrome P450 (CYP) genes and could contribute to gender differences in disease susceptibility. Lastly, I performed studies into the functional role of the Zhx2 target gene Elovl3. A mouse model of HCC revealed that Elovl3 is completely repressed in HCC tumors. Cell viability and cell cycle assays indicate that Elovl3 expression slows cell proliferation and may be important for proper cell cycle checkpoints. Together, these data indicate that Zhx2 and/or its targets could be clinically

relevant in the detection, prevention, or treatment of cardiovascular disease, fatty liver, and HCC.

KEYWORDS: Zinc fingers and homeoboxes 2, gene regulation, lipid metabolism, cancer, cardiovascular disease

Kate Townsend Creasy

Student's Signature

May 8, 2015
Date

ZHX2 REGULATION OF LIPID METABOLISM AND THE BALANCE BETWEEN CARDIOVASCULAR AND HEPATIC HEALTH

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Chapter One

Introduction

The liver is a key metabolic organ in mammals and its proper functioning is necessary to maintain health. The liver is responsible for the metabolism of macronutrients from dietary intake, including maintaining serum glucose balance, synthesizing non-essential amino acids and proteins, regulating lipid utilization and storage, and transporting these molecules through the body (Figure 1). Additionally, the liver is main site for synthesis of cholesterol and steroid hormones, bile acids, and serum transport proteins (i.e. albumin). The diverse functions of the liver continue with its role in detoxification of xenobiotic compounds and the elimination of harmful or excessive byproducts (ammonia, cholesterol) from the body. Most of the described metabolic functions are performed by hepatocytes, the predominant cell type in the liver. Hepatocytes comprise 80% of the liver mass, but only about 60% of the actual cells within the liver. Other liver cell types include Kuppfer cell (resident macrophages) natural killer (NK) cells (also known as Pit cells), as well as hepatic stellate cells, which serve as Vitamin A stores in quiescent states but produce collagen upon liver damage and can lead to fibrosis [1]. The remaining liver cells are sinusoidal and biliary endothelial cells which form a cell barrier between hepatocytes and the blood and bile ducts, respectively [2]. As hepatocytes are the primary contributors to metabolic functions of the liver, they will be the focus of this dissertation.

Liver Lipid Metabolism

Hepatocytes in the liver are the main site of multiple aspects of lipid metabolism and homeostasis. Under fasting conditions, blood glucose levels are low which results in the pancreatic release of the peptide hormone glucagon. Glucagon binds to receptors expressed on hepatocytes to activate

glycogenolysis, the cleavage of glucose molecules from the stored form glycogen. If fasting is prolonged and glycogen stores are depleted, glucagon activates hormone sensitive lipase in adipose tissue to hydrolyze fatty acids for an energy source for most tissues. Fats released from adipose depots undergo beta-oxidation in mitochondria to produce two-carbon acetyl-CoA units that are substrates for the Citric Acid Cycle and ATP synthesis. Fatty acids can also be transported to the liver where they are converted to glucose through gluconeogenesis, and during extreme fasting, they are converted to ketone bodies as an alternate energy source.

In the fed state, short chain fatty acids (<C12), carbohydrates, and proteins are transported from the small intestine directly to the liver through the hepatic portal vein. Longer chain fatty acids, dietary cholesterol, and the fat soluble vitamins A, D, E, and K are packaged into triglyceride-rich chylomicrons that are transported through circulating blood to peripheral tissues. Interaction between apoCII in the chylomicron membrane and the cell surface enzyme lipoprotein lipase (Lpl) in peripheral tissues activates Lpl to hydrolyze dietary triglycerides into monoglycerol and two free fatty acids that can be transported into the cell. Chylomicron remnants or chylomicrons not utilized by peripheral tissues are transported through systemic circulation to the liver, where apoE binds hepatic receptors to deliver cholesterol, fat-soluble vitamins, and fatty acids for processing by hepatocytes. When dietary intake of carbohydrates and/or lipids is in excess of immediate physiologic need, hepatocytes convert them to triglycerides to be stored mostly in adipose tissue, but also to a lesser degree in the liver. Hepatocytes package triglycerides along with cholesterol in VLDL particles which are exported into the circulation, and again taken up by peripheral tissues by Lpl activation and hydrolysis. After triglycerides are deposited to other cells, the remaining cholesterol-containing particles are considered LDL and continue to circulate and transport cholesterol to peripheral tissues and back to the liver through receptor-mediated endocytosis. Hepatocytes synthesize apoAI and apoAII which are incorporated into the membrane of nascent HDL

lipoproteins that facilitate cholesterol uptake from peripheral tissues and transport it back to the liver, where it may be excreted as bile.

Hepatocytes respond to increased glucose availability and insulin signaling through coordinated transcriptional regulation of genes to increase the synthesis of fatty acids and triglycerides. One key event is insulin signaling to activate SREBP1c-mediated induction of lipogenesis in the liver, with resulting fatty acids and triglycerides then transported in VLDL particles to peripheral tissues or adipose for storage [3, 4]. The cellular sterol content regulates the activity of SREBP1a and SREBP2 to induce cholesterol biosynthesis [4]. Cholesterol is an essential cellular component and is incorporated into membranes with roles in signal transduction and transport of molecules. Cholesterol is the precursor to bile acids, which are required for the emulsification and absorption of lipids in the digestive tract. Cholesterol is also the precursor to steroid hormones, including the sex hormones testosterone, estrogen and progesterone [5]. Steroid hormone synthesis involves metabolism by the cytochrome P450 (CYP) family of enzymes. CYPs function as monooxygenases that are involved in both the synthesis and degradation of various compounds, including lipid products and steroids, Vitamin D, bilirubin, ethanol, drugs, and toxic byproducts from nutrient metabolism [6]. There are a large number of CYP enzymes, as each subgroup operates in response to specific stimuli. Due to the metabolism of sex hormones and other substances, many CYPs are expressed in a gender-specific manner. The regulation of sexually dimorphic CYP expression appears to be in response to growth hormone signaling [7, 8].

Disorders of lipid metabolism and health implications

Disruptions in the synthesis and storage of triglycerides can manifest in a variety of detrimental health concerns. A main cause of disregulated lipid homeostasis is diet induced obesity, which has quickly risen to epidemic proportions in the U.S., with current estimates of 34.9% of adults (78.6 million) classifying as obese [9]. The excess energy supply characteristic of obesity is

stored as triglycerides in adipose tissue, which can also accumulate in the liver [10]. Increased hepatic lipid accumulation has been associated with insulin resistance and Type 2 Diabetes, hyperlipidemia, atherosclerosis, hepatosteatosis, non-alcoholic fatty liver disease (NAFLD), and hepatocellular carcinoma (HCC) [10, 11].

NAFLD is a term used to describe the excessive presence of lipids in the liver that is not a result of alcohol consumption. NAFLD has several categories of manifestations with advancing disease states. Hepatic steatosis (fatty liver) is characterized as lipid droplet accumulation in hepatocytes and is the result of an imbalance in the triglyceride synthesis, transport, or utilization pathways [11]. Steatosis is a reversible condition and can be alleviated with dietary and activity modifications [12]. Unfortunately, steatosis is highly prevalent in people with obesity, type 2 diabetes, and metabolic syndrome, and often progresses to a more severe condition, hepatitis [13-15]. Hepatitis is an inflammatory condition marked by infiltration of immune cells and increased necrosis and apoptosis of damaged cells [15]. It has been strongly implicated that the inflammation associated with hepatitis can cause or exacerbate insulin resistance [11, 14, 16]. Hepatic inflammation can interfere with normal insulin response and glucose utilization, which then results in a metabolic switch to induce lipolysis, releasing triglycerides into the blood and causing hyperlipidemia. Dyslipidemia, elevated serum glucose, and obesity are key features of metabolic syndrome, which is a key risk factor in developing cardiovascular disease [17, 18]. There is growing evidence that NAFLD increases atherosclerotic plaque formation and elevates incidence of cardiovascular disease [13, 19, 20]. It is difficult to determine if NAFLD is an independent cause of atherosclerosis because it usually occurs in conjunction with other risk factors. Cardiovascular disease is the leading cause of death among Americans, and the number of people at risk is expected to continue to increase along with the rising obesity rate [21].

In addition to the cardiovascular implications of NAFLD, the inflammation and increasing damage to hepatocytes can result in fibrosis, the development of

scarring that interferes with normal liver function, and cirrhosis. Hepatic stellate cells become activated in response to increasing hepatocyte death. Stellate cells attempt to repair damaged tissue by expanding collagen fibers and extracellular matrix proteins around the injury site [15]. Prolonged damage increases the amount of collagen produced and results in cirrhosis. Cirrhosis is considered end-stage liver disease and results in liver failure and death [22]. Between 5-30% of people with cirrhosis will progress to HCC, depending on the disease severity at diagnosis and other risk factors [23]. HCC is the fifth most common cancer and is the third leading cause of cancer deaths worldwide. The most common causes of HCC are viral infections (hepatitis B and C) and alcohol abuse [15]. Patients with these risk factors can undergo serum screening for elevated alpha-fetoprotein (AFP) and ultrasound imaging for HCC tumor detection, which are currently the best methods of identifying HCC. These methods are not highly specific or accurate since serum AFP can be elevated in response to any type of liver damage, and many people do not present with the traditional risk factors for HCC. In recent years, metabolic syndrome and associated NAFLD have become increasingly linked to HCC. HCC has a high mortality rate and very poor survival prognosis in part because there are few reliable diagnostic markers for HCC, which is an alarming realization when paired with a huge potential increase in people at risk due to obesity and metabolic syndrome. HCC is projected to be the third leading cause of cancer deaths in the United States by 2030 [24]. A better understanding of early events in the development of HCC and better detection methods is critical to preventing this anticipated surge in HCC related deaths.

Zinc Fingers and Homeoboxes 2 (Zhx2)

AFP was the first diagnosed oncofetal gene in that it is normally expressed during fetal life, silenced at birth, and reactivated in cancer [25]. Serum AFP levels is a widely used diagnostic marker to detect liver damage and possible HCC, so understanding its expression is of clinical interest. A screen of various mouse strains revealed very low AFP levels in adult serum [26]. However, the one exception was the BALB/cJ substrain, with serum AFP levels

roughly 20-fold higher than other mouse strains (Figure 2). The persistent AFP expression in BALB/cJ mice was characterized as a monogenic trait and mapped to mouse chromosome 15 [27]. Additional studies indicated that elevated serum AFP levels correlated with increased hepatic AFP mRNA levels, suggesting that difference was occurring at the level of gene regulation [26]. A molecular screen for other genes expressed similarly to AFP (high in fetal liver, silenced at birth, persistent expression in adult BALB/cJ liver) identified H19 as another Zhx2 target [28]. Our lab found that Glypican 3 (Gpc3) is silenced in the perinatal liver but expressed at higher levels in BALB/cJ liver [29], similarly to AFP and H19. Using positional cloning, our lab identified a mutation in the BALB/cJ *Zhx2* allele as being responsible for the persistent AFP and H19 expression in the adult liver of these mice [30]. For confirmation, overexpression of a liver-specific Zhx2 transgene in BALB/cJ mice resulted in normal AFP and H19 silencing after birth [30] (Figure 3). The mutation in BALB/cJ *Zhx2* allele is due to a Mouse Endogenous Retroviral (MERV) insertion element within the first *Zhx2* intron that dramatically reduces, but does not eliminate, Zhx2 expression [30, 31]. Thus, the *Zhx2* mutation in BALB/cJ mice is hypomorphic, resulting in Zhx2 mRNA levels that are roughly 5% of that found in strains with a wild-type *Zhx2* allele. These combined data suggest that Zhx2 functions to repress target genes in the perinatal liver, and the reduction in Zhx2 levels in BALB/cJ livers results in persistent expression of these genes.

Zhx2 is the member of a small family of highly conserved vertebrate-specific genes (Zhx1, Zhx2 and Zhx3). Each Zhx protein contains two amino-terminal C_2H_2 zinc finger domains, typically associated with DNA, RNA, and protein interactions, and four to five carboxy-terminal DNA-binding homeodomains, suggesting that these proteins function as transcriptional regulators [32]. Early studies indicate that ZHX proteins can homodimerize and heterodimerize with each other, and luciferase assays suggest that ZHX proteins function as transcriptional repressors [32, 33]. Chromatin immunoprecipitation (ChIP) and luciferase reporter assays indicate that ZHX2 binds to and regulates the human AFP and Gpc3 promoters [34]. Taken together, these data are

consistent with our model of Zhx2 acting to repress AFP and Gpc3 expression in the adult liver. Further, ChIP analysis in human HCC cell lines indicate that ZHX2 binds to the promoter regions of *CCNA2* and *CCNE1*, the genes coding for Cyclins A and E, respectively, and inhibited their transcription [35]. However, other data suggests Zhx2 may regulate target gene expression at the posttranscriptional level (Martha Peterson, unpublished data, [36]). Additionally, as described in this dissertation, we have recently identified several novel Zhx2 targets, including Elovl3, Cyp2a4, and MUPs, that are regulated by Zhx2. These data indicate that Zhx2 is involved in both gene activation and repression.

Zhx2 targets in HCC and other diseases

The Zhx2 target AFP serves as a model for examining gene expression in fetal development and disease. AFP is expressed at very high levels in the fetal liver, repressed over 1000-fold shortly after birth, and becomes reactivated in liver damage [25, 37]. AFP is a serum transport protein that circulates lipids, steroids, and other substances through the body. Although it is commonly used as a diagnostic marker for liver damage and HCC, the functional relevance of its increased expression in liver disease is not clear. In humans, hereditary persistence of AFP (HPAFP) is considered a benign condition with no known health risks [38] Known cases of HPAFP have indicated a mutation in the hepatocyte nuclear factor-1 (HNF1) binding site in the AFP promoter results in the elevated AFP expression [39, 40]. Although the basis for persistent AFP expression in adult BALB/cJ mice is different than HPAFP in humans, they similarly do not appear to have any adverse consequences associated with increase serum AFP levels.

Since AFP is frequently reactivated in HCC, there is interest in whether other Zhx2 targets and Zhx2 itself are disregulated in HCC. Gpc3 has a similar expression pattern to AFP in that it is expressed in the fetal liver, repressed at birth, and remains silent in normal adult tissue [41]. Gpc3 is a heparin sulfate proteoglycan present on the cell surface and linked to the membrane by phosphatidylinositol [42]. Proteins in this category are associated with cell-cell

interactions and formation of extracellular matrix, and may integrate signals for cell growth and division. Humans with loss of function Gpc3 mutations suffer from Simpson-Golabi-Behmel Syndrome (SGBS), characterized by overgrowth in both prenatal and postnatal development, multiple body dysmorphisms, and frequent death in infancy [43]. Gpc3 knockout mice display many of these maladies, but studies in the mice have not elucidated the mechanism that causes SGBS [44]. Gpc3 is silenced in normal adult tissues but becomes elevated in HCC, making it another candidate HCC diagnostic marker. In fact, some studies suggest Gpc3 is more specific for HCC than AFP and could be used for more accurate diagnosis [34]. Elevated serum Gpc3 is associated with poor prognosis in HCC patients [45] and increased Gpcs3 detected by IHC has over 95% predictive rate of pediatric hepatoblastoma [46]. Gpc3 may increase the metastatic properties of cells through ERK signaling [47]. Antibodies targeting Gpc3 inhibit Wnt signaling and reduce tumor xenografts in nude mice [48], so there is potential for HCC therapeutic treatment by regulating Gpc3.

H19 was the second Zhx2 target to be identified. H19 is a long non-coding RNA and serves as a model of parent-of-origin genomic imprinting [49]. The H19 transcript is processed into microRNA (miR) [50], and may also interact with other miRs and thus contributes to gene regulation [51]. H19 knockout mice appear to develop normally, yet overexpression of H19 in zygotes results in perinatal death [52]. The role of H19 in tumorigenesis has yet to be elucidated. Studies have reported increased H19 expression in pancreatic, ovarian, and bladder cancers [53-55]. Additional studies suggest that H19 is necessary for HCC tumor growth [56], and targeted interference of H19 inhibits cell proliferation and induces apoptosis [57]. In contrast, H19 has also been identified as a tumor suppressor in colorectal cancer and in HCC [58]. Based on these conflicting data, further analysis of the role of H19 in cancer is warranted. In addition to its involvement in cancer, polymorphisms in H19 has been associated with increased risk of coronary artery disease (CAD) in Chinese men [59]. The impact of the polymorphism is unknown, but altered H19 expression increased the risk and severity of CAD in the study subjects.

Lipoprotein lipase (Lpl) is expressed at high levels in the fetal liver, silenced at birth, continues to be expressed at elevated levels in adult BALB/cJ liver, and silenced in BALB/cJ mice expressing a liver-specific Zhx2 transgene (H. Ren, unpublished). This suggests that Lpl is also a target of Zhx2. Increased Lpl expression has been observed in cases of HCC as well as cervical squamous cell carcinoma, chronic lymphocytic leukemia, and non-small cell lung cancer [60-62]. Lpl facilitates the uptake of fatty acids into cells for energy production, which is necessary for the high metabolic requirements of tumorigenic cells [63]. Although its expression is altered in cancer, normal Lpl expression is necessary for fetal development and cardiovascular health in mature organisms. Lpl knockout mice suffer from severe hyperlipidemia and die shortly after birth due to blood saturation of chylomicrons and lack of energy in peripheral tissues [64]. Partial deletion mutations in human LPL are associated with elevated serum lipoproteins [65, 66]. Lpl overexpression in otherwise healthy animal models alters lipid metabolism, results in increased atherosclerosis and skeletal muscle damage due to lipotoxicity [67, 68], and may contribute to insulin resistance [69]. In mouse models of diabetes, increased transgenic Lpl expression has a protective effect and has been shown to reduce diabetic hyperlipidemia and lessen atherosclerotic lesions [70]. The cardioprotective effect may depend on tissue-specific expression, as reduced Lpl in vascular tissue is associated with less lipoprotein infiltration [71].

The disregulation of many Zhx2 targets in HCC leads to question if Zhx2 expression is also altered in liver cancer. Current data regarding Zhx2 expression in HCC tumors are conflicting. A study utilizing sequence screening of tumor and adjacent non-tumor tissue reported that CpG islands in the Zhx2 promoter were hypermethylated in HCC, resulting in reduced Zhx2 expression in tumors [72]. In contrast, another study analyzing tissue samples by immunohistochemistry (IHC) noted that Zhx2 protein was expressed only in HCC samples compared to cirrhotic liver, cholangitis samples, and normal adjacent tissue [73]. This second study also indicated that increased Zhx2 was associated with more advanced

stages of HCC. Dr. Chunhong Ma and our collaborators at Shandong University, China have found that ZHX2 overexpression reduced proliferation of HCC cells as well as reduced the growth of tumor xenografts in nude mice [35]. Further, they showed that the Zhx2 proteins were primarily in the nucleus in non-tumor liver tissue, but localized to the cytoplasm in HCC samples [35]. This data suggests that ZHX2 is excluded from the nucleus in HCC, effectively blocking its ability to control expression of target genes. More recently, our collaborators published data showing again reduced nuclear ZHX2 localization in HCC tumors and a resulting increase in Gpc3 expression in human patient samples [34]. In tumor samples from a mouse model of HCC, my analysis indicates increased mRNA levels of AFP, Gpc3 and Lpl in tumors compared to control liver samples, but no difference in total Zhx2 mRNA expression in normal and HCC samples (Figure 4). My analysis of mouse HCC samples indicates that Zhx2 accumulates in the cytoplasm and is excluded from the nucleus, consistent with published findings. The nuclear exclusion seen in these samples would likely cause the disregulation of target genes without necessarily altering the total Zhx2 mRNA or protein levels. The mechanism causing the altered Zhx2 localization and subsequent disregulation of target gene expression will provide great insight into the progression of liver damage and HCC, and may identify new therapeutic targets.

Zhx2 has also been implicated in the regulation of lipid metabolism genes and cardiovascular disease risk. Serum lipid analysis performed on various mouse strains found that on a high fat diet (HFD), BALB/cJ mice have reduced serum lipid levels and fewer atherosclerotic plaques than other strains. Quantitative trait locus (QTL) mapping identified this trait, known as *Hyperlipidemia2* (*Hylip2*), to a region on mouse chromosome 15 [74]. The Hylip2 phenotype results in hypertriglyceridemia due to reduced clearance of serum triglycerides. The BALB/cJ *Hyplip2* allele confers cardiovascular protection by increased triglyceride clearance and lower circulating lipid levels, resulting in fewer atherosclerotic lesions. In collaboration with the lab of Jake Lusis (UCLA), we identified Zhx2 as the gene responsible for the Hylip2 phenotype [75].

BALB/cJ mice expressing a liver-specific Zhx2 transgene exhibited significantly higher plasma cholesterol and triglycerides and increased atherosclerotic plaques compared to non-transgenic BALB/cJ littermates. Consistent with this mouse data, ZHX2 has been identified in two human GWAS studies with interactions in cardiovascular disease. GWAS analysis of over 31,000 patients in the CHARGE consortium identified single nucleotide polymorphisms (SNPs) in ZHX2 as the strongest predictive factor of lower carotid intima media thickness (cIMT), a subclinical marker of atherosclerosis [76]. A GWAS study analyzing data gathered on black and white males in the Bogalusa Heart Study found that a particular ZHX2 SNP was strongly associated with cIMT in white males [77]. It is not clear how the SNPs evaluated in the study affect the expression or function of ZHX2 in humans, but these data indicate ZHX2 has a role in the risk of developing cardiovascular disease in humans. The significance of Zhx2 involvement in serum lipid levels and cardiovascular disease risk is a new avenue of investigation for our lab. The Lusis lab performed microarray analysis in congenic mice expressing wild-type or the mutated BALB/cJ Zhx2 alleles. This analysis identified over 1000 genes that showed differences in expression in the presence or absence of Zhx2 and are therefore potential new targets of Zhx2.

My dissertation explores the effects of Zhx2 on the regulation of lipid metabolism gene expression and resulting disease risk. Many of these studies have utilized a novel mouse model I developed that utilized mice with a floxed allele of Zhx2 in the BL/6 background; by crossing these mice with Albumin-Cre transgenic mice, I have been able to delete Zhx2 specifically in hepatocytes. I have identified several novel Zhx2 targets and further developed the mechanism by which hepatic Zhx2 deletion increases hepatic lipid accumulation, resulting in lower serum triglycerides and lower risk of cardiovascular disease. I have identified the cytochrome P450 enzyme Cyp2a4 as a Zhx2 target, and discovered that Zhx2 regulates sexually dimorphic CYP gene expression in the adult liver. Additionally, I have characterized the regulation and function of the Zhx2 target Elongation of very long chain fatty acids-like 3 (Elovl3) in cell cycle regulation and HCC progression.

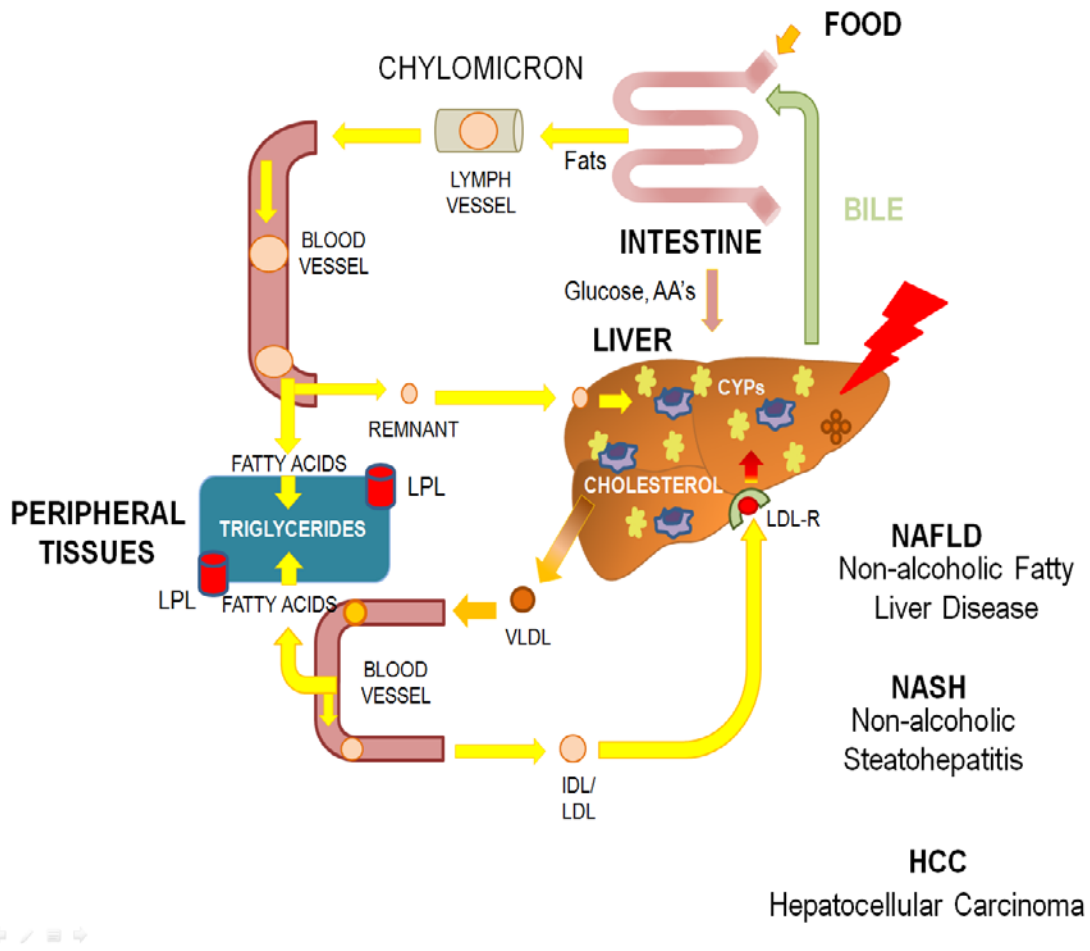


Figure 1. Overview of lipid metabolism in the liver. The liver is the main processing site of nutrient metabolites. The liver synthesizes numerous Cytochrome P450 (Cyp) enzymes which synthesize cholesterol and bile acids, as well as break down drugs, toxins, and other substances to be removed from the body. Bile secretion into the intestine is necessary for the absorption of dietary fats that are packaged into chylomicron particles. Lipoprotein lipase (Lpl) on the surface of peripheral tissues hydrolyzes triglycerides from chylomicrons for uptake into cells. Unused remnants travel back to the liver for processing. Cholesterol is exported from the liver in VLDL particles for delivery of substances to peripheral tissues, and unused particles are reabsorbed by the liver. In cases of excess dietary intake, fat can accumulate in the liver, causing non-alcoholic fatty liver disease (NAFLD). Continued damage resulting in inflammation and macrophage infiltration is characterized as non-alcoholic steatohepatitis (NASH). Prolonged damage to the liver increases the risk of hepatocellular carcinoma (HCC).

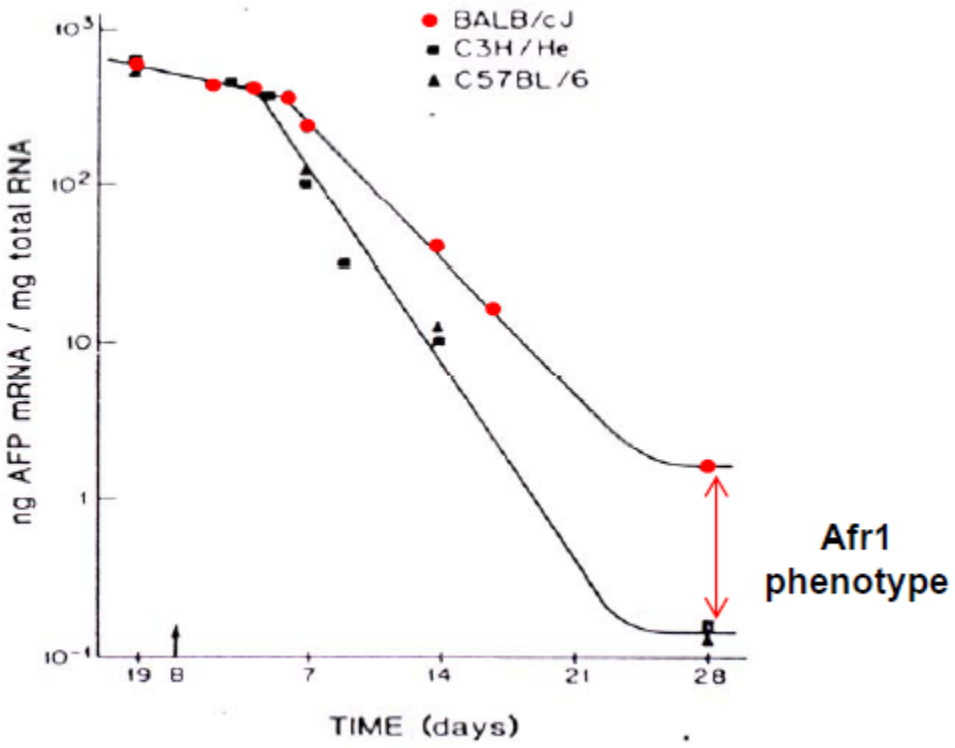


Figure 2. Postnatal AFP repression in inbred mouse strains. RNA was isolated from mouse livers at embryonic day 19 and periodically from birth to day 28. Mice have high AFP expression during fetal development which is repressed after birth. C3H and B6 mice have rapid and dramatic repression of AFP expression. BALB/cJ mice have delayed onset of repression that is incomplete at day 28. BALB/cJ mice continue to express AFP 10 to 20-fold higher than other mouse strains. Copyright 1982 Molecular and Cellular Biology.

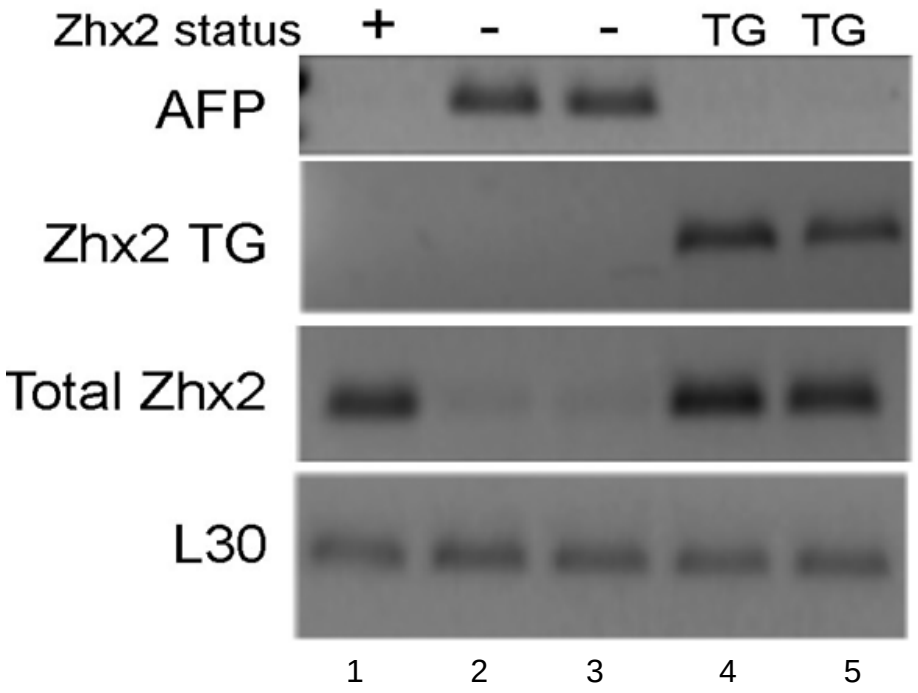


Figure 3. Hepatic Zhx2 transgene expression restores AFP repression. Mouse liver RNA amplified by RT-PCR to detect Zhx2, AFP, and Zhx2 transgene expression. BALB/c mice with wildtype Zhx2 expression (Lane 1) have postnatal AFP repression. BALB/cJ mice (Lanes 2-3) have greatly diminished Zhx2 expression and persistent AFP expression. BALB/cJ mice with liver-specific Zhx2 expression (Lanes 4-5) have elevated Zhx2 and restoration of AFP repression. These data confirm Zhx2 represses AFP in the mouse liver. L. Morford, unpublished data.

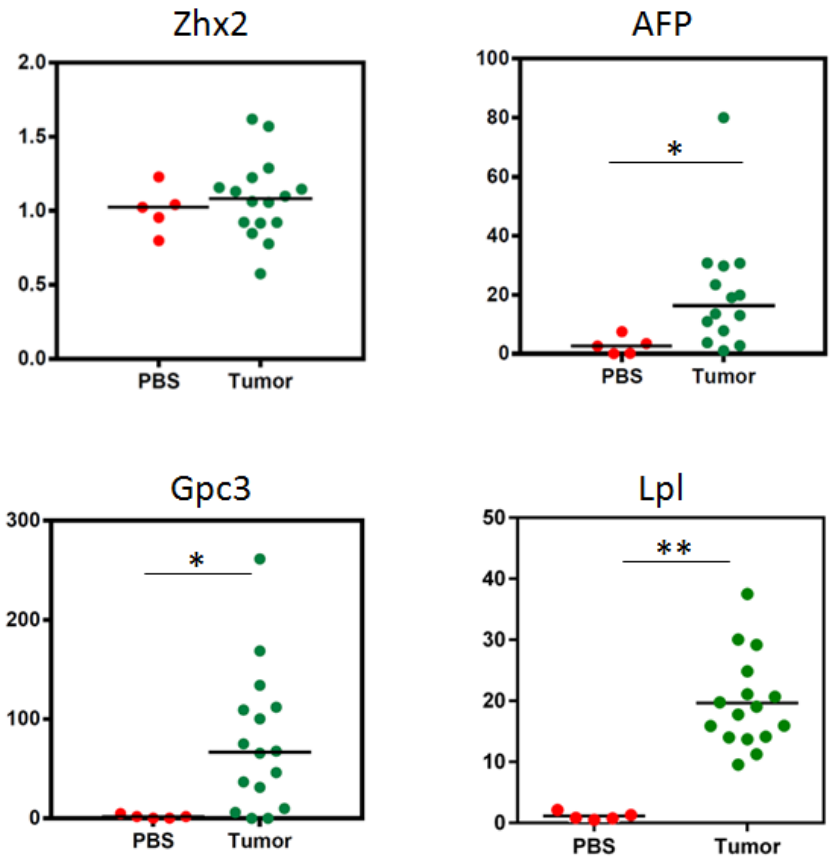


Figure 4. Zhx2 targets, but not Zhx2, are overexpressed in HCC tumors. Male C3H-B6 F1 offspring were injected with PBS (n=5) or DEN (n=16) to initiate liver tumor development in a mouse model of HCC. At 36 weeks post-injection, liver and tumor samples were collected and analyzed by qPCR for expression of Zhx2 (A), and its targets AFP (B), Gpc3 (C), and Lpl (D). AFP, Gpc3, and Lpl have increased mRNA expression in HCC tumors, but Zhx2 expression does not change. Values are normalized expression shown as fold change to controls. * $p<0.05$, ** $p<0.01$.

Chapter 2

Materials and Methods

Mice

All mice were housed in the University of Kentucky Division of Laboratory Animal Research (DLAR) facility and kept according to Institutional Animal Care and Use Committee (IACUC) approved protocols. All mice had *ad libitum* access to food and water and were maintained on a 12/12 hour light/dark cycle.

Generation of hepatocyte-specific Zhx2 knockout (*Zhx2*$^{\Delta hep}$) mice:

Breeding pairs of C57Bl/6 mice with a Zhx2 floxed allele (*Zhx2*^{fl}) were purchased from the Knockout Mouse Project Repository at the University of California-Davis. In these mice, exon 3 has been flanked by loxP sites. Since the entire Zhx2 coding region is found on exon 3, deletion of this exon results in the loss of the entire Zhx2 protein. These mice were crossed with C57/Bl6 mice expressing cre recombinase driven by the liver-specific Albumin promoter (Alb-Cre) (Jackson Labs). Mice were bred to achieve homozygous floxed Zhx2 alleles with Alb-Cre expression (*Zhx2*$^{\Delta hep}$), or without Alb-Cre (*Zhx2*$^{f/f}$) serving as littermate controls. Mice were maintained under normal conditions until 4-8 weeks old, then euthanized by CO_2 asphyxiation for tissue harvest.

Genotyping:

At approximately 10-days of age, each mouse pup was given an ear punch for identification and the tissue was collected. DNA was extracted by incubation in lysis buffer (100mM Tris-HCl pH 8.5, 5mM EDTA, 200mM NaCl) with 2.5 units proteinase K (Sigma) followed by isopropanol precipitation and washing with 95% ethanol. The DNA pellet was resuspended in water, then genotyped by PCR amplification using the appropriate primers (Table 1) with ThermoStart Master Mix according to manufacturer's protocol (ThermoScientific). Samples were analyzed by gel electrophoresis and visualized by a UV lightbox.

Murine model of atherosclerosis with hepatocyte specific Zhx2 knockout mice:
A breeding pair of *LDLr*$^{-/-}$ mice were purchased from Jackson Laboratory. After two weeks of acclimation, *LDLr*$^{-/-}$ mice were bred to *Zhx2*$^{f/f}$ and *Zhx2*$^{\Delta hep}$ mice. Offspring were genotyped and crossbred to obtain homozygous *LDLr*$^{-/-}$ and *Zhx2*$^{f/f}$ with and without Alb-Cre expression. *LDLr*$^{-/-}$, *Zhx2*$^{\Delta hep}$ and control *LDLr*$^{-/-}$, *Zhx2*$^{f/f}$ littermates were weaned onto a high fat, high cholesterol diet (TD.94059, Harlan Teklad; Appendix A) at 21 days old and were maintained on this diet for 18 weeks. Mice were monitored for weight gain every week, and serum collections were obtained every 4 weeks throughout the study.

Serum Collection:
Starting at the second week on the HFD study (mouse age 5 weeks old) and repeated every four weeks, serum collections were obtained by submandibular bleeding. Mice were fasted starting at 8:00am for five hours but had access to water. Mice were manually restrained and punched with a lancet (Goldenrod/Medinet) to penetrate the submandibular vein in the cheek. Blood was collected in a serum separator tube (~20-100ul blood per collection, depending on age of mouse) and incubated at room temperature for at least 30 minutes before centrifugation at 13000 xg rcf for 1 minute. Serum was aspirated by pipetting and transferred to a 1.5ml Eppendorf tube and stored at -80°C.

Euthanasia and Tissue Collection:
Mice were maintained on HFD for 18 weeks with weekly weight measurements and monthly serum collections. After the 18th week, mice were fasted beginning at 8:00am for five hours. Mice were weighed and administered Tribromoethanol (Avertin) by intraperitoneal injection (250 mg/Kg body weight) and monitored for non-responsiveness to sensory stimuli. Mice were dissected and underwent transcardial perfusion with PBS to complete exsanguination. For each mouse, the liver was removed and separated into three portions: one section was placed in OCT media and frozen at -80° (for IHC-F), one section as placed in a tissue

cassette and formalin fixed (for IHC-P), and one portion was flash frozen and stored at -80° (for RNA extraction). The aorta was removed from surrounding tissue and placed with the aortic root perpendicular to the bottom of a tissue cassette and fixed in OCT media and frozen at -80°C.

Developmental timepoint studies:

Female C3H/HeJ (C3H) mice were bred to male C57Bl/6J (Bl/6) mice, and female mice were monitored for vaginal plugs to estimate the time of fertilization. Pregnant females were euthanized by CO_2 asphyxiation at 17.5 days post-conception and the amniotic sac removed. Neonatal pups were euthanized by decapitation, and pups aged more than 14 days were euthanized by CO_2 asphyxiation. All pups were dissected and livers were isolated, frozen, and stored at -80° C.

Liver Regeneration:

Mouse liver regeneration was induced by a single intraperitoneal injection of carbon tetrachloride (CCl_4). Adult male C3H and Bl/6 mice were administered either 0.05 ml mineral oil (MO, n=5) or 0.05 ml 10% CCl_4 diluted in MO (n=5). After 3 days, animals were euthanized by CO_2 asphyxiation, the livers were removed, and samples were analyzed by quantitative Real-Time PCR of genes indicative of hepatic injury.

Murine model of hepatocellular carcinoma:

Female C3H mice were bred to male Bl/6 mice and monitored until pups were born. At 14 days of age, male offspring were injected with either diethylnitrosamine (DEN, n=16) or PBS (n=5) at a dose of 10ul/g body weight. Mice were weaned at 21 days of age and maintained under normal conditions for 36 weeks. Mice were euthanized by CO_2 asphyxiation and examined for HCC tumor development.

RNA extraction, cDNA synthesis, and Quantitative Real-Time PCR:

Samples were stored at -80° C until needed and then thawed on ice. Approximately 100 mg of tissue or 10^6 cells were homogenized in 1 ml RNAzol RT (Sigma #R4533) and mRNA was extracted according to the product technical bulletin. Rehydrated RNA was quantified by OD measurement using a NanoDrop Spectrophotometer (Thermo Scientific). cDNA was synthesized from 1 ug RNA by the iScript cDNA Synthesis Kit (BioRad #170-8891) per manufacturer's protocol. Quantitative Real-Time PCR (qPCR) reactions using SYBR Green PCR Supermix (BioRad #172-5275) were performed with a CFX96 Touch Real-Time PCR Detection System and results were analyzed with the CFX Manager software (BioRad). Oligonucleotide primer sequences are listed in Table 2. qPCR Ct values were normalized to the ribosomal protein L30 and is reported as Normalized Expression of the indicated gene. Data shown as Fold Change was normalized to L30, then values were calculated using the $\Delta\Delta$Ct method.

Immunohistochemistry:

Dissected mouse tissues were embedded in OCT media (#), flash frozen, and stored at -80°C. Frozen tissues were acclimated to -20°C and sectioned (10um thick) using a Microm HM505 N cryostat. Sections were placed onto glass slides, air dried for 10 minutes, then fixed in 4% paraformaldehyde for 10 minutes. Slides were washed twice in cold 1xPBS buffer then blocked with the appropriate serum blocking buffer at room temperature for one hour. Primary antibody detecting Zhx2 (abcam #96083, 1:250) was incubated overnight at 4°C, washed twice with 1xPBS, then incubated with secondary antibody (SouthernBiotech #4010-13, 1:200)for 1.5 hours at room temperature. Slides were mounted with aqueous mounting media (Dako #2013-05) and covered. Slides were imaged with a Nikon Eclipse 80i upright microscope with NiS Elements software.

Oil Red O Staining

Frozen mouse tissues embedded in OCT were sectioned at 10 um thick using a cryostat. Section were fixed in 10% buffered formalin for 10 minutes then washed in distilled water for 5 minutes. Slides were submerged in 60% isopropanol for 10 minutes to dehydrate the tissues. Oil Red O stain (Sigma #O0625, 0.5% in 100% isopropanol) was diluted 3:2 in distilled water and filtered. Slides were stained for 15 minutes while rocking, then washed with 60% isopropanol and counterstained with hematoxylin. Slides were mounted and covered and visualized Nikon Eclipse 80i upright microscope with NiS Elements software.

Cell Culture:

Cryopreserved HEK293, Huh7, and HeLa cells were removed from liquid nitrogen, thawed, and grown in T25 flasks with Dulbecco's minimal eagle's media (DMEM, Corning Cellgro #10-017-CV) supplemented with 10% fetal bovine serum (FBS, Fisher #03-600-511) and maintained in an incubator at 37°C and 5% CO_2.

Expression Plasmid Cloning:

A full-length expression vector for mouse Elovl3 was generated by PCR amplification (Forward primer: GCCACCATGGACACATCCATGAATTTCTCAC; Reverse primer: GGATCCTTGGCTCTTGGATGCAACTTTG) of mouse liver cDNA. Amplicons were cloned into the pGEM-T Easy vector (Promega #A1360), sequenced, excised using EcoRI and BamHI restriction digests, and cloned into the pcDNA3.1 expression vector (Invitrogen V790-20). Elovl3 and empty vector pcDNA3.1 expression plasmids were transformed into competent E. coli cells using a standard cell transformation protocol. Plasmid preparations were performed using a Plasmid Max Kit (Qiagen #12165). DNA was quantified by OD measurement using a NanoDrop Spectrophotometer.

Transfection of Expression Plasmids:

Cells were seeded onto 10 cm^2 plates and cultured for 24 hours to obtain 70-80% confluence. Transfection reactions using Turbofect reagent (Thermo

Scientific #R0531) were performed according to the manufacture's protocol. Cells were co-transfected with a GFP expression plasmid to visualize transfection efficiency prior to further experimentation.

Growth in Soft Agar:

Transfected Huh7 cells were cultured for 24 hours, then washed with PBS and treated with trypsin to lift cells from the plates. Cell suspensions were centrifuged at 1400 x g for 10 minutes at 4°C, decanted, washed with 1 ml PBS and centrifuged again, then resuspended in 1 ml PBS. Cells were counted and then seeded in 96-well plates with media supplemented agar as described [78]. A feeder layer consisting of 25 ul growth media mixed with 25 ul warm 1.2% agar (RPI cat #9002-18-0) was added to each well of a 96-well plate and allowed to solidify. A cell layer of 25 ul 0.8% agar mixed with 5000 cells suspended in growth media was seeded on top of the feeder layer and allowed to solidify. Another layer 25 ul growth media with 25 ul 1.2% agar was gently added on top of the cell layer. The plate was covered and cultured in the cell incubator for one week. Each sample was plated in triplicate wells, and the experiment was repeated three times. Cell viability was measured by CellGlo Titer Luminescent Cell Viability Assay (Promega #G7570) according to the product protocol and results were read on a luminometer. Sample luminescent values were normalized to wells containing media and agar layers without cells to account for background. Luminescent values for control cells were set to 100% and compared to values of Elovl3 transfected cells in three separate experiments.

Cell Synchronization and Analysis of Cell Cycle:

GFP and Elovl3 or pcDNA3.1 co-transfected cells were cultured for 24 hours then synchronized by blocking cell growth using a double Thymidine block. Thymidine dissolved in DMEM was added to plates to a final concentration of 2.5 mM. After incubation for 17 hours, cells were washed three times with PBS and supplied with fresh growth media for 9 hours. At this time, cells were again blocked with 2.5 mM Thymidine solution for 15 hours, then washed with PBS.

Cells were collected at this time (time 0) and at subsequent hourly timepoints. Cells were fixed overnight in 70% ethanol then washed with 1 ml ice cold PBS and centrifuged at 1400 x g for 10 minutes at 4°C three times. Cells were resuspended in 1 ml cold PBS and treated with RNAse A (Sigma #R-5125) at 37°C for 30 min. Cells were stained with propidium iodide (Roche #11348639001) and strained to remove cell aggregates immediately prior to analysis by Flow cytometry. Cells were gated for GFP expression then analyzed for DNA content to estimate the number of cell in each phase of the cell cycle. Flow cytometry analysis was performed by the Flow Cytometry & Cell Sorting core facility at the University of Kentucky.

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 and between three or more groups by ANOVA followed by Tukey's test. A p-value less than or equal to 0.05 was considered significant. Data was graphed and analyzed using GraphPad Prism 6 software.

Table 1. Primer Sequences, Genotyping.

Gene	Sequence (5')
Cre Recombinase	F: ACCTGAAGATGTTCGCGATTATCT R: ACCGTCAGTACGTGAGATATCTT
LDLr Neo Cassette	F: AGGTGAGATGACAGGAGATC R: ACCCCAAGACGTGCTCCCAGGATGA R: CGCAGTGCTCCTCATCTGACTTGT
Zhx2 Floxed Allele	F: GGACCGAATCTCACTATTTAACTCA R: ACAACGGGTTCTTCTGTTAGTCC

Table 2. Primer Sequences, qRT-PCR

Gene	Forward	Reverse
AFP	CCGGAAGCCACCGAGGAGGA	TGGGACAGAGGCCGGAGCAG
CD36	AAAGTTGCCATAATTGAGTCCT	AAAGTTGCCATAATTGAGTCCT
ChREBP	CATCTCCAGCCTCGTCTTC	CTTGGTCTTAGGGTCTTCAGG
CPT1A	ACTCGCTGAAGGTGCTGCTCTC	GTGCTGTCATGCGTTGGAAGTC
CPT1B	TTCAACACTACACGCATCCC	GCCCTCATAGAGCCAGACC
CPT2	CACAGCATCGTACCCACCAT	TGTCTTCCTGAACTGGC TGTC
CYP1A1	CCGGCATTCATCCTTCGT	GCCATTCAGACTTGTATCTCTTGTG
CYP1A2	ATCCTTTGTCCCCTTCACCAT	GGGAATGTGGGAAGCCATTCA
CYP2A4	GGAAGACGAACGGTGCTTTC	TTC CCA GCA TCA TTC TAA GA
CYP2A5	GGA AGACGAACG GTG CTT TT	TTC CCA GCA TCA TTC GAA GC
CYP2B9	CCTCGACTACATTGCCCATAG	GTTCTGGTGATGGAACTCTGTG
CYP2B13	GCTTTTCTACCCTTCTCCACAG	ATGTCCTTAGAAGCAACAGGGC
CYP2C40	TGGAAGAGGAAGGATTCCGG	TCACTGTGAAGACCCTTGTGG
CYP2D9	AGAAGTCTCTGGCTTAATTCCTG	GTGGTCCTATTCTCAGTCAACAC
CYP2D10	GAAGGTCTTCCAAGGTCAGAAG	CAGCATTCCCCTTTACCTTCTC
CYP3A16	GATGCCCTCTTTTTGGTTCTGTTGGC	TCAGGTTGGAATTCTTCAGGCTCTGG
CYP3A25	TCCTCTTCACCGAAATCCTGAG	TCCTGGGTCCATTTCCAAAGG
CYP4A10	TCCTTAATGACCCTAGACACTG	TGAAAGATATTCCTCACACGGG
CYP4A12	ATCCTTCTCGATTTGCACCAGG	TTCATCGCAAACTGTTTCCCAATG
CYP7A1	GGGCTGTGCTCTGAAGTTCGG	CACAGAGCATCTCCCTGGAGGG
CYP8B1	CAGAGAAAGCGCTGGACTTC	GGCCCCAGTAGGGAGTAGAC
CYP39A	TGGCTCCTGGCGCTGTTTGAG	TGGACTGTATTGACGTGTTTCCGTCT
Elovl1	GTGGCCCAGCCCTACCTTTGG	TGGTAGTTGCAGCTGGGCATGA
Elovl2	TCACCACGCGTCCATGTTCAACA	AAGCTGTTCAGGGTGGGTCCAA
Elovl3	CCTCTGGTCCTTCCTGGCA	CGGCGTCATCCGTGTAGATGGC
Elovl4	GTGGGTGGCTGGAGGCCAAG	AGCTGCAGCATGGTCAGGTATCG
Elovl5	ACTGGGTTCCCTGCGGCCAT	TTCCACCAGAGGTAGGGACGCA
Elovl6	TCCTGTTTTCTGCGCTGTACGCT	GCACCAGTTCGAAGAGCACCGA
Elovl7	TCATGGAGAACCGGAAGCCCTT	AACCTGTACCCCAGCCAGACA
HNF4α	GGAAGCTGTCCAAAATGAGCG	ATG TCG CCA TTG ATC CCA GAG
L30	ATGGTGGCCGCAAAGAAGACGAA	CCTCAAAGCTGGACAGTTGTTGGCA
Lpl	TGGCTACACCAAGCTGGTGGGA	GGTGAACGTTGTCTAGGGGGTAGT
MAT1a	GGCTGAAATTCCTCAAGGAGTCA	GGGCAAAGAGGGAGATAGCG
PPARγ	GATTCATGACCAGGGAGTTCCT	GGTTGTCTTGGATGTCCTCGAT
STAT5a	CGCTGGACTCCATGCTTCTC	GACGTGGGCTCCTTACACTGA
STAT5b	GGACTCCGTCCTTGATACCG	TCCATCGTGTCTTCCAGATCG
SREBP1a	CCGAGATGTGCGAACTGGAC	GGGAAGTCACTGTCTTGGTTG
SREBP1c	GGAGCCATGGATTGCACATTTG	CCTGTCTCACCCCCAGCATA
SREBP2	GGCGTTCTGGAGACCATG	AGGGAAGGAGCTACAAAGTTG
Zhx2	AGGCCGGCCAAGCCTAGACA	TGAGGTGGCCCACAGCCACT
hCyclin A	CTGCATCTCTGGGCGTCTTT	GTGCAACCCGTCTCGTCTTC
hCyclin B	TGGTGAATGGACACCAACTCT	TAGCATGCTTCGATGTGGCA
hCyclin D	GGATGCTGGAGGTCTGCGA	TAGAGGCCACGAACATGCAAGT
hCyclin E	CCCCATCATGCCGAGGGAG	TTATTGTCCCAAGGCTGGCT
hB2M	GACTTTGTCACAGCCCAAGATAG	TCCATTCCAAATGCGGCATCTTC

Chapter 3

Hepatic Zhx2 regulates lipid metabolism genes that alter risks of fatty liver and cardiovascular disease

Introduction

Obesity in the United States is growing in prevalence, with over one-third of adults and almost one-third of children and adolescents classified as obese [9]. Obese people face a multitude of adverse health effects, such as dyslipidemia, diabetes, hypertension, and Metabolic Syndrome, conditions that increase the risk and prevalence of cardiovascular disease (CVD) and non-alcoholic fatty liver disease (NAFLD). CVD is the leading cause of death of Americans with close to 800,000 deaths reported annually, and costing an estimated $320 billion in treatment as of 2011 [21]. Obesity and its comorbidities have strong associations with high fat diet, physical inactivity, smoking, gender, race, and age, yet there are numerous contributing genetic factors that are poorly understood.

Obesity-related NAFLD is characterized by excess lipid accumulation in the liver, which can lead to inflammation and hepatocyte necrosis and apoptosis, classified as non-alcoholic steatohepatitis (NASH). Continuing damage and increased fibrosis in the liver can progress to cirrhosis with declining liver function. Cirrhosis is a risk factor for the development of hepatocellular carcinoma (HCC), which is one of the fastest growing cause of cancer deaths in America [24]. It is estimated that roughly 30% of the American population has NAFLD, and around 3% of these cases will progress to NASH, then cirrhosis or HCC [79]. However, almost 90% of obese people have NAFLD, and alarmingly, the mortality rate from HCC is five times higher in obese men than those of normal weight [80]. Even without the disease progression to cancer, fatty liver presents a significant health threat because it contributes to insulin resistance and diabetes which are features of metabolic syndrome and risk factors for CVD.

Given the dramatic increase of obese Americans, it is imperative to gain better understanding of the metabolic disruptions involved in both CVD and NAFLD.

Zinc fingers and homeoboxes 2 (Zhx2) is a eukaryotic transcription factor identified by the Spear lab as a regulator of gene expression. A natural hypomorphic mutation in the BALB/cJ *Zhx2* gene is responsible for the *Hyplip2* phenotype in this strain characterized by lower serum triglycerides and fewer atherosclerotic plaques when placed on a high fat diet compared to what is seen in other mouse strains [74]. BALB/cJ mice expressing a liver-specific Zhx2 transgene have elevated serum lipid levels similar to other strains, confirming that hepatic Zhx2 expression contributes to lipid homeostasis and impacts CVD risk [75]. Studies in our lab have shown that BALB/cJ mice exhibit increased lipid accumulation and liver damage than other strains on HFD (Clinkenbeard Thesis, 2011).This is an interesting finding in that BALB/cJ mice develop fatty liver and yet are protected from CVD, although the two conditions are usually concordant. Understanding Zhx2 regulation and identification of its targets will provide better insight into the switch between cardiovascular and hepatic protection seen in differing Zhx2 status. Notably, two recent human Genome Wide Association Study (GWAS) identified ZHX2 as an important risk factor for carotid intima media thickness (cIMT), a subclinical marker of atherosclerosis, although the functional role of Zhx2 in these cases was not identified [76, 77].

Microarray data indicate Zhx2 may regulate numerous genes involved in lipid metabolism and transport, including lipoprotein lipase (Lpl), HMG CoA synthase, Fatty Acid Synthase (FAS), and CD36, among many others. The findings in BALB/cJ transgenic mice suggest that hepatic Zhx2 expression is sufficient to reverse the cardio-protective phenotype normally seen in these mice. As the liver is the major site of lipid metabolism and coordinates many metabolic processes, it is logical to examine the impact of hepatic Zhx2 on lipid metabolism genes. However, further exploration of Zhx2 regulation of lipid metabolism in the liver is limited by the fact that the mutation in BALB/cJ mice is hypomorphic, not null [81]. BALB/cJ mice continue to express low levels of Zhx2, but only around

5% of what is seen in other strains of mice. Additionally, Zhx2 is expressed ubiquitously and therefore Zhx2 and Zhx2-target genes in other tissues could contribute to the *Hyplip2* phenotype. To elucidate the role of hepatic Zhx2 expression on lipid gene regulation, I have developed a hepatocyte-specific Zhx2 knockout mouse line (*Zhx2*$^{\Delta hep}$). The studies described here analyze the impact of Zhx2 deletion from hepatocytes on lipid metabolism and the resulting impact on CVD and fatty liver.

Results

Confirmation of hepatocyte Zhx2 knockout

To confirm the establishment of the Zhx2 hepatocyte-specific knockout mouse line, male mice were genotyped for the expression of Albumin-Cre. *Zhx2*$^{\Delta hep}$ and *Zhx2*$^{f/f}$ control littermates were housed under normal condition until 6 weeks of age, then euthanized for tissue sample collection. Whole liver RNA was analyzed by qPCR for the expression of Zhx2 (Figure 5A). *Zhx2*$^{\Delta hep}$ mice had 97% decrease in Zhx2 mRNA compared to littermates. The remaining Zhx2 expression in the RNA sample is likely from other cell types, as Zhx2 remains to be expressed in Kupffer cells and other non-parenchymal cells in the liver (B Spear, unpublished obs.). Immunofluorescence staining of frozen liver sections with antibodies demonstrate Zhx2 proteins localized to the nuclei of hepatocytes in *Zhx2*$^{f/f}$ livers (Figure 5C). In contrast, no Zhx2 staining is observed in hepatocytes of *Zhx2*$^{\Delta hep}$ mice, although nuclear staining is still evident in non-parenchymal nuclei (Figure 5D). To confirm further the validity of *Zhx2*$^{\Delta hep}$ mice, we monitored AFP expression, AFP is known to be repressed by Zhx2 and continues to be expressed in synthesized in adult BALB/cJ liver. RT-qPCR indicates that AFP mRNA levels are roughly 10-fold higher in *Zhx2*$^{\Delta hep}$ mice compared to Zhx2$^{f/f}$ controls (Figure 5B). These results are similar to what is observed in BALB/cJ mice, confirming *Zhx2*$^{\Delta hep}$ mice provide a valid model to study hepatic Zhx2 gene regulation.

Zhx2 regulates Cyp2a4, Elovl3, and MAT1a

The cytochrome P450 member Cyp2a4 is a female-specific enzyme that catalyzes the hydroxylation of testosterone [82]. Male BALB/cJ have elevated expression of Cyp2a4 [83], so we were interested to investigate a connection with Zhx2. Male *Zhx2*$^{\Delta hep}$ mice have a 5-fold increase in hepatic Cyp2a4 expression (Figure 6A). Interestingly, female *Zhx2*$^{\Delta hep}$ mice have moderately reduced Cyp2a4 compared to wildtype female littermates, indicating Zhx2 has a role in the gender-specific hepatic gene expression (Figure 12, Chapter4). This is discussed further in Chapter 4.

A potential Zhx2 target gene is *Elongation of very long chain fatty acids-like 3* (*Elovl3)*. Elovl3 synthesizes mono-unsaturated and saturated fatty acids 22-24 carbons in length [84]. Elovl3 knockout mice are resistant to diet induced obesity, which is attributed to increased fatty acid oxidation to support thermogenesis [85, 86]. Elovl3 expression is reduced by more than 50% in *Zhx2*$^{\Delta hep}$ mice (Figure 6B), making Elovl3 the first identified target of Zhx2 to be positively regulated. Elovl3 is further described in Chapter 5.

Methionine adenosyltransferase Ia (Mat1a) is involved in the synthesis of S- adenosylmethionine (SAMe), the sole carbon donor source for DNA methylation, which is important for epigenetic gene regulation. Mat1a is not detectable in fetal liver, but its expression increases during development and is expressed in adult liver. Mat1a is repressed in the regenerating liver and is greatly reduced in HCC [87]; other Zhx2 targets are also disregulated in these conditions. Under normal treatment, Mat1a knockout mice develop NASH by 6 months and have spontaneous HCC by 18 months of age [88]. Mat1a mRNA levels are reduced in the livers of *Zhx2*$^{\Delta hep}$ mice, indicating that the Mat1a gene is positively regulated by Zhx2 (Figure 6C).

Hepatic Zhx2 regulates lipid transport genes Lpl, Cpt1a, and Cd36

Microarray data from livers of BALB/cJ congenic mice with or without the wild-type Zhx2 gene identified numerous genes involved in lipid metabolism and

homeostasis as putative Zhx2 targets [75]. We are interested in evaluating these target genes that are implicated in the control of lipid metabolism. Lpl was of interest due its known role in lipid disorders in humans. Lpl is usually expressed on the surface of adipose, skeletal muscle, and other non-hepatic tissues but not normally expressed in the adult liver. Lpl is activated by ApoCII to hydrolyze fatty acids from chylomicrons and VLDL particles for energy use or triglyceride storage within those tissues. qPCR of liver tissue shows that hepatic Lpl increases nearly two-fold in the absence of Zhx2 (Figure 7A). Lpl is not highly expressed in the liver, so the increase seen in *Zhx2*$^{\Delta hep}$ mice could result in fatty acid clearance from the serum via increased uptake in the liver. This disregulation of hepatic Lpl expression provides a possible mechanism for the BALB/cJ *Hyplip2* phenotype.

Studies in our lab have shown that BALB/cJ mice on a HFD have significantly increased liver lipid accumulation and liver damage compared to BALB/c mice, which have a normal Zhx2 gene (Clinkenbeard Thesis, 2011). This suggests that the reduction of Zhx2 results in increased lipid retention in the liver and a concomitant reduction in serum lipid levels, leading to CVD resistance. The transport of long chain fatty acids across membranes requires the activity of various enzymes. The carnitine shuttle consists of three enzymes with coordinated processes to move long chain fatty acids across the mitochondrial membrane for beta-oxidation [89]. Carnitine palmitoyltransferase-1 (Cpt1) replaces the CoA group on long chain fatty acids with carnitine, and the resulting acyl-carnitine traverses the outer mitochondrial membrane to the inner membrane space. Carnitine translocase shuttles acyl-carnitine through to the inner mitochondrial membrane in exchange for a free carnitine recycling back to the inner membrane space. The carnitine palmitoyltransferase-2 (Cpt2) enzyme then cleaves carnitine and substitutes CoA to yield fatty acyl-CoA, which is a substrate for acyl-CoA dehydrogenase in the beta-oxidation pathway. Cpt1a has two isoforms: Cpt1a is generally expressed in liver, whereas Cpt1b is expressed in muscle. *Zhx2*$^{\Delta hep}$ mice have roughly half the wildtype hepatic expression levels of Cpt1a (Figure 7C). Cpt1b and Cpt2 expression did not differ between *Zhx2*$^{\Delta hep}$

and *Zhx2*$^{f/f}$ mice (Figure 7D, 7E). Cpt1a activity is necessary for the proper utilization of fats and provides another mechanism to explain the increased liver lipid accumulation in BALB/cJ mice.

CD36, a fatty acid translocase, is another lipid transport enzyme involved in the uptake of lipids, including oxidized LDL particles and long chain fatty acids [90]. CD36 is expressed in hepatocytes and Kupffer cell in the liver, but is typically more abundant in adipocytes, skeletal muscle, endothelial cells, and blood cells [91]. CD36 in macrophages facilitates the uptake of long chain fatty acids and lipoproteins, notably oxidized LDL particles which initiates foam cell formation and atherogenesis [92]. Expression of hepatic CD36 in *Zhx2*$^{\Delta hep}$ mice is reduced about 45% compared to wildtype mice (Figure 7B).

Zhx2 interactions with other regulators of lipid metabolism

Sterol regulatory element binding-proteins (SREBPs) are critical regulators of genes that control lipogenesis and therefore of interest as potential contributors to the *Hyplip2* phenotype. SREBP1a activates LDL receptor expression for increased cholesterol uptake into cells and SREBP1c integrates insulin signals with lipogenic gene transcription [4]. SREBP2 is a key inducer of cholesterol biosynthesis [93]. Mutations in the SREBP genes have been associated with NASH and metabolic syndrome [94]. While these functions might suggest that SREBP genes could be Zhx2 targets, none of these genes is altered in *Zhx2*$^{\Delta hep}$ mice (Figure 8A-C). Similar to SREBP1c, the carbohydrate response element binding protein (ChREBP) is a regulator of lipid synthesis in the liver, though it is activated in response to glucose availability instead of insulin [3, 4] . ChREBP expression is reduced roughly 50% in *Zhx2*$^{\Delta hep}$ mice (Figure 8D).

Zhx2 represses transcription factors HNF4α, STAT5a and STAT5b, and PPARγ

Hepatocyte nuclear factor 4-alpha (HNF4α) is a member of the nuclear receptors superfamily and is among the most abundant transcription factor in the liver [95]. HNF4α is a key regulator of lipid metabolism, glucose homeostasis, and cell differentiation [96, 97] . Because of its importance in the regulation of

metabolic genes in the liver, we were interested to see if Zhx2 status had any impact on its expression. Indeed, Zhx2 deletion in hepatocytes lowers Hnf4α (Figure 9A). Interestingly, liver-specific Hnf4α knockout mice have reduced serum cholesterol and triglycerides and increased liver lipid accumulation, as is seen in BALB/cJ mice on HFD [96].

The peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor known for its role in adipocyte differentiation and increased lipid storage capacity [98]. PPARγ is increased in HFD-induced steatosis [99] and adipose PPARγ deletion may be protective against HFD insulin resistance [100]. PPARγ responds to long chain fatty acids to increase triglyceride synthesis and storage [101]. PPARγ is upregulated in the livers of *Zhx2*$^{\Delta hep}$ mice (Figure 9B). This could contribute to the increased liver damage seen in BALB/cJ on HFD.

We also investigated the impact of Zhx2 on the transcription factors in the Signal Transducer and Activator of Transcription 5 family, STAT5a and STAT5b. These factors were identified as essential mediators of growth hormone signaling and sex-specific gene expression patterns [7]. The disregulation of Cyp2a4 and other CYP genes in male BALB/cJ [93] and *Zhx2*$^{\Delta hep}$ mice (Chapter 4) suggested a possible interaction with Zhx2. Livers of *Zhx2*$^{\Delta hep}$ mice had a modest but significant reduction in both STAT5a and STAT5b compared to *Zhx2*$^{f/f}$ mice (Figure 9C, 9D).

Zhx2$^{\Delta hep}$ *mice on a HFD (preliminary data)*

Analysis of altered hepatic gene expression in *Zhx2*$^{\Delta hep}$ mice has provided insight into the *Hyplip2* phenotype in BALB/cJ mice. Because BALB/cJ mice continue to express low levels of Zhx2, one objective of my research is to examine the impact of complete hepatocyte Zhx2 deletion on cardiovascular disease risk in mice on HFD. To accomplish this, I bred *Zhx2*$^{\Delta hep}$ and *Zhx2*$^{f/f}$ mice onto *LDLr*$^{-/-}$ background. *LDLr*$^{-/-}$, *Zhx2*$^{\Delta hep}$ and control *LDLr*$^{-/-}$, *Zhx2*$^{f/f}$ littermates were weaned onto HFD and maintained on the diet for 18 weeks. This study is ongoing, but preliminary data reveals the hepatic consequences of Zhx2 hepatic

knockout on HFD. Both cohorts steadily gained weight over the course of the study. Although not statistically significant, *LDLr*$^{-/-}$, *Zhx2*$^{\Delta hep}$ have a slightly greater weight increase than controls (Figure 10A). Completion of the study with more mice may reveal a difference in weight gain among the groups. H&E staining of mice livers shows lipid droplets, hepatocyte ballooning, and distorted morphology in both *LDLr*$^{-/-}$, *Zhx2*$^{\Delta hep}$ and *LDLr*$^{-/-}$, *Zhx2*$^{f/f}$ mice (Figure 10B). Oil Red O staining indicates *LDLr*$^{-/-}$, *Zhx2*$^{\Delta hep}$ have dramatically increased lipid accumulation compared to *LDLr*$^{-/-}$, *Zhx2*$^{f/f}$ mice, as expected (Figure 10C). Further analysis in this study will continue to evaluate the liver phenotype and characterize the cardiovascular phenotype, which will include serum lipid profiling, aortic root examination for atherosclerotic lesion formation and lipid accumulation, and IHC to detect CD68 for macrophage infiltration into cardiac tissue.

Discussion

BALB/cJ mice, which have a natural hypomorphic mutation in the Zhx2 gene, have been instrumental in understanding liver gene regulation and lipid homeostasis. Studies described in this chapter demonstrate that the *Zhx2*$^{\Delta hep}$ mice will also be valuable in understanding the role of Zhx2 in CVD and fatty liver disease. Overlapping and sometimes opposing functions of Zhx2-target genes identified here make understanding the underlying mechanisms of the *Hyplip2* phenotype complicated. BALB/cJ mice on a HFD have reduced serum triglycerides and cholesterol, yet the experience much greater liver lipid accumulation and damage (Clinkenbeard Thesis, 2011). Increase in hepatic Lpl expression seen in BALB/cJ and *Zhx2*$^{\Delta hep}$ mice presumably have increased fatty acid uptake into hepatocytes. The decrease in Cpt1a suggests lipids are not efficiently metabolized in these mice, which could also contribute to increased lipid accumulation. Additionally, when lipids are not utilized for mitochondrial beta-oxidation, long chain fatty acids may be shunted to peroxisomes for degradation [102]. These pathways produce more damaging oxidative byproducts and could contribute to an inflammatory state and increased liver

damage in these mice. The impact of decreased CD36 expression in *Zhx2*$^{\Delta hep}$ mice is not as obvious. CD36 null mice on a HFD have increased serum HDL attributed to increased hepatocyte cholesterol and lipoprotein efflux, conferring a cardio-protective phenotype in these mice [103]. Another study in CD36 null mice showed they were resistant to steatosis on a high carbohydrate and alcohol diet [104]. CD36 null mice under LXR activation exhibited less liver lipid accumulation than controls [90]. In contrast, CD36 null mice on ob/ob background had reduced VLDL secretion and greater steatosis [91]. The VLDL output has not been measured in *Zhx2*$^{\Delta hep}$ mice, but future investigation could help elucidate the impact of diminished CD36. The decrease in Mat1a likely also contributes to the NAFLD in BALB/cJ mice. In addition to spontaneous NASH and HCC development, Mat1a null mice also have altered plasma lipid levels marked by impaired VLDL secretion [87]. The combined impact of these alterations in gene expression is the increased clearance of serum triglycerides by elevated hepatic Lpl activity, blockage of beta-oxidation and fat usage for energy, and diminished secretion of lipids in VLDL. The consequence of these is reduced serum lipids and corresponding accumulation of lipids in the liver (Figure 11).

Data reported in this study suggest cross-talk between Zhx2 and other transcription factors that are known regulators of lipid metabolism, adipose accumulation, liver injury and inflammation. These results implicate Zhx2 as a contributor to these main lipid regulatory networks and hint to a broader role in metabolic homeostasis than originally anticipated. The SREBP genes play key roles in regulating lipid and cholesterol homeostasis in the liver. The fact that they are not altered in *Zhx2*$^{\Delta hep}$ mice is curious but not unexpected as we have no indications of altered serum glucose levels or insulin sensitivity in these mice. We have not directly examined these characteristics but would predict that BALB/cJ mice would not exhibit cardiovascular protection on a HFD if they had fatty liver as well as altered glucose/insulin responses.

Studies in *ob/ob* mice, as a model of fatty liver and insulin resistance, report that ChREBP and SREBP1c synergistically increased after feeding,

resulting in increased lipogenesis and eventual steatosis [105]. Knockdown of ChREBP in these mice improved insulin sensitivity and lowered hepatic fat accumulation and serum triglycerides. Hepatic overexpression of ChREBP in B6 mice maintained insulin sensitivity when placed on HFD, yet had increased steatosis [106]. The basis for decreased expression in ChREBP in *Zhx2*$^{\Delta hep}$ mice is not clear; analysis of possible changes in serum glucose or insulin response may provide insight into the relationship between ChREBP and Zhx2.

PPARγ is a well characterized transcriptional activator of genes involved in adipogenesis and is primarily expressed in adipocytes. An interesting relationship exists between PPARγ and Elovl3, in that VLCFAs produced by Elovl3 stimulate PPARγ activity, and PPARγ may increase Elovl3 expression [107]. Elovl3 expression is ablated in *Zhx2*$^{\Delta hep}$ livers, so it would be interesting to see if it is upregulated in other tissues to compensate for decreased production in the liver. There is some overlap in substrate response with the other elongase members, so it is likely that other elongases expressed in the liver are able to synthesize the VLCFAs that stimulate PPARγ.

HNF4α is well established as a master regulator of hepatic gene expression and therefore metabolic processes. It is unclear whether HNF4α responds directly to fatty acid binding or if it is activated by other mechanisms. The similarity in fatty liver and lower serum cholesterol and triglycerides in HNF4α liver knockout mice and BALB/cJ mice is consistent with our data that *Zhx2*$^{\Delta hep}$ mice have reduced HNF4α expression. Further analysis of the interaction of genes regulated by ChREBP, PPARγ, and HNF4α and Zhx2 will help explain the relationship between these transcriptional regulators and resulting changes in expression of lipogeneic and adipogenic genes. It will be interesting to see if Zhx2 directly regulates the expression of these other transcription factors and thereby is a master regulator of hepatic gene expression.

To understand the integrated effects of the alterations in these transcription factors in the livers of *Zhx2*$^{\Delta hep}$ mice, a more thorough metabolic

analysis must be completed. Serum panels to assess insulin and glucose repose and lipid abundance and content will clarify the role of hepatic Zhx2 in nutrient sensing responses and lipoprotein production/export. *Zhx2*$^{\Delta hep}$ mice should be analyzed for differences in food intake, absorption and excretion, as well as alterations in activity level, oxidative capacity, and thermogenesis. Detailed analysis of the complete metabolic profile of *Zhx2*$^{\Delta hep}$ mice will help identify the impact of altered transcription factor expression seen in the mice and understand how Zhx2 integrates into established lipid metabolism regulatory networks. Since Zhx2 is expressed in many tissues, the role of Zhx2 in other tissues, particularly metabolic tissues such as muscle and adipose, will provide further insight into Zhx2 and lipid homeostasis.

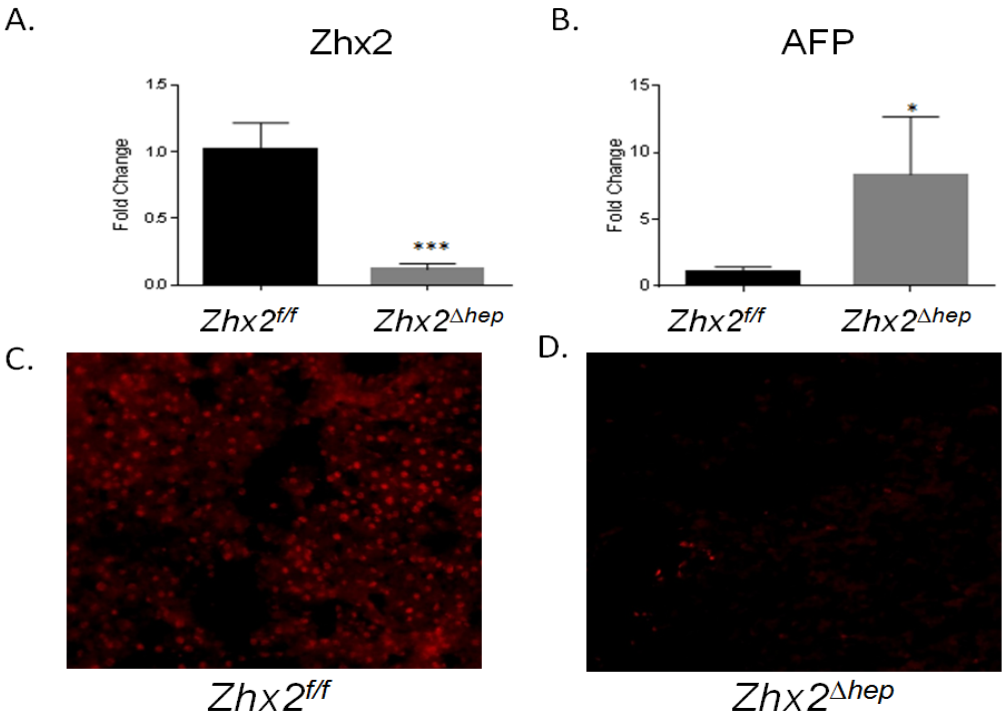


Figure 5. Hepatocyte Zhx2 Knockout Mouse Line. Total liver RNA from *Zhx2*$^{f/f}$ and *Zhx2*$^{\Delta hep}$ littermates was analyzed by qPCR for Zhx2 expression (A) and AFP (B). Elimination of Zhx2 expression in hepatocytes is confirmed by immunofluorescence staining which detected positive nuclear Zhx2 in *Zhx2*$^{f/f}$ (C) compared to *Zhx2*$^{\Delta hep}$ (D). **p<0.05, ***p<0.001.*

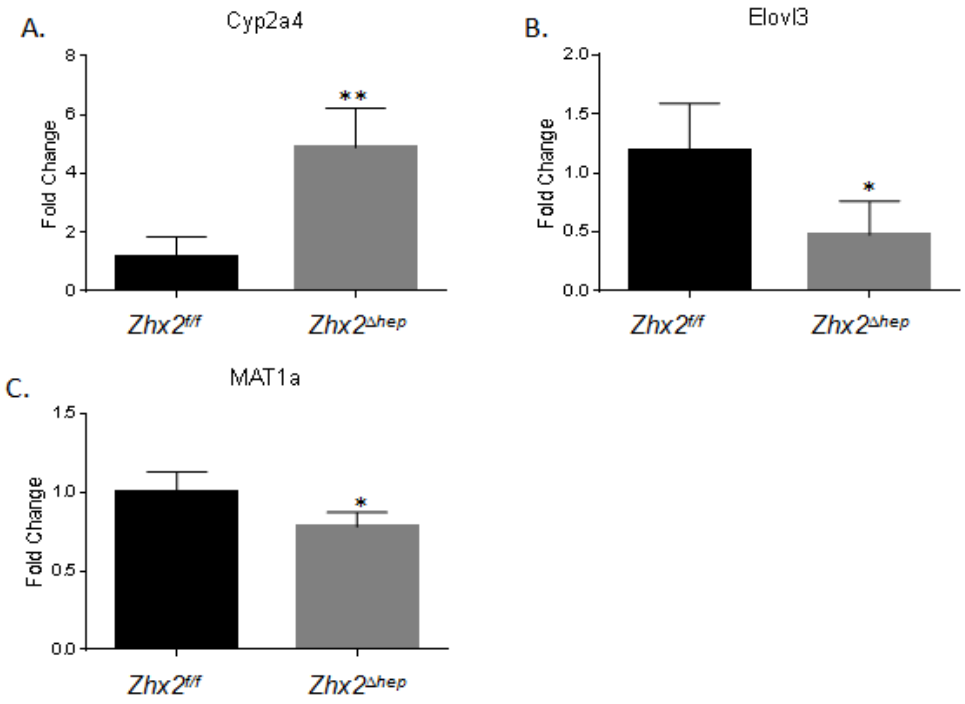


Figure 6. Zhx2 regulates hepatic expression of Cyp2a4, Elovl3, and MAT1a. *Zhx2*$^{\Delta hep}$ mice have increased expression of Cyp2a4 (A), normally expressed at very low levels in male mouse liver. Elovl3 is repressed in *Zhx2*$^{\Delta hep}$ (B) and is the first identified Zhx2 that is positively regulated. MAT1a (C) is repressed in *Zhx2*$^{\Delta hep}$. **p<0.05, **p<0.01*.

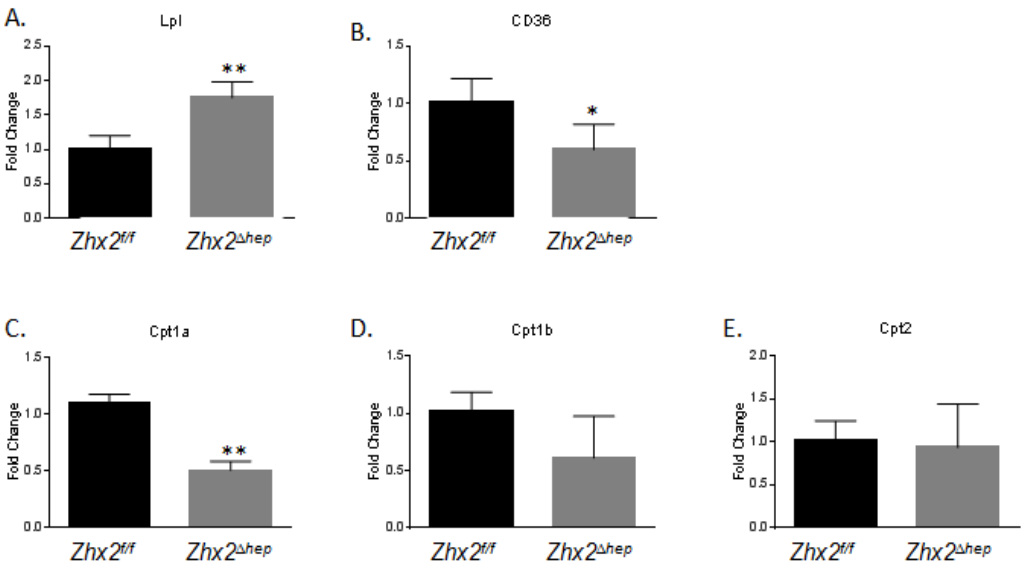


Figure 7. Zhx2 regulates lipid transport genes. *Zhx2*$^{\Delta hep}$ mice have increased expression of Lpl (A), and decreased expression of CD36 (B) and Cpt1a (C). The alterations in these genes are consistent with the fatty liver phenotype in BALB/cJ mice. Cpt1b (D) and Cpt2 (E) do not exhibit significant changes in *Zhx2*$^{\Delta hep}$ liver. **p<0.05, **p<0.01*.

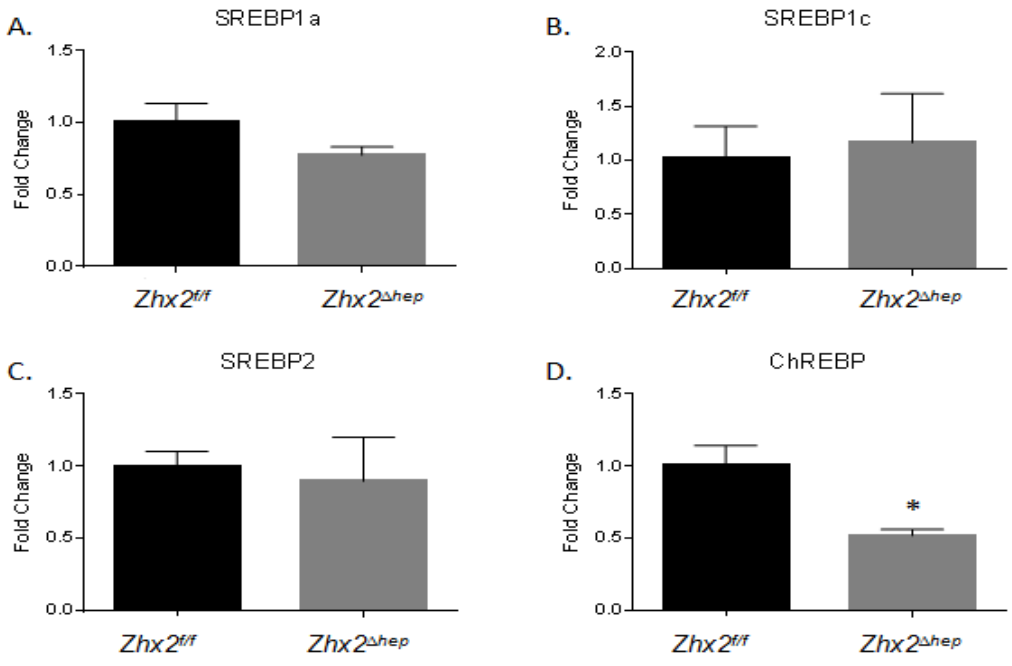


Figure 8. Zhx2 regulates other regulators of lipid metabolism. Hepatic Zhx2 expression does not alter the expression of SREBP1a (A), SREBP1c (B), or SREBP2(C). The glucose-responsive transcription factor ChREBP is significantly reduced in the livers of *Zhx2*$^{\Delta hep}$ mice (D). **p<0.05*.

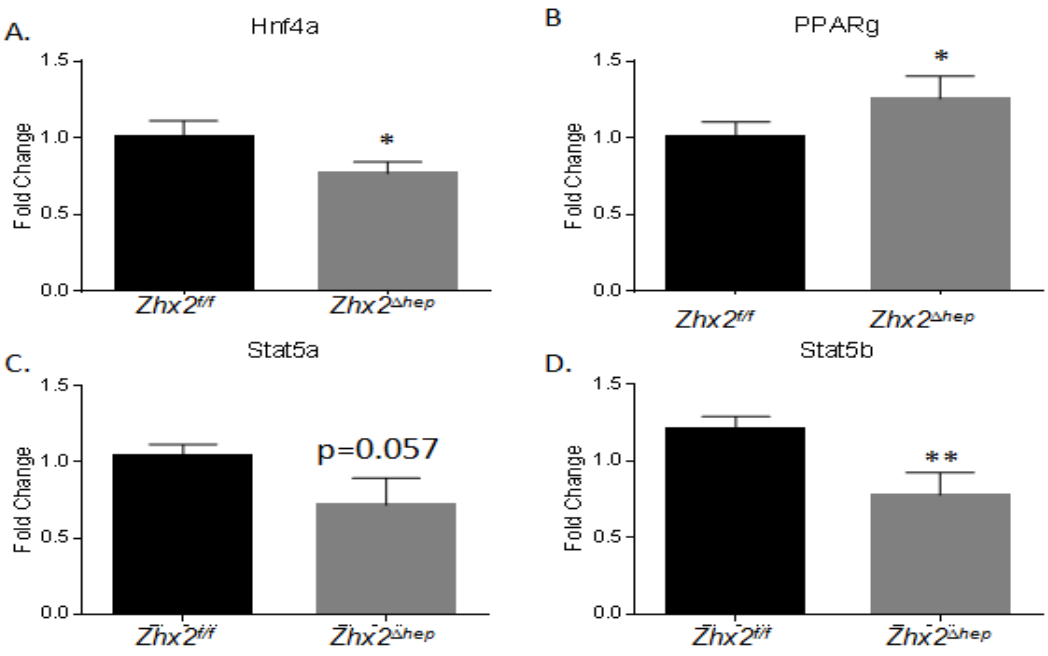


Figure 9. Zhx2 regulates expression of several hepatic transcription factors. The liver-enriched factor HNF4α is decreased in the absence of Zhx2 (A), while PPARγ expression is enhanced (B). Signaling effecter genes STAT5a (C) and STAT5b (D) are modestly reduced in *Zhx2*$^{\Delta hep}$ mice. **p<0.05, **p<0.01*.

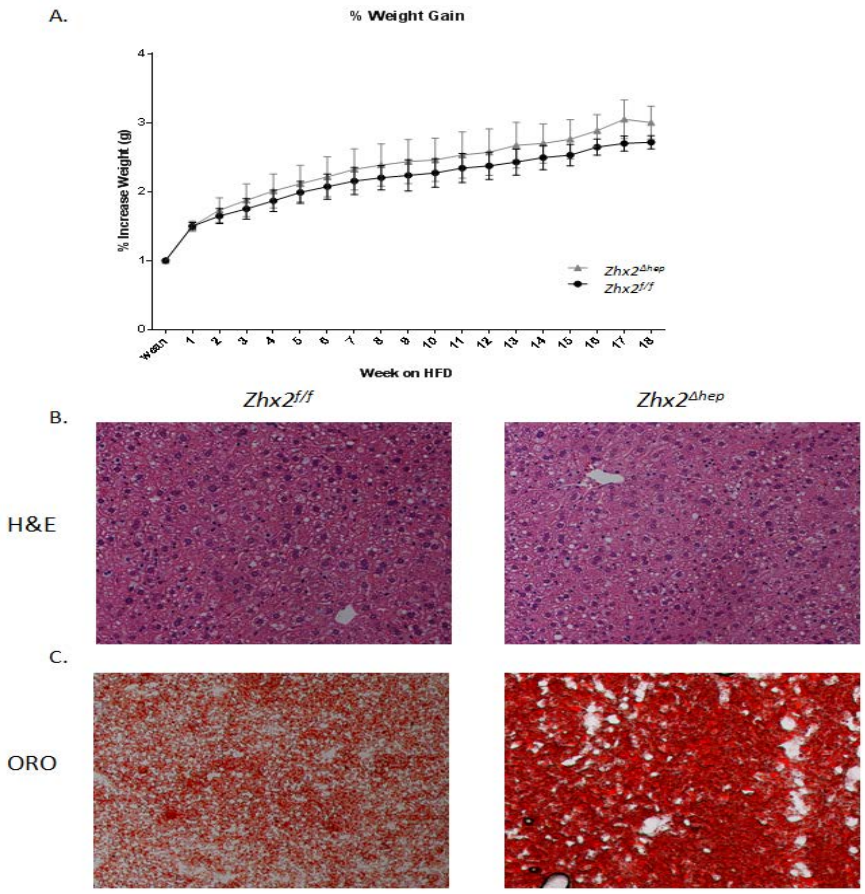


Figure 10. Preliminary data of *LDLr*$^{-/-}$, *Zhx2*$^{\Delta hep}$ and *LDLr*$^{-/-}$, *Zhx2*$^{f/f}$ mice on HFD. *LDLr*$^{-/-}$, *Zhx2*$^{\Delta hep}$ and *LDLr*$^{-/-}$, *Zhx2*$^{f/f}$ littermates were fed HFD for 18 weeks. Weight gain is similar in both groups (A). H&E staining of livers shows ballooning of hepatocytes and triglyceride accumulation indicative of fatty liver in both cohorts (B). Noticeably increased lipid accumulation is detected in *LDLr*$^{-/-}$, *Zhx2*$^{\Delta hep}$ livers by Oil Red O staining (C).

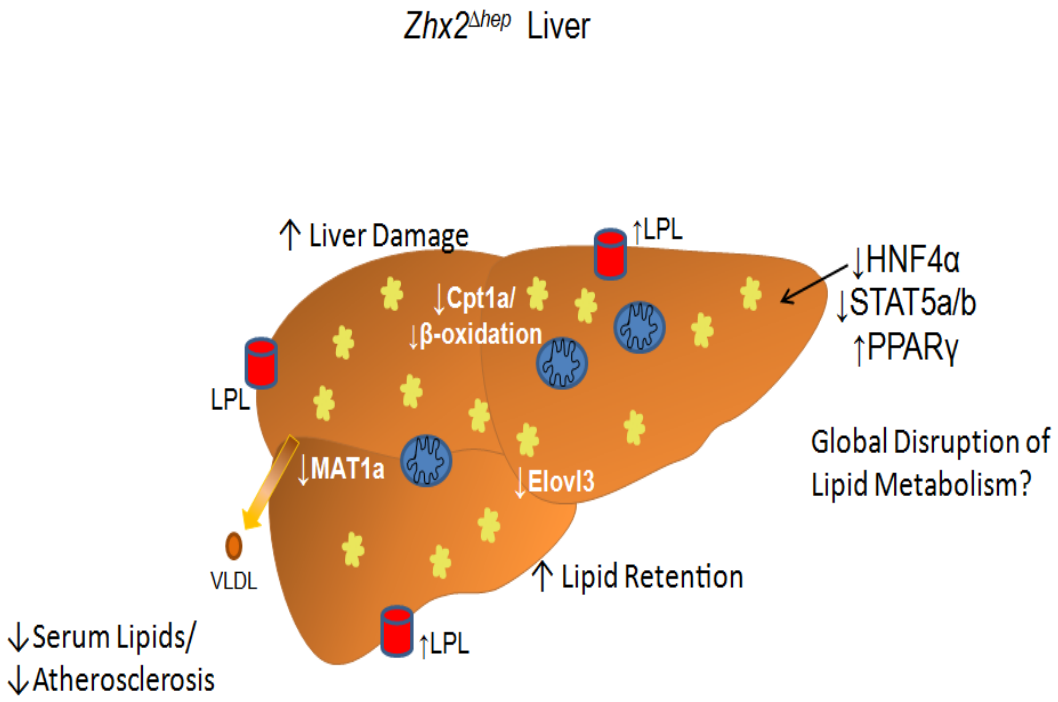


Figure 11. Impact of Hepatic Zhx2 Deletion. *Zhx2*$^{\Delta hep}$ mice have altered expression of numerous genes important for lipid metabolism. Hepatic Lpl expression increases triglyceride uptake to the liver. Repression of Cpt1a and Elovl3 reduces hepatic capacity to metabolize and export lipids and cholesterol from the liver, increasing retention and damage. Lowered Mat1a may independently contribute to NAFLD development. Decrease in HNF4α and increase in PPARγ expression could alter normal hepatic gene regulation on a global scale. The increase accumulation and reduced export of lipid in the liver effectively lower serum lipids and reduce atherogenesis.

Chapter 4

Zhx2 regulates sexually dimorphic CYP gene expression in the adult mouse liver

Introduction

The cytochrome P450 (CYP) supergene family is one of the largest and most diverse gene families in eukaryotes [108, 109]. CYP genes encode structurally related enzymes that catalyze a variety of metabolic reactions, including metabolism of steroid-based hormones, lipids, drugs and environmental chemicals [110]. In humans, mutations in CYP genes contribute to a variety of metabolic diseases [111], and polymorphisms in CYP genes are a major contributor to variations in susceptibility to xenobiotics [112, 113]. The CYP supergene family arose through numerous duplication events. In vertebrates, the number of CYP genes and pseudogenes vary dramatically between different species. Analysis of genome databases suggests that humans contain 57 functional CYP genes and 58 pseudogenes, whereas mice contain 102 functional CYP genes and 88 pseudogenes.

CYP genes exhibit a variety of expression patterns. Some CYP genes are expressed in numerous tissues, whereas expression of other CYP genes is more highly restricted to one or several tissues. The liver has the highest level of CYP expression, which is not surprising since ingested xenobiotics enter the liver before circulation elsewhere in the body. Numerous drugs and xenobiotics can induce CYP gene expression; much of this is governed by members of the nuclear receptor family, including the Constitutive Androstane Receptor (CAR), aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), and peroxisome proliferator activated receptor α (PPARα) [114]. As with structural polymorphisms in CYP enyzmes, variation in the expression and induction of CYP enzymes in response to xenobiotics can lead to different responses to these agents. These variations must be considered when comparing the metabolism and toxicity of drugs and environmental chemicals between different humans as well as different species such as mice and humans [115].

One common feature of many vertebrate CYP genes is sexual dimorphic expression [116]. Many CYP genes, including Cyp 2d9 and Cyp4a12, are expressed primarily in males [117]. In contrast, other CYP genes such as Cyp2b9 and 2b13 show a female-biased pattern of expression [117]. The degree of sex-biased expression varies considerably. Differences in circulating Growth Hormone (GH) levels play an important role in this sexually dimorphic expression [118]. In the liver, the transcription factors STAT5a/b and hepatocyte nuclear factor 4α (HNF4α) help govern gender-specific expression [8, 119, 120].

A dramatic example of sexually dimorphic expression is seen with the Cyp2a4 gene in mice. In normal adult liver, the mouse Cyp2a4 gene is expressed abundantly in female livers with very low hepatic expression in adult males [121]. Curiously, Cyp2a4 expression in adult male BALB/cJ mice is almost equal to what is seen in female mice [83] . The high Cyp2a4 mRNA levels observed in BALB/cJ males is an autosomal recessive trait. This led to the suggestion that the absence of Cyp2a4 expression in male mice is due to a transcriptional repressor, and that a mutation resulting in the loss of this putative repressor in BALB/cJ mice results in de-repression of Cyp2a4 in adult BALB/cJ male liver.

Several years ago, we identified Zinc fingers and homeoboxes 2 (Zhx2) as a regulator of gene expression in the postnatal liver. Several Zhx2 target genes, including alpha-fetoprotein (AFP), H19, and Glypican 3 (Gpc3) are expressed abundantly in the fetal liver and dramatically repressed in the first several weeks after birth [29, 81]. In contrast to most strains of mice, AFP, H19 and Gpc3 continues to be expressed in the adult liver of BALB/cJ mice. The continued expression of these genes in BALB/cJ liver, where adult liver AFP mRNA levels are 10- to 20-fold higher than other mouse strains, is an autosomal recessive trait regulated by a locus on Chromosome 15. Using positional cloning, we found that elevated AFP, H19 and Gpc3 mRNA levels in BALB/cJ livers was due to a hypomorphic mutation in the Zhx2 gene that dramatically reduces Zhx2 levels. Interestingly, when placed on a high fat diet, BALB/cJ mice have fewer serum

triglycerides and reduced atherosclerotic lesions compared to other mouse strains on the same diet. This strain difference is also due to the Zhx2 mutation, and several proteins involved in lipid/cholesterol homeostasis are disregulated in BALB/cJ livers. Taken together, these data indicate that Zhx2 is an important regulator of numerous hepatic genes in the adult liver.

Zhx2 is a member of a small gene family that also contains Zhx1 and Zhx3. The Zhx proteins contain two amino-terminal C2-H2 Zinc fingers and four (or five, in the case of Zhx1) carboxy-terminal homeodomains. *In vitro* studies suggest that Zhx proteins function as transcriptional repressors. This, along with the fact that the Zhx2 gene is mutated in BALB/cJ mice, led us to investigate whether the mutation in Zhx2 could account for elevated Cyp2a4 expression in adult male BALB/cJ mice. Using several mouse models, our data indicate that Cyp2a4 is a target of Zhx2 repression. In contrast to Cyp2a4, expression of the highly related Cyp2a5 gene shows little change in the absence of Zhx2. Furthermore, we demonstrate that numerous other sex-biased CYP enzymes exhibit altered expression in the absence of Zhx2. In general, female-biased CYP enzymes exhibit increased expression in males but male-biased CYP enzymes show little change in either sex. Levels of Cyp7a1 mRNA, which is present at high levels in both male and female liver, are increased in males and reduced in females in the absence of Zhx2. This data indicates that Zhx2 is an important regulator of sex-specific expression of CYP enzymes in the liver.

Results

Cyp2a4 mRNA levels are higher in the liver of male Zhx2$^{\Delta hep}$ mice

Cyp2a4 is normally expressed at high levels in adult liver of female mice but not expressed in adult male liver. An exception to this is seen in BALB/cJ mice, in which Cyp2a4 mRNA levels are almost equal in both male and female adult liver. We asked whether Zhx2, which is mutated in BALB/cJ mice, could account for this strain-specific trait. To accomplish this, we analyzed Cyp2a4 expression in BL/6 mice with a hepatocyte-specific deletion of Zhx2. Mice with a floxed Zhx2

allele (described in Chapter 2) were bred with Albumin-Cre (Alb-Cre) transgenic mice to generate mice that were homozygous for the floxed Zhx2 gene and Alb-Cre+ (*Zhx2*$^{\Delta hep}$); floxed homozygous littermates that did not contain the Alb-Cre transgene (*Zhx2*$^{f/f}$) were used as controls. Zhx2 is efficiently silenced in the hepatocytes of *Zhx2*$^{\Delta hep}$ mice but still expressed in non-parenchymal cells (Chapter 3, Figure 5). In control *Zhx2*$^{f/f}$ mice at 5 weeks of age, Cyp2a4 is expressed in females but barely detectable in males as expected. In contrast, Cyp2a4 mRNA levels are increased nearly 90-fold in age matched Zhx2$^{\Delta hep}$ male mice to levels that are comparable to those seen in female *Zhx2*$^{\Delta hep}$ adult liver, which show a ~40% reduction in Cyp2a4 levels compared to *Zhx2*$^{f/f}$ females (Figure 12A, B). These data indicate that Zhx2 is responsible for the low Cyp2a4 levels in adult male mice. Interestingly, Cyp2a5, which is highly related to Cyp2a4, does not exhibit sex-biased expression or significant differences between *Zhx2*$^{\Delta hep}$ and *Zhx2*$^{f/f}$ mice.

Cyp2a4 levels exhibit gender-specific changes in the postnatal liver

Previously identified Zhx2 targets, including AFP, H19 and Gpc3, are expressed abundantly in the fetal liver and silenced after birth. In contrast to Cyp2a4, these genes do not exhibit gender-biased expression. This led us to investigate Cyp2a4 in the fetal liver and postnatal patterns of Cyp2a4 expression in male and female wildtype mice. In both sexes, Cyp2a4 levels are very low in the e17.5 liver and show a gradual increase during the first four weeks after birth. A modest increase in Zhx2 mRNA levels is also seen at this time (Figure 13A, B). However, the gender bias in the liver is evident at p28, with female Cyp2a4 levels roughly 8-fold higher than that seen in males (Figure 13C, D). A dramatic change in this pattern occurs between four weeks and eight weeks of age. In male mice, Zhx2 levels increase roughly 6-fold during this period (Figure 13A), whereas Cyp2a4 levels are dramatically reduced to barely detectable levels (Figure 13C). In contrast, Zhx2 and Cyp2a4 levels show modest changes in the female liver during this time period (Figure 13B, D). This data supports the possibility that Zhx2 contributes to Cyp2a4 silencing in the adult male liver, and that full

repression is associated with sexual maturity that occurs between 4 and 8 weeks of age.

A number of CYP enzymes in addition to Cyp2a4 exhibit gender-specific expression, with some being expressed at higher levels in male and others in females. Since male-specific Cyp2a4 mRNA levels increased in *Zhx2*$^{\Delta hep}$ mice, we evaluated expression of other hepatic Cyp enzymes in the presence or absence of Zhx2. Eight Cyp genes that are normally expressed at higher levels in females were examined. In general, the loss of hepatic Zhx2 resulted in increased expression in adult male liver (Figure 14). The increase was robust in several cases (27-fold and 9-fold for Cyp2b13 and Cyp2b9, respectively) and more modest for other enzymes (2- to 3-fold for Cyp4a10, Cyp2c40, Cyp 3a16 and Cyp39a). Zhx2 did not affect expression of Cyp1a1 or Cyp1a2 in males. Expression of these genes was the same or slightly less in females *Zhx2*$^{\Delta hep}$ mice than in *Zhx2*$^{f/f}$ only This pattern is similar to what was seen with Cyp2a4 (Figure 12). A different pattern was seen with several male-biased Cyp enzymes. In two that were analyzed, Cyp2d9 and Cyp8b1, showed little change in either sex in *Zhx2*$^{\Delta hep}$ liver compared to *Zhx2*$^{f/f}$ liver (Figure 15). Cyp4a12 was repressed in female *Zhx2*$^{\Delta hep}$ mice. Three Cyp enzymes that are expressed equally in both sexes were examined (Figure 16). Cyp3a25 and Cyp2d10 did not respond to the loss of Zhx2 whereas Cyp7a1 was slightly increased in males and decreased in females. Interestingly, Cyp7a1 codes for the rate-limiting enzyme that converts cholesterol to bile acids so alterations in its expression likely affects cholesterol metabolism.

Cyp2a4 mRNA levels are increased in HCC

Previously identified genes that are repressed by Zhx2, including AFP, H19 and Gpc3, are silent in the healthy adult liver but frequently reactivated in HCC. Since Cyp2a4 also appears to be negatively regulated by Zhx2, we asked whether it was also disregulated in HCC. Analysis of RNA from normal C3B6F1 male mouse liver and male HCC tumors induced by DEN (described in Chapter 2) indicated that Cp2a4 levels were significantly increased in tumors (Figure 17).

Discussion

Data in this chapter provide the first evidence that Zhx2 contributes to gender-biased gene expression in the adult liver. The sexually dimorphic expression of CYP genes is well documented, but the involvement of Zhx2 regulation of genes in a sex-specific manner provides a link between developmental gene regulation and gender-biased expression. Zhx2 represses targets AFP, H19, and Gpc3 in normal adult mouse liver, and these genes have similar expression levels in males and females. Zhx2 positively regulates Elovl3 in mouse liver during development (discussed in Chapter 5). After sexual maturity, Elovl3 is more abundant in male liver, and is expressed in a circadian rhythm only in male liver [122]. Data presented here shows that Zhx2 positively regulates Cyp2a4 in male and female liver during development, but between 4-8 weeks of age, Zhx2 is required for full Cyp2a4 repression in male liver.

Cyp2a4 and other Cyp enzymes, along with Elovl3, exhibit circadian patterns of expression. Cyp2a4 circadian regulation in male mice is attributed to integration of growth hormone (GH) signaling via STAT5 activation [8, 123]. STAT5b expression is necessary for expression of male-specific Cyp genes, whereas both STAT5a and STAT5b expression are required for female gene patterning [119]. The expression of the appropriate STAT5 isoforms coordinates the expression of genes in a sex-specific manner in response to GH signaling. GH is expressed in a pulsating manner in males while females have more steady GH circulation [120]. HNF4α is a key regulator of gene expression in the liver. Almost half of actively transcribed hepatic genes containing response elements [95], suggesting that HNF4α affects more target genes than other hepatic transcription factors. Studies in HNF4α-deficient mice showed this factor to be necessary for the expression of several sex-specific Cyps [124]. A current model of integration of HNF4α with GH signaling suggests the pulsating spike in GH in male mice activates STAT5b, and possibly HNF4a, which translocate to the nucleus and increase transcription of male specific Cyp genes (Figure 18).

Increased HNF4a suppresses HNF6 and HNF3β which are associated with expression of female Cyps. The continuous GH levels in females correspond with increased HNF6 and HNF3β activity and fail to activate STAT5b and therefore fail to activate male Cyp expression [124, 125]. These combined studies indicate that GH pulses and activation of STAT5b and HNF4α are critical to male Cyp expression. Interestingly, qPCR data in $Zhx2^{\Delta hep}$ mice suggest hepatic Zhx2 deletion reduces the expression of HNF4α, STAT5a, and STAT5b (Chapter 3). As each of these transcription factors is a critical component of sex-specific gene regulation, the reduction in $Zhx2^{\Delta hep}$ mice could help explain the disregulation of multiple Cyp genes in the livers of both male and female $Zhx2^{\Delta hep}$ mice. Further examination into the potential interaction of Zhx2 with HNF4α and STAT5 is warranted by these findings, and could elucidate the mechanism by which sex-biased genes are regulated in the liver.

We provide evidence that Cyp2a4 is activated in HCC, similarly to other Zhx2 targets. Disregulated expression of various CYP genes has been associated with human liver disease risk. Data from human tissue arrays of HCV infected, HCV infected with HCC, and non-HCV normal livers identified differential expression of 27 different CYPs depending on disease state and severity [126]. The pattern of expression varies; CYP2C9 is lower in HCC cases with larger tumors, whereas CYP51A1 increases with tumor size. CYP2B6, CYP2C9, CYP2C19, CYP3A5, CYP4F3, CYP27A1, CYP2E1, and CYP4F2 were lower in HCC with greater vascularization and thus more advanced progression [126]. A qRT-PCR panel of numerous CYPs revealed that HCC tissues generally have lower CYP expression than non-tumor tissue; however, HCC samples that overexpress one CYP gene usually exhibit higher expression of several CYPs [127]. Diminished CYP2E1 has been associated with increased tumor aggression and poor prognosis in HCC patients [128]. These studies confirm that CYP expression is often disregulated in HCC and the degree of disregulation could correlate with the advancement of disease. Data from our collaborator [35] indicates that nuclear Zhx2 levels are reduced in HCC samples compared to non-tumor liver tissue, suggesting that this differential localization of Zhx2 could account for the

lack of repression of Zhx2 targets in HCC. Additional studies will be needed to further explore whether reactivation of CYP enzymes in cancer is due, at least in part, to changes in Zhx2.

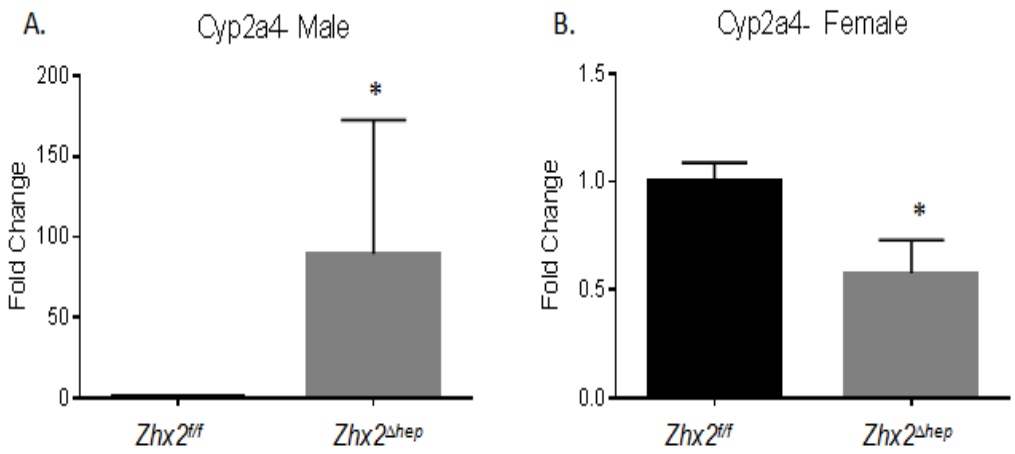


Figure 12. Zhx2 regulates Cyp2a4 in a gender-specific manner. Adult male *Zhx2*$^{\Delta hep}$ mice have 90-fold increase in Cyp2a4 expression (A), whereas female *Zhx2*$^{\Delta hep}$ have about 40% reduced expression (B) when compared to *Zhx2*$^{f/f}$ controls. This data suggests Zhx2 positively regulates Cyp2a4 in female mouse liver and represses it in adult male liver. *$p<0.05$.

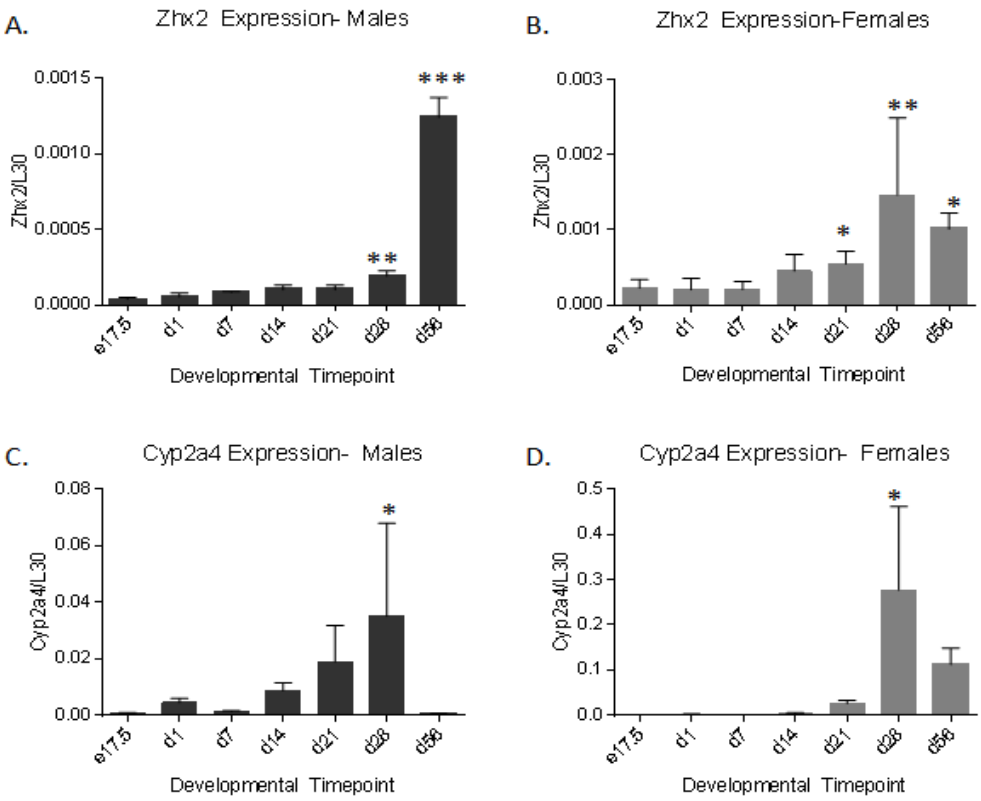


Figure 13. Zhx2 regulation of Cyp2a4 during development. Liver RNA extracted from C3B6F1 offspring at development timepoints was analyzed by qRT-PCR. Zhx2 expression increases with age in both male (A) and female (B) offspring until d28. Zhx2 levels continue to increase in male mice from d28 to d56. Cyp2a4 expression is positively regulated in males (C) and females (D) until d28. Around sexual maturity at d56, male mice have almost complete repression of Cyp2a4 while females continue to express it. Developmental expression compared to e17.5, **p<0.05, **p<0.01, ***p<0.001*.

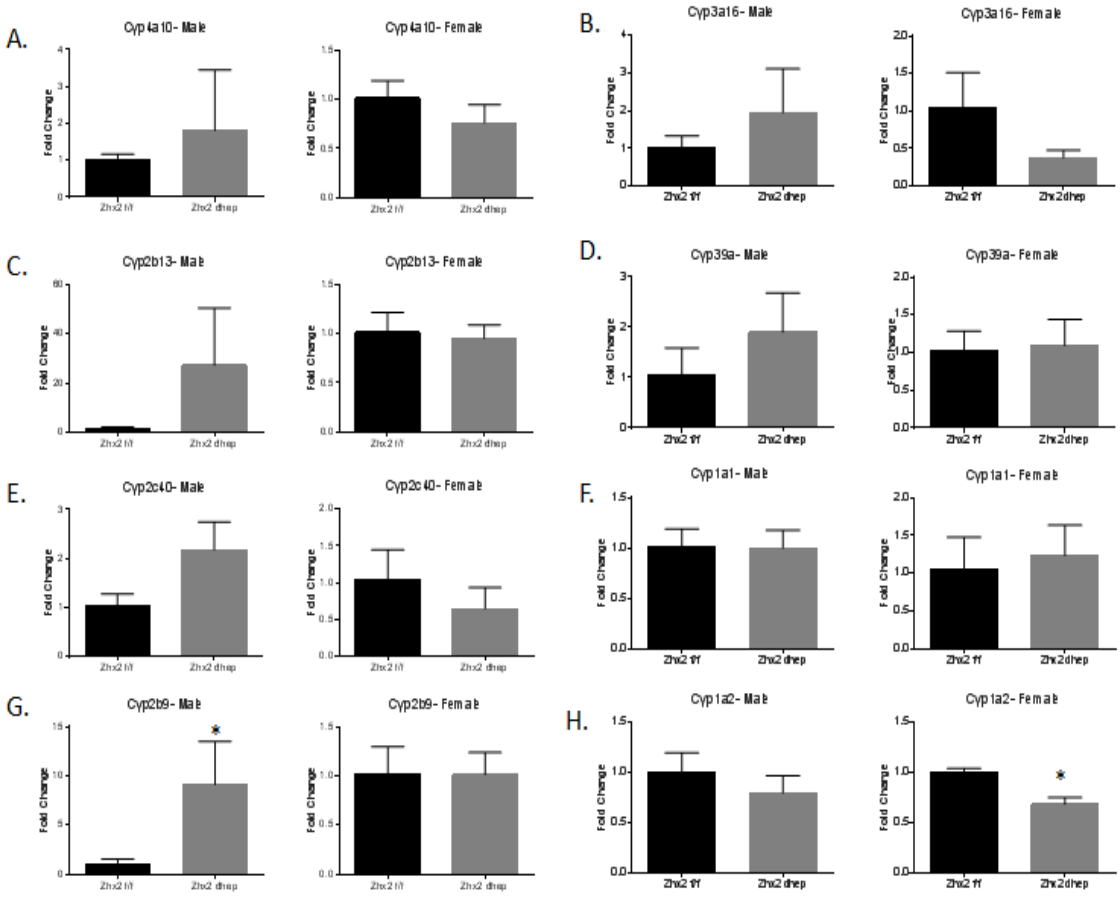


Figure 14. Zhx2 regulation of female-specific Cyps. Eight Cyp genes characterized as being more abundant in females were analyzed. Liver mRNA was analyzed by qRT-PCR in *Zhx2*$^{f/f}$ and *Zhx2*$^{\Delta hep}$ littermates. Although not statistically significant, expression trends are apparent. Two genes, Cyp2b13 (C) and Cyp2b9 (G) increased dramatically in male *Zhx2*$^{\Delta hep}$ mice. Cyp4a10 (A), Cyp3a16 (B), Cyp39a (D) and Cyp2c40 (E) increased modestly in male *Zhx2*$^{\Delta hep}$ mice. Cyp1a1 (F) and Cyp1a2 (H) did not differ with Zhx2 status. Cyp1a2 (H) was lower in female *Zhx2*$^{\Delta hep}$ mice; hepatic Zhx2 expression did not affect expression of other female biased CYPs. *$p<0.05$.

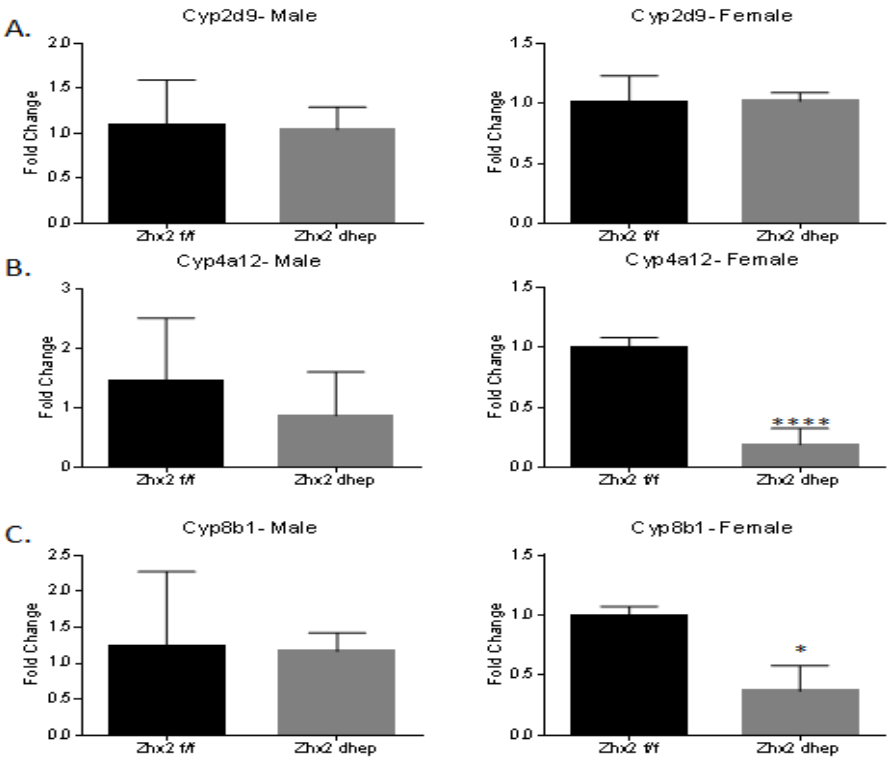


Figure 15. Zhx2 regulation of male-specific Cyps. Three Cyp genes typically more abundant in males were analyzed by qRT-PCR. There is no difference in expression in male *Zhx2*$^{\Delta hep}$ mice from controls. Female *Zhx2*$^{\Delta hep}$ mice had lower expression of Cyp4a12 (B) and Cyp8b1 (C). **p<0.05 , ****p<0.0001.*

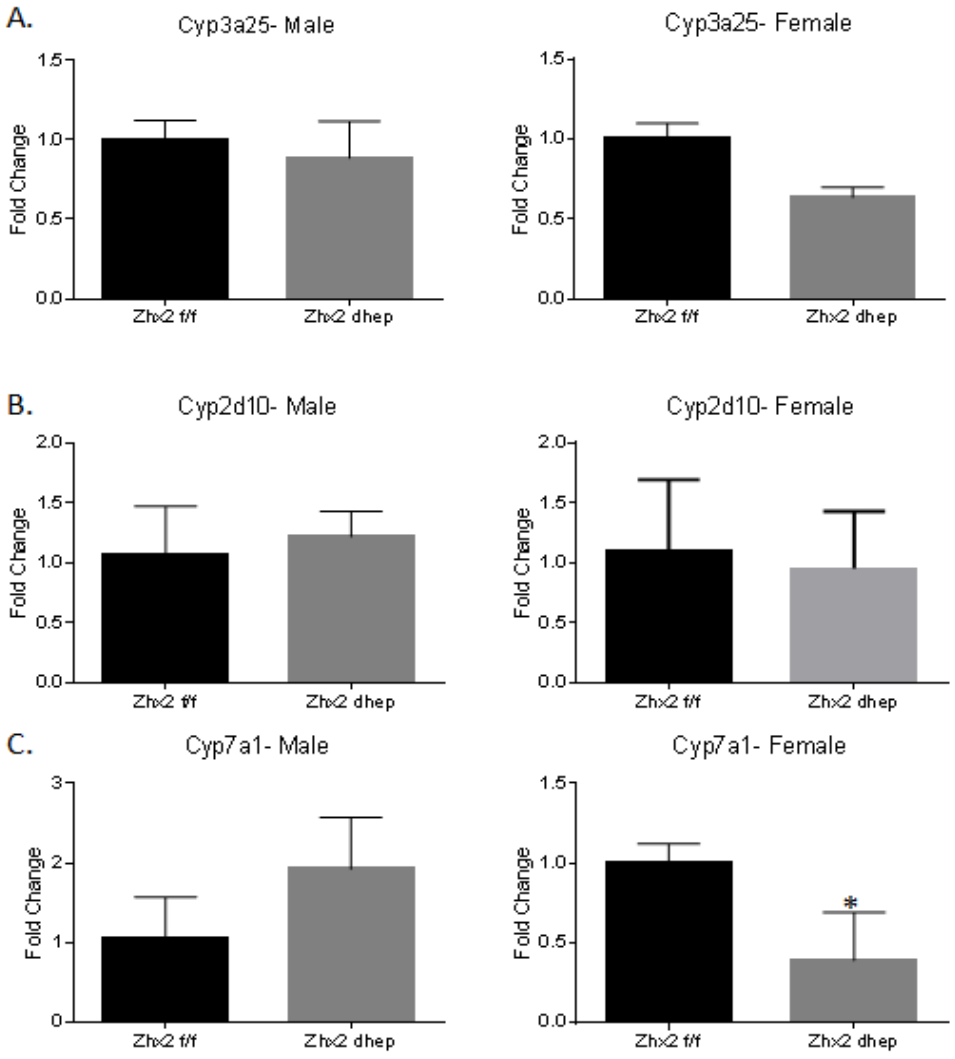


Figure 16. Zhx2 regulation of gender-neutral Cyps. Analysis by qRT-PCR of Cyp genes expressed equally in males and females indicated hepatic Zhx2 does not alter their expression in males. Cyp7a1 (C) was reduced in female *Zhx2*$^{\Delta hep}$ mice. **p<0.05*.

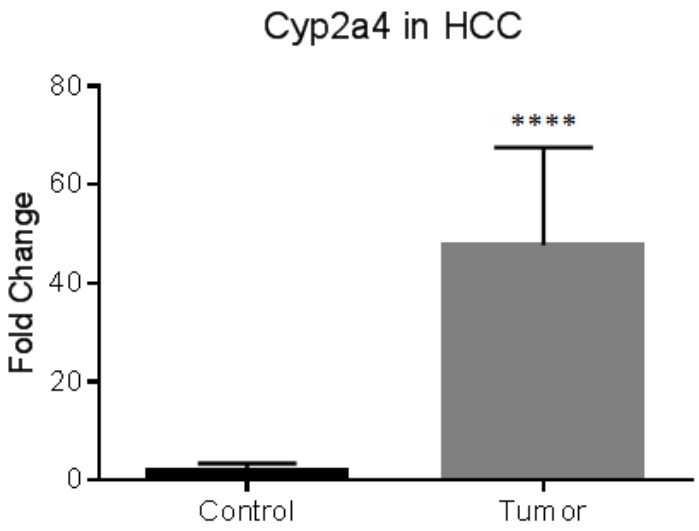


Figure 17. Cyp2a4 is disregulated in HCC. B6C3F1 male mice were injected with DEN (n=15) or PBS (n=5) to induce HCC tumor formation. Samples were collected 36 weeks post-injection. Tumor samples had 40-fold higher Cyp2a4 than control liver. ****$p<0.0001$.

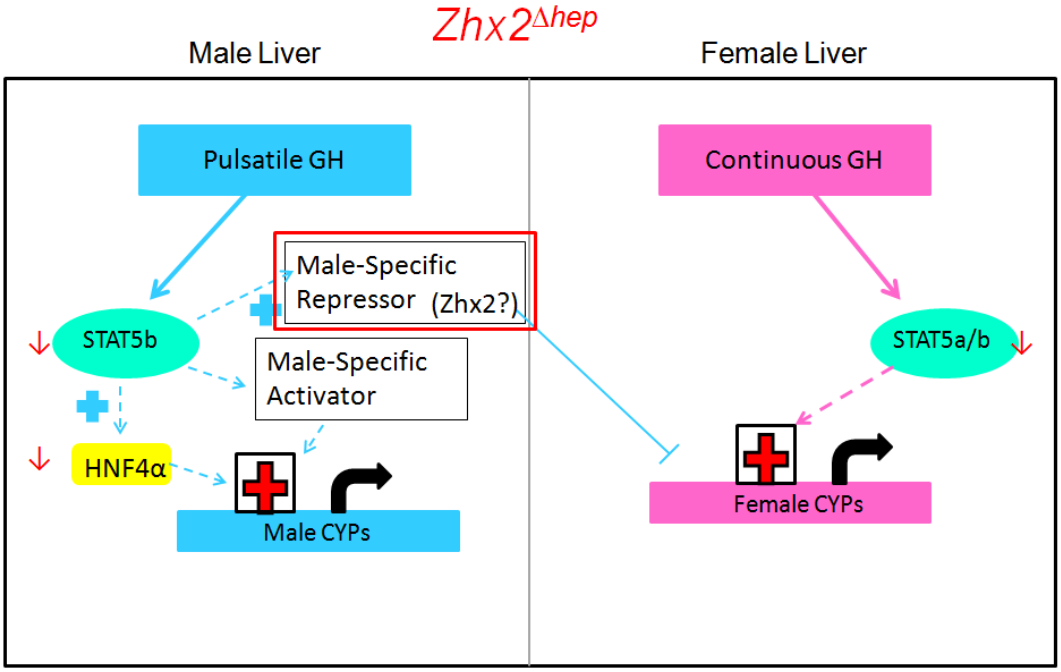


Figure 18. Zhx2 involvement in Growth hormone-STAT5 signaling to regulate gender-specific CYP expression. Female mice have continuous growth hormone (GH) signaling that activates STAT5a and STAT5b heterodimers to promote female-specific CYP expression. Male mice have GH pulses that activate STAT5b homodimers, possibly coordinated with HNF4α, to activate male-specific CYP genes and cause repression of female-specific CYPs. Our data suggest Zhx2 may be the male-specific repressor of female CYPs, explaining the elevated female-specific CYP expression in exhibited in male *Zhx2*$^{\Delta hep}$ mouse livers.

Chapter 5

Zhx2 regulates hepatic Elovl3, a fatty acid elongase with tumor suppressor qualities

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common type of cancer globally, and is the third leading cause of cancer deaths [129]. The prevalence of HCC in the United States is relatively low compared to other cancers and is primarily attributed to alcoholic liver disease and infection with Hepatitis C Virus (HCV) and, to a lesser extent, with Hepatitis B Virus (HBV) [130]. In the past several decades, there has been a surge in both cases and deaths from HCC in the U.S., and a large portion are correlated with the increasing rates of obesity and Type 2 Diabetes [15, 131] With the expected increase of obesity and associated clinical manifestations, the incidence of HCC is expected to increase and be the third leading cause of cancer deaths in the United States by 2030 [24]. The high death rate resulting from HCC is due in part to a lack of early detection methods and poor understanding of early events in the progression of the disease. Currently, the most commonly used detection method for liver damage is serum screening for elevated alpha-fetoprotein (AFP). AFP has been used as a diagnostic marker for liver damage and possible HCC for over 40 years [132]. AFP is expressed abundantly in the fetal liver, dramatically repressed at birth, and reactivated in liver damage and liver cancer, and thus was the first identified and well-characterized HCC oncofetal protein.

In contrast to other mouse strains, AFP continues to be expressed in the adult liver of BALB/cJ mice. This recessive trait has provided the basis for studies on postnatal AFP regulation and enabled us to identify the genetic basis for persistent AFP expression [26]. By positional cloning, we found that the loss of postnatal AFP repression was due to a hypomorphic mutation in the BALB/cJ *Zinc fingers and homeoboxes 2* (*Zhx2*) gene [31, 81]. Several other genes that

are expressed abundantly in the fetal liver and repressed at birth, including H19, Glypican 3 (Gpc3), and Lipoprotein lipase (Lpl, unpublished), are also expressed in BALB/cJ adult liver; these genes are also targets of Zhx2 [29, 81]. Interestingly, all of these targets are also frequently reactivated in HCC. While AFP and Gpc3 are used clinically as diagnostic markers, a functional role for these proteins in HCC cause or progression has not been demonstrated. Due to the fact that AFP, H19 and Gpc3 are reactivated in HCC, there is interest in identifying other Zhx2 targets that might contribute to HCC progression and/or serve as biomarkers of liver disease.

Microarray data provided by our collaborator, Jake Lusis (University of California, Los Angeles) identified *Elongation of very long chain fatty acids-like 3* (*Elovl3*) as a potential Zhx2 target. Elovl3 is a member of a family of seven mammalian fatty acid elongases (Elovl1-7) that are required for de novo synthesis of very long chain fatty acids (VLCFAs) up to 24 carbons in chain length [84]. Each elongase in this family has distinct tissue distribution and substrate specificity, making them important regulators of cellular lipid composition as well as specific cellular functions [133]. The Elovl3 enzyme synthesizes C20-C24 saturated and monounsaturated fatty acids and is most abundantly expressed in skin sebaceous glands, brown and white adipose, and the liver [133]. Prior reports indicate Elovl3 in the skin is imperative for proper barrier function [134] and is important in brown fat for activation of thermogenesis in response to cold exposure [135]. However, the role of Elolv3 in the liver has not been investigated. Here, using several mouse models, we tested whether Elovl3 is a target of Zhx2. Our data indicate that Elovl3 is controlled by Zhx2. In contrast to other Zhx2 targets that are silenced in the postnatal liver and repressed by Zhx2, hepatic Elovl3 mRNA levels increase after birth and are positively regulated by Zhx2. This led us analyze Elovl3 mRNA levels in regenerating liver and in HCC. We find that Elovl3 levels decrease in a mouse models of liver regeneration and in mouse liver tumors. Based on these data, we investigated whether the loss of Elovl3 has a causal role in HCC progression. We have found that forced Elovl3 expression reduces growth of human hepatoma

cells in soft agar. Consistent with this, flow cytometry indicates that Elovl3 alters cell cycle progression and expression of cyclins. Studies by our collaborators in China indicate that Elovl3 mRNA levels are significantly decreased in human HCC samples compared to adjacent non-tumor regions. Taken together, this data indicates that Elovl3 might function as a tumor suppressor in HCC and is a novel biomarker of HCC in humans.

Results

Hepatic Elovl3 mRNA levels are reduced in the absence of Zhx2.

Microarray data suggested that Elovl3 might be a target of Zhx2. However, in contrast to previous Zhx2 targets that are higher when Zhx2 levels are reduced, Elovl3 levels appear to be decreased when Zhx2 levels are lower. This suggests that Elovl3 is positively regulated by Zhx2. To explore this further, studies were carried out in mice in which the Zhx2 gene was deleted in hepatocytes (*Zhx2*$^{\Delta hep}$) mice and littermate controls (*Zhx2*$^{f/f}$) (described in Chapters 2 and 3). To confirm the reduction in Zhx2 levels, qPCR was performed with cDNA from *Zhx2*$^{\Delta hep}$ and control *Zhx2*$^{fl/fl}$ mice (Figure 19). Zhx2 mRNA levels in 5 week old *Zhx2*$^{\Delta hep}$ mice were roughly 3% the levels seen in *Zhx2*$^{f/f}$ littermates, consistent with deletion of the *Zhx2* gene in hepatocytes (Figure 19A). The remaining Zhx2 expression in *Zhx2*$^{\Delta hep}$ liver is likely due to Zhx2 expression in non-parenchymal cells where Alb-Cre is not active. Elovl3 mRNA levels were also significantly reduced in *Zhx2*$^{\Delta hep}$ livers (Figure 19B), indicating that Elovl3 is the first gene positively regulated by Zhx2.

Elovl3 is developmentally activated in the perinatal mouse liver.

Previously identified Zhx2 targets, including AFP, H19 and Gpc3, are silenced in the liver after birth and repressed by ZHx2. In contrast to these genes, Elovl3 appears to be positively regulated by Zhx2. This suggests that Elovl3 might be developmentally activated during the perinatal period. To test this, livers were removed from C3B6F1 mice at e17.5 and postnatal day 1 (p1), p7, p14, p21, p28 and p56. Since Elovl3 is expressed at higher levels in adult

male mice than in female mice, studies were performed in both sexes. RNA was extracted and analyzed by RT-qPCR for Zhx2 and Elovl3. Hepatic Zhx2 mRNA expression is very low but detectable at e17.5 and gradually increased in both male and female livers until p28 (Figure 20A, B). However, Zhx2 levels remain relatively constant in female mice for the next four weeks, but increased roughly 6-fold in male mice between p28 and p56, suggesting that this male-specific Zhx2 increase between four and eight weeks coincides with sexual maturity. Elovl3 in male liver also showed a gradual but modest increase between e17.5 and p28 (Figure 20C). In female livers, Elovl3 levels remain relative constant until p21 but increased somewhat by p28 (Figure 20D). Interestingly, at p28, Elovl3 mRNA levels are 10-fold higher in female than in males. However, between p28 and p56, hepatic Elovl3 levels increase ~10-fold in males and decrease ~20-fold in females. The dramatic rise in Elovl3 in male mice between p28 and p56 coincides with the increase in Zhx2, whereas Elovl3 levels decrease in females during this period even though Zhx2 levels stay relatively constant. This data indicates that male-biased Elovl3 expression is established between 1 and 2 months of age.

Zhx2 regulation of elongase family members in adult liver.

The seven mammalian elongases (Elovl1-7) are similar in sequence and structure, and there is some overlap and redundancy in their enzymatic activities [84, 133]. Due to the strong impact Zhx2 expression has on Elovl3, we examined whether expression of the other elongases at four weeks of age changed in the absence of Zhx2. Elovl1 mRNA levels were significantly lower in *Zhx2*$^{\Delta hep}$ liver. Elovl5 and Elovl6 showed this same trend but the difference between *Zhx2*$^{\Delta hep}$ and *Zhx2*$^{f/f}$ livers did not reach significance. Elovl2 showed no change of expression. Elovl4 and Elovl7 are not normally expressed in the liver and were not detected in these samples (Figure 21 and data not shown).

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

Hepatocytes are normally quiescent in the adult liver, but have the ability to re-enter the cell cycle after injury to restore the liver to its original size and function [136]. This regenerative capacity is of great importance since a number of drugs and environmental chemicals are toxic to hepatocytes. Carbon tetrachloride (CCl_4) is a well-studied hepatotoxin that induces liver regeneration. In adult mice, an intraperitoneal injection of 5 ul of CCl_4 will kill roughly 70% of hepatocytes, and over a 5-7 day period after this insult the liver will regenerate to its normal size. All known targets of Zhx2, including AFP, are silent in the healthy adult liver but are transiently reactivated during liver regeneration, with peak expression occurring ~3 days after injury. Reactivation of these Zhx2 targets is lower in BL/6 mice than in other mouse strains. This low AFP induction in BL/6 mice is a dominant trait governed by a single locus on mouse Chromosome 2 called Alpha-fetoprotein regulator 2 (Afr2). Since every known target of Zhx2 is regulated by Afr2, we tested whether Elovl3 mRNA levels changed in regenerating liver and also be regulated by Afr2. Liver regeneration was initiated in C3H and BL/6 mice by a single IP injection of CCl_4 in mineral oil, or mineral oil alone as control. Mice were killed and livers removed 72 hours post-injection. As expected, AFP levels were low in both strains when treated with mineral oil, whereas the AFP induction was much higher in C3H mice than in BL/6 mice (Spear lab, unpublished). Elovl3 levels were about the same in both strains treated with mineral oil. After CCl_4 treatment, Elovl3 mRNA levels were reduced but the extent of reduction was greater in C3H than in BL/6 mice. Since Elovl3 regulation by Zhx2 is opposite what is seen with AFP, It is not surprising that Elovl3 levels decreased, rather than increased, in regenerating liver. The more robust repression of Elovl3 in C3H compared to BL/6 liver indicates that Elovl3 is also regulated by Afr2 during liver regeneration.

Elovl3 is repressed in a mouse model of HCC.

While the liver regeneration in response to a single insult is a short-term proliferative response, persistent damage and/or treatment with mutagenic

agents can lead to tumor formation [137]. AFP and all other Zhx2 targets are classified as oncofetal genes in that they are expressed in fetal tissues, developmentally silenced at birth, and reactivated in cancer, including hepatocellular carcinoma (HCC). The dramatic reduction in Elovl3 during liver regeneration led us to ask if Elovl3 is also repressed in HCC. Male B6C3F1 mice were treated with the tumor initiator DEN. After 36 weeks, mice were killed. Those receiving no DEN had no tumors, whereas all DEN-treated mice had multiple tumors. qRT-PCR analysis showed an almost complete repression of Elovl3 expression in tumors (Figure 22A). Other elongase family members also exhibited altered expression in tumors (Figure 22B), but not to the same extent as Elovl3. Elovl2 expression increased over two-fold in tumor samples, whereas Elovl6 expression was decreased roughly 60%.

Elovl3 expression reduces anchorage-independent cell growth and stalls cell cycle progression in S phase.

Loss of Elovl3 mRNA and protein expression during liver regeneration and in HCC tumors led us to consider whether Elovl3 may influence cell proliferation. Anchorage independent cell growth is a noted feature of transformed cells, which can be measured by growth in soft agar. We examined the effect of Elovl3 expression in Huh7 cells, a human HCC cell line that has negligible endogenous Elovl3 expression. Cells transfected with expression plasmids for either Elovl3 or pcDNA3.1 empty vector were seeded at 5000 cells per well in media-supplemented soft agar, cultured for one week, and measured by a luminescent viability assay. Elovl3 transfected cells exhibit 35% reduced growth compared to pcDNA3.1-transfected control cells (Figure 23). A similar (~30%) reduction in cell growth was seen in Elovl3-transfected Huh7 cells grown as monolayers (data not shown).

This data led us to look more closely at Elovl3 and cell cycle progression. Hela cells were transfected with a GFP expression plasmid along with Elovl3 expression vector or control pcDNA3.1 and synchronized. Cells were then analyzed by flow cytometry at various times after release from the double-

thymidine block. Propidium iodide (P.I.) staining of GFP-positive cells indicated that as early as one hour after release, Elovl3 expression results in fewer cells in G2 and an increased proportion of cells in S phase when compared to pcDNA3.1-transfected cells (Figure 24). This trend continued to hold for the first 8 hours after release; control cells begin to progress to G2 roughly three hours after release whereas Elovl3 cells do not show noticeable G2 progression until 5 hours, suggesting that Elovl3 stalls cells in the S phase.

The cell cycle is a tightly regulated process that requires the coordinated expression of several cyclins at varying points in the cycle, as well as the association of the appropriate cyclin-dependent kinase (CDKs) [138]. Previous studies have shown that Zhx2 regulates the expression of cyclins E and A [35], which are necessary for the proper progression of cells from G1 to S-phase and S-phase to G2, respectively. As Zhx2 regulates Elovl3 and our data indicate that Elovl3 reduces cell proliferation and alters the cell cycle, we next examined the effect of Elovl3 on cyclin expression at the hourly timepoints after cell synchronization. Cyclins D, E, A, and B mRNA levels are similar in Elovl3-transfected and pcDNA3.1-transfected control cells until hour 3 (Figure 25). At this time, control cells show a surge in expression of Cyclins D, E, and A, and a modest increase in Cyclin B. The increased cyclin expression coincides with the increased number of cells progressing to G2 at this timepoint. In contrast, the surge in Cyclins E, A and B in Elovl3-transfected cells is delayed until hour 5. Elovl3-transfected cells do not exhibit a surge in Cyclin D that is seen in control cells.

Discussion

Identification of Zhx2 was based on the continued expression of AFP and other fetal genes in the adult liver of BALB/cJ mice. This and additional studies indicate that Zhx2 is a repressor of AFP and other target genes that are silenced at birth. Data shown in this chapter indicate that Elovl3 is also a target of Zhx2. In contrast to other targets, Elovl3 is the first gene that appears to be positively regulated by Zhx2. Our data show that Elovl3 levels are significantly reduced in

Zhx2$^{\Delta hep}$ mice compared to *Zhx2*$^{f/f}$ control littermates (Figure 19). Consistent with this data, Elovl3 levels are higher in BALB/cJ mice that express a liver-specific Zhx2 transgene than in non-transgenic BALB/cJ littermate controls (Spear lab, unpublished). In addition, Elovl3 mRNA levels increase during the first four weeks after birth, a time when Zhx2 mRNA levels are also increasing (Figure 20). In vitro studies by our collaborators in China indicate that endogenous Elovl3 mRNA levels in human hepatoma cell lines increase in Zhx2-transfected cells, whereas these Elovl3 levels decrease when Zhx2 levels are reduced by miRNA knock-down (Ma et al, unpublished). While these studies indicate that Elovl3 is positively regulated by Zhx2, they do not distinguish whether this regulation is direct or indirect. Further studies, including ChIP, will be needed to distinguish between these possibilities and to identify the cis-acting site(s) in the Elovl3 regulatory region required for Zhx2 responsiveness. It should also be noted that nuclear run-on studies performed in Dr. Peterson's lab indicate that Zhx2 might act, at least in part, at the post-transcriptional level. Thus, further studies will be needed to fully understand the mechanism by which Zhx2 regulates target gene expression.

It is of great interest that Elovl3 is the first positively regulated Zhx2 target and is expressed in normal adult mouse livers. The increasing levels of Zhx2 and Elovl3 in the perinatal period matches the physiological need for proper skin and fur development [134], thermal regulation [135], and maintenance of storage triglyceride pools [133]. While our studies have focused on liver, future studies should evaluate Zhx2 and Elovl3 in other tissues, particularly skin and brown adipose tissue.

Previous mouse studies have shown that Elovl3 is expressed at higher levels in males than in females [122]. The transcriptional basis for this gender-biased expression is not fully understood. We found that Elovl3 levels increase in both sexes in the first month after birth, but were puzzled to find that Elovl3 mRNA levels were roughly 10-fold higher in female liver than in male liver at p28. However, we observed a dramatic increase and decrease of Elovl3 in male and

female liver, respectively, between p28 and p56, resulting in male-biased expression by 2 months of age that is consistent with other reports. Since this Elovl3 increase in males coincides with a dramatic increase in Zhx2 levels and occurs at a time of sexual maturity, it is possible that male hepatic Elovl3 expression is regulated by growth hormone signaling. Whether Zhx2 is also regulated by growth hormone will require further study. It should also be noted that Elovl3 displays a circadian expression pattern in males, whereas female mice appear to have constant, low-level Elovl3 expression [122, 139]. Zhx2 also exhibits a circadian pattern of expression (K. Esser, Personal communication), suggesting a possible role for Zhx2 in diurnal changes in the expression of Elovl3 and other target genes.

While Elovl3 levels are low in adult female mice, this basal Elovl3 expression is still sufficient to have physiological impact. Zadravec et al. [86] reported that Elovl3 ablation resulted in resistance to diet-induced obesity and was much more pronounced in females than males. The sex difference in the magnitude of expression is similar in the other elongase family members as well. Although the potential impact is unknown, it would be interesting to determine if the constant elongase expression in females result in a favorable lipid pool concentration that confers protective effects and reduces disease risks in women more so than men.

Previously identified Zhx2 targets are disregulated in HCC. Most, but not all studies suggest that AFP and H19 do not have functional role in HCC progression, but there is strong data that Gpc3 can promote cancer [46, 47]. This raises the question whether Zhx2 regulates liver tumor formation. Data from Dr. Chunhong Ma and colleagues at Shandong University in China, with whom we have had a longstanding collaboration, suggest that Zhx2 proteins are primarily in the nucleus in normal hepatocytes but are localized in the cytoplasm in HCC samples. This would suggest that Zhx2 is disregulated in HCC, and that this is occurring at the post-translational level. Irrespective of Zhx2, we have found that Elovl3 is repressed in mouse HCC samples. Preliminary analysis of human

samples at Shandong University show no significant differences in Elovl3 mRNA levels between human HCC samples and surrounding non-tumor tissues, but further analysis is warranted.

The dramatic downregulation of Elovl3 in liver regeneration and HCC samples in mice led us to investigate a causal role for Elovl3 in cell proliferation. By transfections in cultured cells, our data indicate that Elovl3 can repress Huh7 growth in soft agar and in cell monolayers and can alter Hela cell cycle progression and Cyclin expression. A functional consequence of reduced Elovl3 levels could include a diminished supply of saturated and monounsaturated VLCFAs that would disrupt cell membrane integrity and fluidity, and the synthesis of sphingolipids [140]. Elovl3 expression in the liver is necessary for the generation of C20:1, C22:1, and C24:1 VLCFAs. Lipids of these categories are generally incorporated into sphingolipids in the plasma membrane and have been implicated in roles of signaling events, proliferation, and apoptosis [141, 142]. Future studies should explore the relationship between Elovl3 and these lipids and how this might influence cell proliferation.

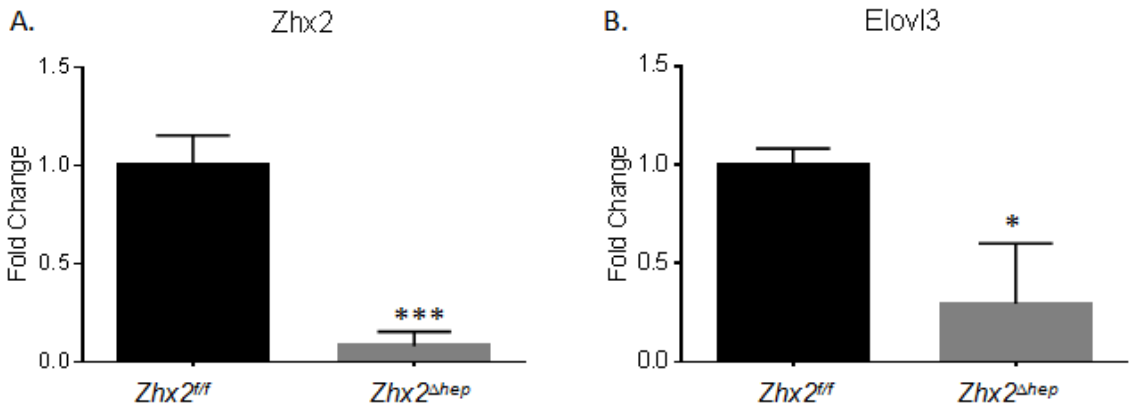


Figure 19. Zhx2 positively regulates Elovl3 expression in adult mouse liver. Liver RNA from male *Zhx2*$^{f/f}$ and *Zhx2*$^{\Delta hep}$ littermates analyzed by qRT-PCR. Zhx2 deletion from hepatocytes (A) results in reduction of Elovl3 expression nearly 75% (B). **p<0.05, ***p<0.001*.

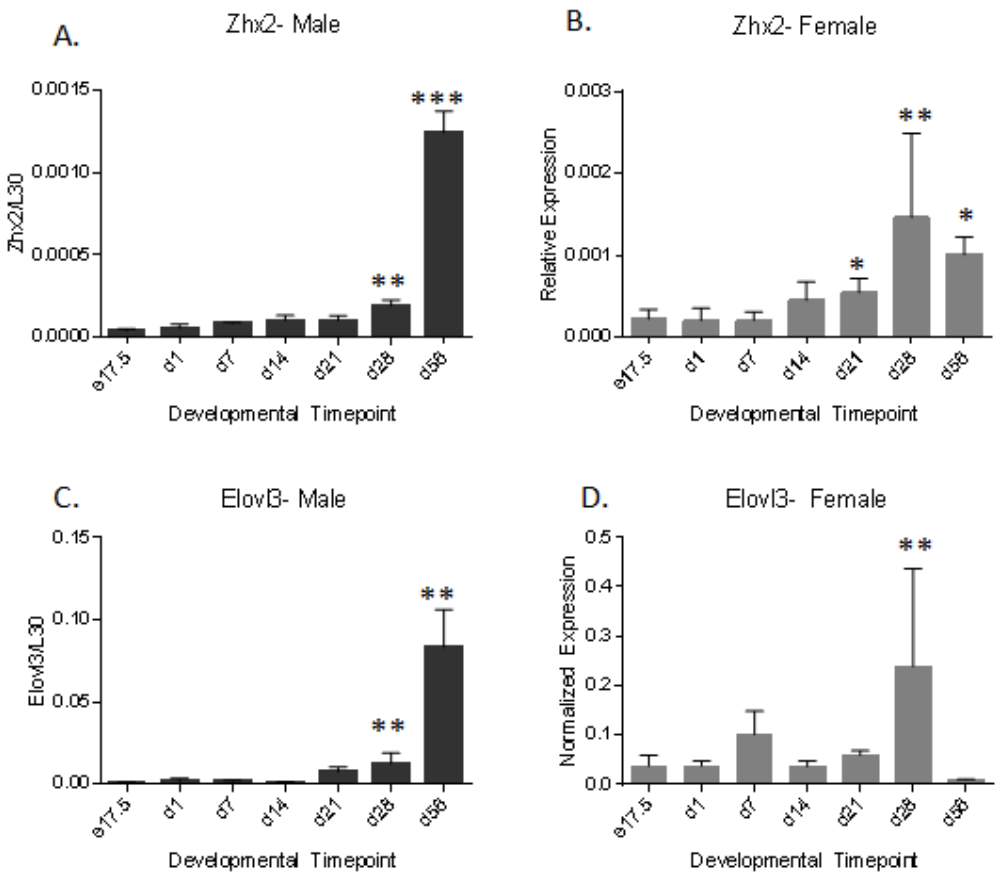


Figure 20. Elovl3 is developmentally activated in the perinatal mouse liver, but silenced in female adult liver. Liver RNA extracted from B6C3F1 offspring collected at developmental timepoints was analyzed by qPCR. Zhx2 expression increases with age (A, B) and Zhx2 increases with sexual maturation in male mice by d56. Correspondingly, Elovl3 expression increases during development (C, D). At d56, female mice have ~20-fold Elovl3 repression while males have ~10-fold increase. Developmental expression compared to e17.5, **p<0.05, **p<0.01, ***p<0.01*.

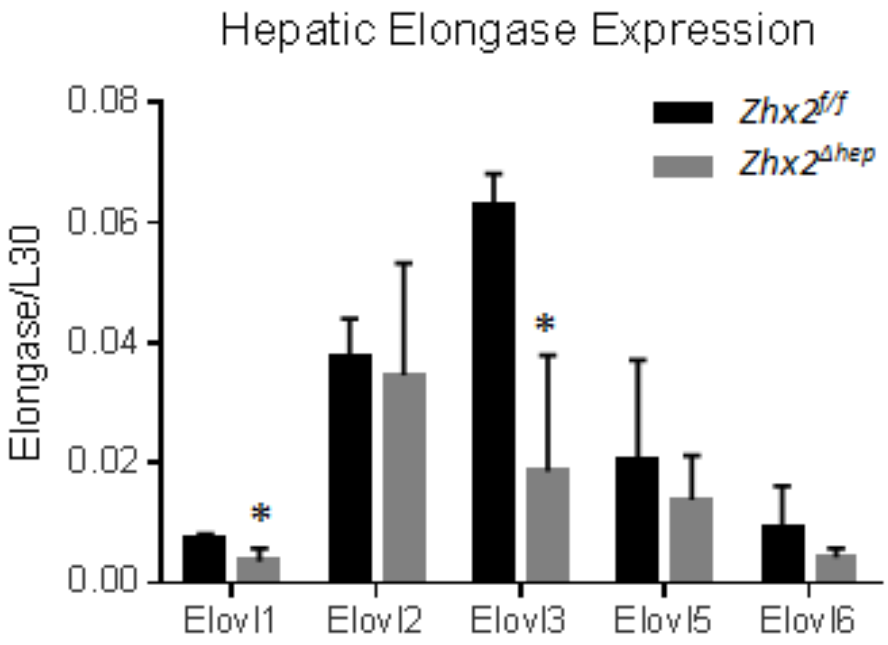


Figure 21. Expression of elongase genes in male liver. Liver RNA from 5-week old *Zhx2*$^{f/f}$ and *Zhx2*$^{\Delta hep}$ littermates was analyzed by qRT-PCR for relative elongase expression. Elovl3 and Elovl2 are the most abundantly expressed elongases in the liver. Elovl1 has low hepatic expression and is reduced in *Zhx2*$^{\Delta hep}$ mice, as is Elovl3 expression. *$p<0.05$.

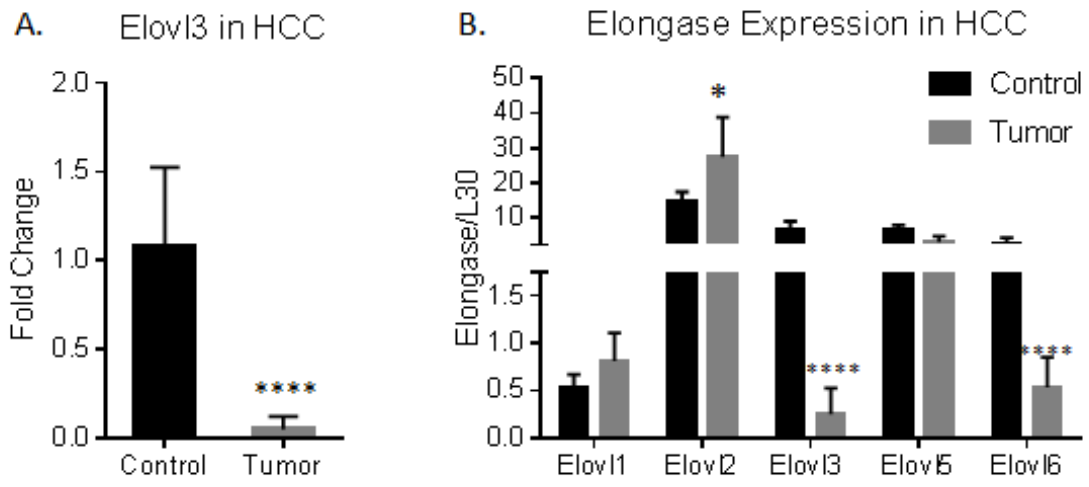


Figure 22. Elovl3 is repressed in a mouse model of HCC. B6C3F1 male mice were injected with DEN (n=15) or PBS (n=5) to induce HCC tumor formation. Samples were collected 36 weeks post-injection. Elovl3 expression was dramatically reduced in tumors (A). Other elongases are also disregulated in HCC (B); Elovl2 is activated and Elovl6 is repressed. **p<0.05, ****p<0.001*.

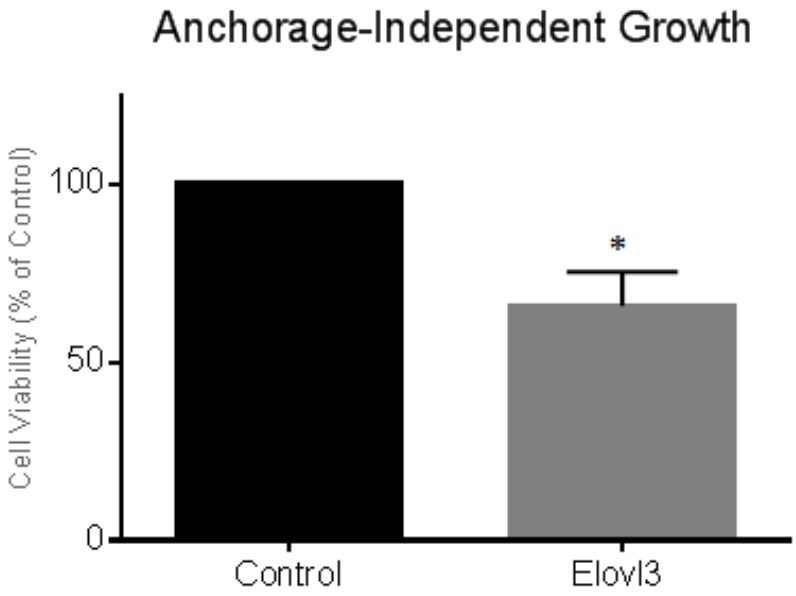
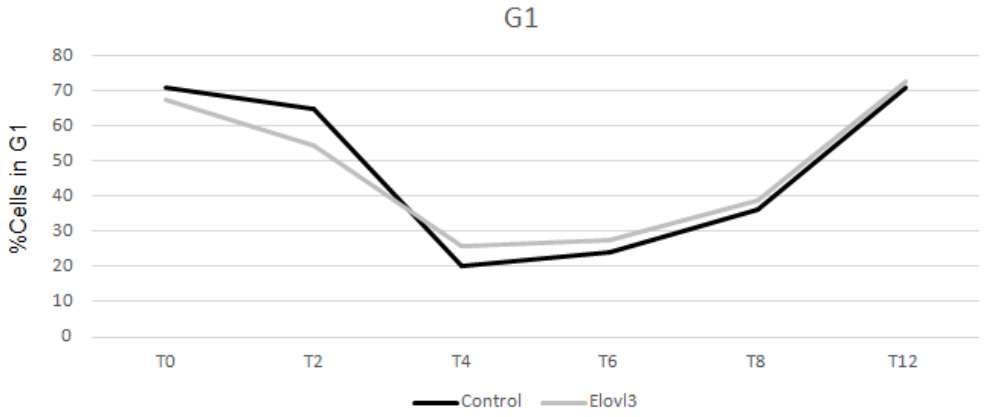
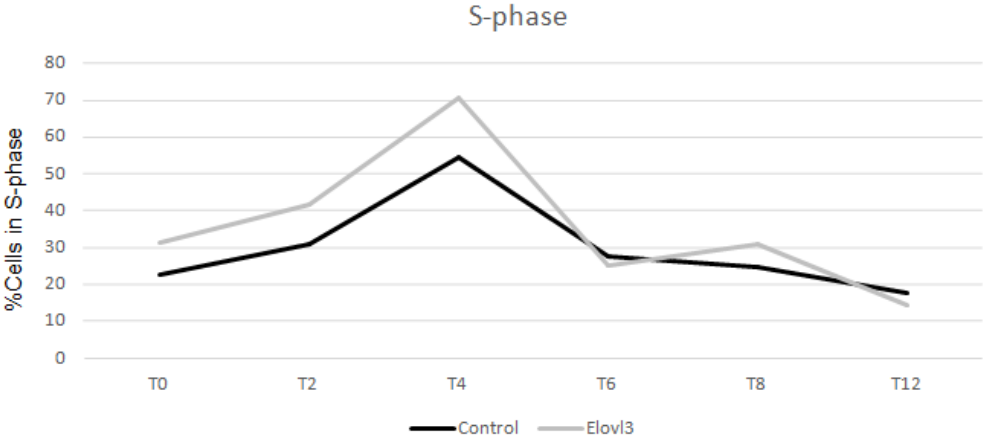


Figure 23. Elovl3 suppresses HCC cell anchorage independent growth. Huh7 cells were transfected with either pcDNA3.1 empty vector (control) or Elovl3 expression plasmid, plated in media-supplemented soft agar and incubated for one week. Cell viability was measured using a luminescent DNA binding dye. Controls were set to 100%. Elovl3 expression reduced cell growth by 30%. *$p<0.05$.

A.


G1
%Cells in G1
80
70
60
50
40
30
20
10
0
T0
T2
T4
T6
T8
T12
Control
Elovl3

B.


S-phase
%Cells in S-phase
80
70
60
50
40
30
20
10
0
T0
T2
T4
T6
T8
T12
Control
Elovl3

C.

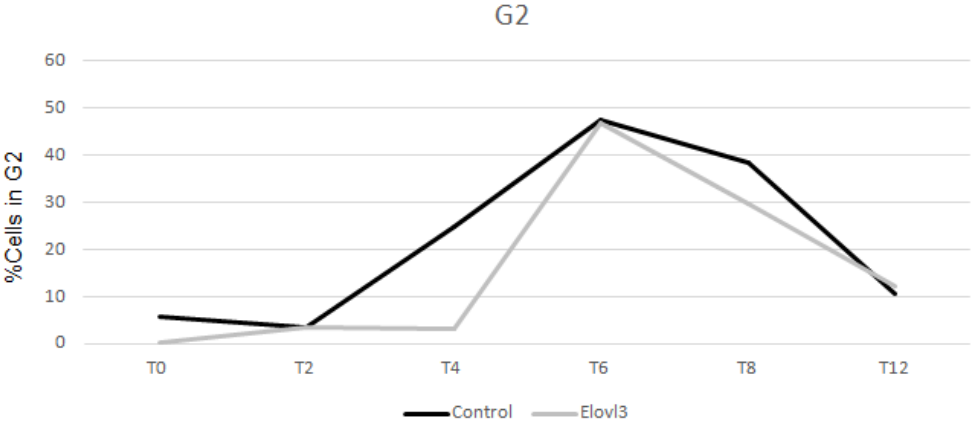
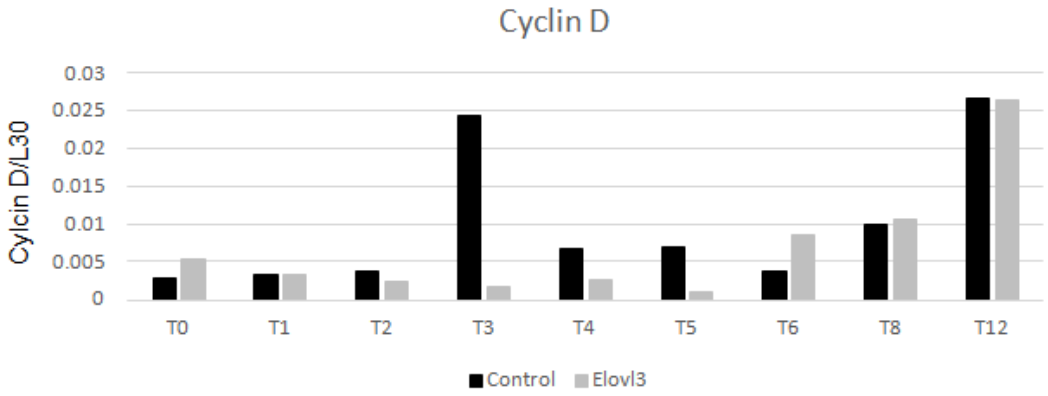
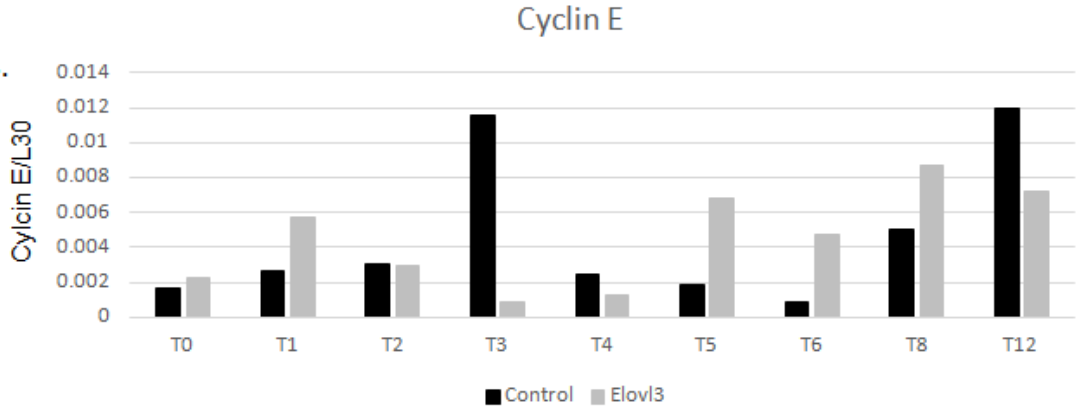

G2
%Cells in G2
60
50
40
30
20
10
0
T0
T2
T4
T6
T8
T12
Control
Elovl3

Figure 24. Elovl3 expression stalls cell in S-phase. Hela cells were co-transfected with GFP and either pcDNA3.1 empty vector (Control) or Elovl3 expression plasmid. Cells were synchronized in G1 then collected at hourly timepoints after release. GFP positive cells were analyzed by FACS to determine the proportion of cells in each cell cycle phase. Elovl3 and control cells are not different in progression through G1 (A). Elovl3 expression increases the number of cells in S-phase (B) which accounts for fewer cells in G2/Mitosis (C). This data suggests that Elovl3 reduces cell proliferation by stalling cells in S-phase.

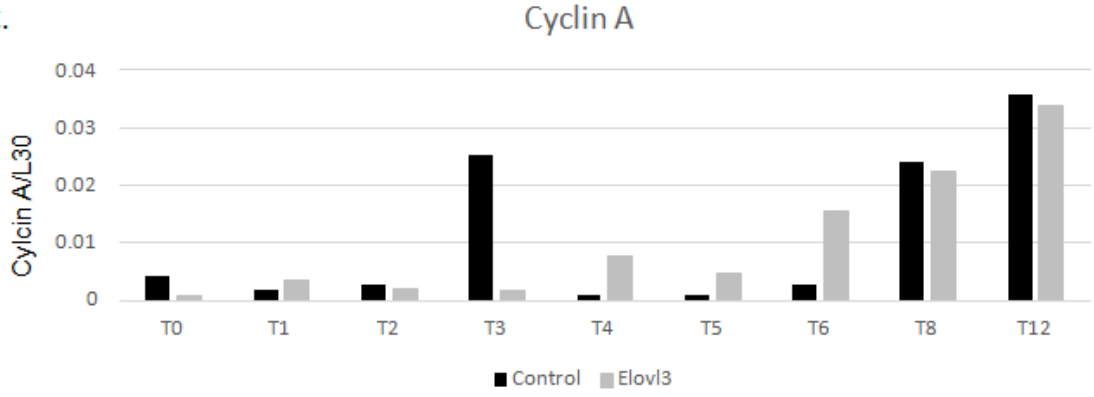
A.


Cyclin D
Cylcin D/L30
0.03
0.025
0.02
0.015
0.01
0.005
0
T0
T1
T2
T3
T4
T5
T6
T8
T12
Control
Elovl3

B.


Cyclin E
Cylcin E/L30
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
T0
T1
T2
T3
T4
T5
T6
T8
T12
Control
Elovl3

C.


Cyclin A
Cylcin A/L30
0.04
0.03
0.02
0.01
0
T0
T1
T2
T3
T4
T5
T6
T8
T12
Control
Elovl3

D.

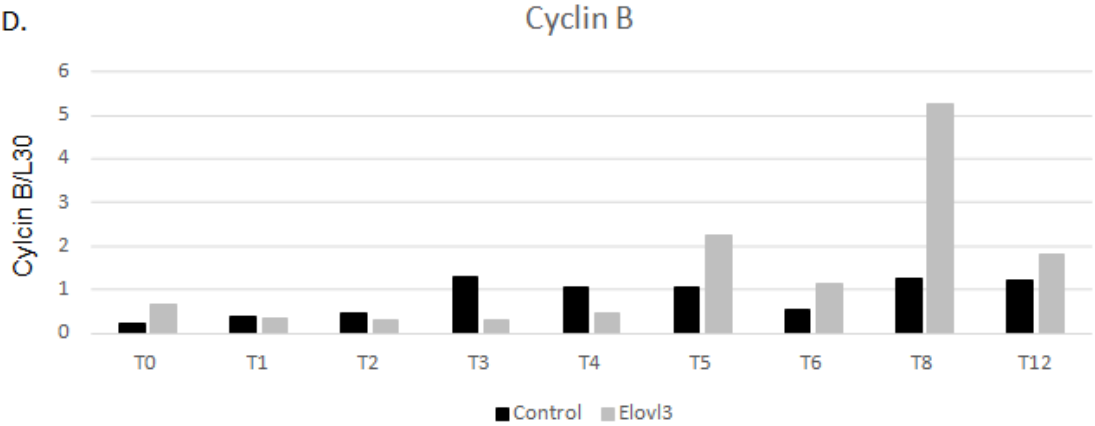

Cyclin B
Cylcin B/L30
6
5
4
3
2
1
0
T0
T1
T2
T3
T4
T5
T6
T8
T12
Control
Elovl3

Figure 25. Elovl3 expression alters expression of Cyclins. Cell RNA was collected at hourly timepoints after release from G1 synchronization and analyzed by qRT-PCR for expression of Cyclin D, E, A, and B. Control cells increase expression of Cyclins D, E, and A three hours after release (A, B, C). Expression of Cyclins is delayed and blunted in Elovl3 transfected cells. Altered expression of Cyclins could contribute to the delay of cell progression through the cell cycle.

Chapter Six

Summary and Future Directions

The data reported in this dissertation characterize Zhx2 as a transcriptional regulator of genes involved in lipid, steroid and xenobiotic metabolism, sex-specific gene patterning, and cell cycle regulation. Zhx2 has previously been reported to act directly on some of its targets to repress expression (AFP, Cyclins A and E, Gpc3) [34, 35, 143]. To date, the mechanism by which Zhx2 regulates many of its targets has not been determined. The fact that some targets are positively regulated while others are expressed suggests Zhx2 may part of a much larger signaling and regulatory complex.

This dissertation describes the first analysis using mice with a floxed Zhx2 allele. My studies in $Zhx2^{\Delta hep}$ mice have identified new Zhx2 targets in entirely new areas of metabolism and cell function. Based on findings in studies with BALB/cJ mice, we expected that Zhx2 regulated many genes in lipid and cholesterol metabolism. I found that Zhx2 regulates ChREBP, Cpt1a, MAT1a, and CD36. The functional parameters of these genes align with characteristics of previously identified targets. Interestingly, Zhx2 appears to impact levels of transcription factors HNF4α and PPARγ. HNF4α, in particular, is a major regulator of liver gene expression. HNF4α is critical to liver function; deletion of the gene is embryonic lethal [96, 144] and loss of expression increases cell proliferation and HCC [145]. My finding that Zhx2 deletion in hepatocytes reduces HNF4α expression implicates Zhx2 involvement, at least by indirect means, of a large portion of genes and metabolic functions in the liver. To gain better insight into the function and impact of Zhx2, ChIP-seq analysis should be performed to identify targets with which Zhx2 has direct interaction, and RNA-seq data will provide a better understanding of the broader effects of Zhx2 on gene expression. Multiple HNF4α binding sites have been found in the Zhx2 promoter (K. Schroeder, personal communications), suggesting potential cross-talk between these factors that govern similar hepatic functions.

Another interesting facet of Zhx2 is its role in developmental gene expression. Zhx2 targets that we have investigated in detail in mouse development (AFP, Cyp2a4, Elovl3) display an expression pattern from embryonic to young adulthood that corresponds with increasing Zhx2 levels during this timeframe. This development regulation has become a hallmark of Zhx2 targets. In addition to HNF4α, the Zhx2 targets Lpl, Gpc3, and Cpt1a are necessary for proper fetal development and mutations in these genes can cause perinatal death. Since $Zhx2^{\Delta hep}$ mice could be obtained, liver-specific Zhx2 expression is not absolutely essential. However, while not included in this dissertation, breeding $Zhx2^{\Delta hep}$ mice revealed an extremely unusual phenotype in that some offspring were born underdeveloped and missing internal organs, and many others dies within 24 hours of birth. Perinatal death in $Zhx2^{\Delta hep}$ mice could be the consequence of its disregulated targets that are important for metabolism. The early developmental abnormalities (absence of posterior half of embryos, lack of internal organs) seen in some of the offspring are quite puzzling, and the physical deformities were more severe than what has been characterized in other gene deletions. These defects are not due to the lack of Zhx2 in the livers of offspring, but are a consequence of the lack of hepatic Zhx2 in the mothers. While we do not know the molecular basis for this phenomenon, it is possible that the maternal hepatic Zhx2 deletion alters nutrient availability and/or ineffective signaling molecules essential for early embryonic patterning. This raises the question of whether maternal Zhx2 expression and proper regulation of Zhx2 targets is required for proper fetal development in humans. Additional research into this topic is needed.

My results identifying Zhx2 as a regulator of Cyp2a4 during sexual maturity of male mice reveals a new role for Zhx2. The dramatic increase in Zhx2 expression seen only in male mice from 4 weeks of age to 8 weeks of age suggests Zhx2 is activated by steroid hormone signaling during sexual maturation. Increased Zhx2 in sexually mature male mice could increase expression of STAT5b and HNF4α, as my data indicates they are both positively regulated by Zhx2 expression in 5 week old mice, but this needs to be further

examined in older mice. My data provides compelling evidence that Zhx2 is an important component of the GH-STAT5-HNF4α signaling axis that is necessary for sex-specific gene expression in the liver. The difference in the expression of multiple Cyp enzymes in males and females has great implications in disease risks. In mice and in humans, males are generally at higher risk than women for fatty liver, liver damage, and HCC . In contrast, women are at higher risk of liver damage from alcohol use [146]; incidentally, women may have lower expression of Cyp2e1, the enzyme that metabolizes alcohol [147, 148]. CVD is the primary cause of death for both men and women in America, but the characteristics differ between sexes. CVD onset is delayed in women with complications appearing about 10 years later than the average age of men [149]. Menopause and hormonal perturbations coincide with the timing of CVD presentation in women. Since GWAS studies have confirmed Zhx2 as a risk factor for CVD in humans [76, 77], it is tempting to speculate that Zhx2 may contribute to the gender differences in disease susceptibility through its coordination of hormone signaling and sex-specific gene patterning. Differential Cyp gene expression could contribute to altered steroid and xenobiotic metabolism and directly impact the development of both fatty liver and cardiovascular disease, as well as the efficacy of therapeutic interventions for these conditions. Therefore, the cardioprotective phenotype seen in BALB/cJ mice might be due in part to the “feminization” of the liver caused by diminished Zhx2 and altered sex-specific expression of Cyps and other proteins. An extension of this observation would be to see if hepatic Zhx2 deletion causes a switch in gender-associated disease, i.e. if female $Zhx2^{\Delta hep}$ mice have increased liver damage and HCC compared to males. Further, I have established a role for Zhx2 in the sexual maturity of male mice, and our lab has shown its role in gene regulation during development. As sex hormones diminish with age, it is logical to examine the expression of Zhx2 in aging mice as well. It will be interesting to explore the role of sex hormones on Zhx2 expression, hepatic gene regulation, and the resulting risk of both liver damage and cardiovascular disease throughout the life cycle in mice.

Zhx2 regulation of Elovl3 provides novel insight into the role of Zhx2 in lipid signaling and cellular metabolism. AFP and H19 are known to be disregulated in HCC and are clinically relevant as biomarkers, but it is not clear if or how their increased expression is involved in tumor formation. Gpc3, another Zhx2 target, is a cell surface proteoglycan that has been shown to influence liver tumor formation [150]. However, my data showing a link between Zhx2, Elovl3, and cell proliferation represents metabolism as another means by which Zhx2 might influence HCC. Elovl3 synthesizes VLCFAs which often are incorporated into cell membranes, constitute sphingolipids, and are conductors of signaling events.[141, 142] There is growing interest in the role of sphingolipids, and ceramide metabolites, in regulating important cell fate processes such as growth and proliferation, apoptosis, and autophagy, among others. Ceramides and their analogues are being tested as therapeutics for cancers due to their role in cell cycle arrest and inducing apoptosis [151, 152]. My data shows that Elovl3 expression contributes to cell cycle arrest, but the mechanism is unknown. It is possible that treatment of cancerous cell lines with Elovl3 lipid products could recapitulate the results of my study. Work is being done in our lab to isolate, culture and transfect primary hepatocytes, which will be an ideal model to perform these studies. Transfections with Elovl3 expression plasmids in these cells followed by LCMS analysis could identify the ceramide or sphingolipid compounds resulting from Elovl3 activity under normal condition. These lipids or synthetic analogs could be used to treat cells exposed to carcinogenic toxins to evaluate the effectiveness in regulating cell proliferation in a disease model. Further understanding of the role of Elovl3 in cell cycle regulation could provide a new avenue for therapeutic potential.

Zhx2 regulates genes involved diverse functions that are important for health and normal metabolism. This makes Zhx2 a potential target for manipulation and mutation in disease. Many of the processes that are disregulated in $Zhx2^{\Delta hep}$ mice parallel activities attributed to the mTOR pathway. mTOR complex 1 (mTORC1) receives signals from insulin and growth factors and senses nutrient status through AMP/ATP ratio and amino acid abundance

[153]. mTORC1 activation results in new gene transcription, increased de novo lipogenesis, increased cell growth and proliferation, and inhibition of autophagy. mTOR is regulated by feedback inhibition from diminished nutrient availability (AMPK), amino acid deprivation, and glucocorticoid signals. When disregulated, mTOR activity proceeds without inhibition and can lead to diseases, including cardiovascular disease and cancer [153, 154]. Zhx2 is integral in conducting signals from GH and effecting gene expression regulation in response. My data suggests that disregulated Zhx2 function disengages nutrient sensing of lipids in hepatocytes resulting in excess fat accumulation, a condition that is exacerbated by faulty lipid export. In HFD conditions, the fatty liver environment in $Zhx2^{\Delta hep}$ mice provides abundant lipid substrate required by cancer cells for membranes and signaling molecule in rapidly dividing cells [155]. The apparent overlap of mTOR activity and Zhx2 regulation of lipid synthesis, growth signaling, cell proliferation, and associations with CVD, cancer, and multiple other disorders suggests Zhx2 could be a part of or affected by mTOR pathways. Future studies into a relationship between mTOR and Zhx2 disregulation in lipid metabolism and cell growth could provide new insight into key events in early stages of CVD and liver disease, including HCC.

Teklad Custom Research Diet Data Sheet

TD.94059 Purina #5015, Cocoa Butter, Cholesterol, etc.

Formula	g/Kg
5015, PMI Mouse Diet	750.0
Casein	75.0
Dextrose, monohydrate	30.0
Sucrose	16.25
Dextrin	16.25
Cocoa Butter	75.0
Cholesterol	12.5
Cellulose	12.5
Mineral Mix, AIN-76 (170915)	8.75
Vitamin Mix, Teklad (40060)	2.5
Choline Chloride	1.25

Footnote

A modification of TD.88051/TD.90221 (Paigen diet) without sodium cholate. The diet contains approx. 15.8% fat (half from cocoa butter) and 1.25% cholesterol.

Selected Nutrient Information[1]

	% by weight	% kcal from
Protein	19.7	20.4
Carbohydrate[2]	41.2	42.7
Fat	15.8	36.9
Kcal/g	3.9	

[1] Values are calculated from ingredient analysis or manufacturer data
[2] Estimated digestible carbohydrate

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- Use within 6 months (applicable to most diets)
- Box labeled with product name, manufacturing date, and lot number
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  - 2 weeks non-irradiated
  - 4 weeks irradiated

Product Specific Information

- 1/2" Pellet or Powder (free flowing)
- Minimum order 3 Kg
- Irradiation available upon request

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My dissertation research has focused on the transcription factor Zinc fingers and homeoboxes 2 (Zhx2) and its regulation of genes involved in lipid metabolism and homeostasis. I have investigated the role of Zhx2 by generating a conditional knockout mouse model and by using in vitro assays to identify Zhx2 targets, characterize the phenotypes associated with their deregulation, and examine the resulting effects on fatty liver, liver cancer, and atherosclerosis.

Post-Baccalaureate Study August 2008 – May 2010
University of North Carolina, Greensboro Greensboro, NC
Human Nutrition & Dietetics GPA: 4.0

Bachelor of Science August 2001 - May 2005
Greensboro College Greensboro, NC
Major: Business Administration/Economics GPA: 4.0
Minor: International Studies Summa Cum Laude

Fellowships and Academic Awards

Graduate School Academic Year Fellowship July 2014- June 2015
- University of Kentucky: $15,000 + Tuition

NIH T32 Predoctoral Training Fellowship December 2012 – July 2014
- Nutrition and Oxidative Stress
- $23,500/year (2 years maximum support)
-

Daniel R. Reedy Quality Achievement Award August 2012 – May 2015
- $3000/year supplemental funding award (renewed 3 years)

Publications

Gedaly R, Angulo P, Chen C, **Creasy KT**, Spear BT, Hundley J, Daily MF, Shah M, Evers BM. *The Role of PI3K/mTOR Inhibition in Combination with Sorafenib in Hepatocellular Carcinoma Treatment.* Anticancer Res. 2012 Jul; 32(7):2531-6.