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INSIGHTS INTO HEPATIC ALPHA-FETOPROTEIN GENE REGULATION
DURING LIVER DEVELOPMENT AND DISEASE

THESIS

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

By

Erica Leigh Clinkenbeard

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

Lexington, Kentucky

2011

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ABSTRACT OF DISSERTATION

INSIGHTS INTO HEPATIC ALPHA-FETOPROTEIN GENE REGULATION DURING LIVER DEVELOPMENT AND DISEASE

The liver is an essential organ for cholesterol homeostasis. If this process becomes dysregulated, cardiovascular disease (CVD) develops. Zinc-fingers and homeoboxes 2 (*Zhx2*) as an important hepatic gene regulator and contributes to CVD. BALB/cJ mice, with mutant *Zhx2* allele, have fewer atherosclerotic plaques compared to other strains on a high fat diet. In my dissertation, I focused on the liver phenotype in BALB/cJ mice on a high-fat diet and found increased liver damage compared to wild-type *Zhx2* mice. These data indicates that reduced *Zhx2* in the liver leads to CVD resistance, but increases liver damage. Therefore, *Zhx2* has an important role in lipid metabolism and liver function.

Hepatic alpha-fetoprotein (AFP) is expressed abundantly in the fetal liver and repressed after birth regulated through three enhancers (E1, E2, and E3). E3 activity is restricted to a single layer of hepatocytes surrounding central veins (pericentral region) along with glutamine synthetase (GS). In my dissertation, I explore pericentral gene regulation in the adult liver. A GS enhancer (GS^c) also exhibits pericentral activity which, along with E3, is regulated by the β -catenin signaling pathway. Orphan receptors, Rev-erb α , Rev-erb β , and ROR α , contribute to E3 activity elucidating a potential mechanism for zonation.

Keywords: AFP, *Zhx2*, zonation, β -catenin, cholesterol

Erica Leigh Clinkenbeard
January 30, 2012

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DURING LIVER DEVELOPMENT AND DISEASE

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CHAPTER 1

Introduction

Liver Cell Composition

The liver is the largest internal organ in mammals and it plays a key role in metabolic homeostasis, biosynthesis and xenobiotic clearance [1]. Gestational events in the fetal liver are crucial for proper development of all hepatic cell types [2]. In mice, the liver bud forms from hepatoblasts that migrate from the foregut endoderm and invade the septum transversum mesenchyme; this process begins at about embryonic day 8.5 (e8.5) and is essential for generating all cell types in the adult liver [3, 4]. Mesenchymal cells include progenitors for hepatic stellate cells and hepatic fibroblasts [5]. Signals from these cells, as well as cardiac myocytes, promote the proliferation of hepatoblasts [6] and induce fetal expression of hepatic genes, including albumin and alpha-fetoprotein (AFP), through the activation of transcription factors such as Hepatocyte Nuclear Factor 3 (Foxa) and CCAAT/enhancer-binding protein (C/EBP) [7]. β -catenin is a key signaling molecule (described below in greater detail) important for cell proliferation and expansion of the liver bud. Several groups have shown the absence of β -catenin early during liver development is embryonic lethal, demonstrating a critical role for β -catenin signaling at this early stage for the liver and other tissues [8-10]. At roughly e13, bipotential hepatoblasts begin to differentiate into mature hepatocytes [albumin-positive] or biliary epithelial cells [cytokeratin-19 (CK19)-positive] which are localized to a region that surrounds the portal vein [11]. As liver maturation continues, biliary epithelial cells form tubular ducts that collect bile, which is later transported to the gall bladder [11]. β -catenin protein levels decrease later in liver development, leading to a reduction in proliferation and ultimately quiescent mature hepatocytes [8]. Gene expression profiles differ substantially between the neonatal and adult liver, which is due, in large part, to different hormonal and nutritional signals [12, 13].

Hepatocytes account for 60-70% of the normal liver parenchyma and are involved in the majority of liver functions, including synthesis of glutamine, bile acid and amino

acids, gluconeogenesis, urea production, glycogen storage, and xenobiotic detoxification. In the adult liver, hepatocytes are generally quiescent although they are capable of proliferation in response to hepatocyte loss [14]. Hepatic stellate cells, in addition to providing growth signals during development, become the major sites of vitamin A storage in adults [15]. In response to persistent damage, due to a number of factors including viral infection and alcohol toxicity, stellate cells are activated and transform into collagen-depositing fibroblasts [16]. In this regard, stellate cells are important in chronic injury that leads to liver fibrosis [17]. While activation of stellate cells leads to injury, depletion of stellate cells reduces the liver's ability to regenerate and decreases survival due to hepatocyte apoptosis [18]. Resident macrophages of the liver, called Kupffer cells, account for about 50% of the macrophages in adult mammals [19]. Kupffer cells can engulf dietary and bacterial toxins, limiting their presence in the enterohepatic circulation and reducing inflammation [20]; another critical role of Kupffer cells is to phagocytose aged erythrocytes [21]. While there is some debate regarding stem cells in the liver, it is generally agreed that a small population of cells in the portal triad region, called oval cells, are bi-potent progenitor cells that can give rise to hepatocytes and biliary epithelial cells [22, 23]. Liver damage caused either by partial hepatectomy or treatment with hepatotoxic agents such as carbon tetrachloride or acetaminophen, elicits a coordinated response by all liver cells to induce proper regeneration through proliferation of quiescent hepatocytes and oval cells [24, 25]. In summary, a variety of cells in the mature liver must function in a coordinated manner for the liver to function properly and respond to stimuli; any imbalance can lead to disease including fibrosis and cirrhosis.

Liver Architecture and Zonation

Proper liver function requires hepatocytes to carry out a myriad of functions. To facilitate this, the adult liver has a unique structure of repeating hexagonal units called lobules (Figure 2A). The middle of each lobule contains a central vein, whereas each of the six corners consists of the portal triad, containing the hepatic artery, portal vein and bile ducts [1]. Oxygen-rich blood via the hepatic artery and nutrient-rich blood via the

portal vein enter the liver and flow along sinusoids towards the central vein, where the blood exits the liver (Figure 2B). Specialized highly fenestrated endothelial cells filter nutrients into the space of Disse, which separates the baso-lateral sides of hepatocytes from the sinusoids [26, 27]. Tight junctions between neighboring hepatocytes create the bile canaliculi which is necessary for the collection of bile released by the apical side of hepatocytes. Bile flows towards the intrahepatic bile ducts, opposite of blood flow, towards the portal triad where they are transported via bile ducts to the gall bladder for storage before release into the small intestine [28]. Kupffer cells reside primarily in the sinusoids nearer the portal vein where they can sense and phagocytose bacteria, damaged red blood cells and macromolecules from the circulation [29]. Vitamin A-storing stellate cells are anchored on the endothelial sinusoid cells in the space of Disse and are more abundant in regions surrounding the portal triad (periportal regions) [17].

The liver carries out a number of opposing metabolic functions, many of which cannot occur simultaneously within the same cell. To overcome this, the liver has developed heterogeneity of gene expression, a fascinating phenomenon called zonal gene regulation [30]. Certain genes are expressed in specific regions along the portocentral axis, with some genes expressed solely in pericentral hepatocytes (those surrounding the central vein) and others expressed in periportal hepatocytes [31] (Figure 2B). Expression of some of these genes is highly restricted to a layer of one to two hepatocytes in a particular zone, whereas others have a more diffuse gradient pattern of zonal expression. The first gene found to exhibit zonal expression was glutamine synthetase (GS) [32]. GS is expressed in liver, brain and kidney and drives the production of glutamine through a reaction between ammonia and glutamate [33]. GS is expressed in all hepatocytes in the neonatal liver but this expression becomes highly restricted in the adult liver to one to two layers of hepatocytes surrounding the central vein [34]. This pattern of GS expression is invariant and not influenced by hormones or nutrients so its pattern does not alter, although the expression of other zonal genes can change in response to external stimuli. The bulk of ammonia is converted to urea in periportal hepatocytes by carbamoyl-phosphate synthetase (CPS1), which exhibits higher expression in periportal hepatocytes [35]. The absence of periportal ammonia-metabolizing enzymes results in death due to ammonia toxicity [36] and patients who are deficient in CPS1 develop life

threatening hyperammonemia [37]. Ammonia detoxification forming urea and glutamine are not the only metabolic pathways that exhibit zonal activity. Gluconeogenesis, required to generate energy, occurs in periportal hepatocytes where the rate limiting enzyme of the pathway, phosphoenolpyruvate carboxykinase (PEPCK), is zonally expressed. PEPCK expression is not static but changes in response to blood-borne nutritional signals [38, 39]. In contrast, Glucose-6-phosphatase is found in pericentral hepatocytes where it converts glucose to glycogen for storage when energy is not needed [40, 41]. A variety of Cytochrome P450 (CYP) enzymes are crucial proteins for xenobiotic metabolism and clearance and many are found at highest levels in the liver. Most CYP enzymes are zonally expressed, mainly in pericentral hepatocytes, but this can change during uptake of chemicals, such as ethanol or phenobarbital, that must be metabolized by a specific CYP [42, 43].

Regulation of liver zonation

Although many enzymes exhibit a zonal pattern of expression, the mechanism of zonal control is not fully understood. One hypothesis is that blood flow along the portocentral axis creates a gradient of oxygen and/or nutrients that can lead to differential gene regulation along this axis [44]. Any mechanism must account for how certain enzymes, including GS, remain static whereas others are dynamic which is found to be regulated, to a large extent, at the level of transcription. Early transgenic studies showed that the 2.5 kb region upstream of the GS gene could confer pericentral regulation to a linked reporter gene. This region contains an enhancer located 2520 to 2148bp upstream of the transcription start site; other studies identified another enhancer within the first GS intron [45]. While no obvious trans-factor binding sequence was identified in the 5' enhancer, this element, when linked to a heterologous promoter, increased reporter gene activity in human hepatoma HepG2 cells. PEPCK, the classically studied periportal gene due to its involvement in energy homeostasis, is regulated by many factors. Glucocorticoids stimulate PEPCK expression through cyclic AMP (cAMP) binding [46] as well as glucocorticoid receptors binding to response elements in the promoter [38]. PEPCK is also activated by the hormones glucagon and repressed by insulin during

starvation and refeeding periods, respectively [47]. However, none of these multiple factors have been identified as being responsible for zonal regulation of the 1kb PEPCK promoter region.

Investigation into known hepatic transcription factors yielded some potential insight regarding mechanisms of zonal regulation. Most of these factors show no change in zonal expression across the liver lobule. However, HNF4 α (hepatocyte nuclear factor 4 α) was expressed mainly in pericentral hepatocytes [48]. HNF4 α is an orphan nuclear receptor that functions during development to promote liver specific gene expression in hepatoblasts and repress mesenchymal genes [49]. Loss of HNF4 α in adult hepatocytes altered zonal gene expression in the adult liver. In particular, GS and several other pericentral genes were expressed in non-pericentral hepatocytes when HNF4 α was deleted. Zonality of PEPCK was also disrupted with the loss of HNF4 α , suggesting a possible role for this factor in zonal regulation [50]. However, HNF4 α regulates many other genes that are not found to have zonal patterns, indicating that HNF4 α is not the sole factor responsible for this regulation [51, 52].

β -catenin

β -catenin, the downstream target of the canonical wnt signaling pathway, is involved in a number of biological processes including the control of target genes [53] and function of adherens junctions with E-cadherin [54, 55]. In the absence of signaling cytosolic β -catenin is post-translationally modified by an inhibitory complex [56] that includes adenomatous polyposis coli (APC), axin, casein kinase 1 (CK1) and glycogen synthase kinase B (GSK3B). This complex binds and phosphorylates β -catenin at specific serine and threonine residues which target it for ubiquitin-mediated proteolysis [56]. Wnt protein binding to a Frizzled receptor activates the signaling pathway, in which the protein disheveled inactivates the inhibitory complex, allowing β -catenin to become stable and accumulate in the cytoplasm [57-59]. Through a mechanism that is not fully understood, the stable β -catenin translocates to the nucleus to activate gene expression. While it does not bind directly to DNA, β -catenin associates with T-cell factor/Lymphoid

enhancer factor (TCF/LEF) transcription factors already bound to sites in target genes [53, 60]. In the absence of β -catenin, TCF/LEF recruits co-repressors, including Groucho, to target genes to actively repress their expression (reviewed in [61]). Upon entering the nucleus, β -catenin interacts with TCF/LEF, resulting in the dissociation of Groucho and recruitment of CBP/p300 coactivators to activate target gene transcription [62], including those involved in cell cycle and cell migration [63, 64].

β -catenin activation is important for the development of the liver as well as many other tissues. Before the foregut invades the septum transversum (STM), a gradient of wnt activation is needed for the appropriate segmentation of the endoderm. At this stage, wnt signaling is inhibited by fibroblast growth factor (FGF) signaling from the STM to promote foregut gene expression [65]. β -catenin activity is re-established along with other signals to promote proliferation and expansion of hepatoblasts in the liver bud [10]. At e14 in mice, β -catenin levels begin to decrease, leading to undetectable levels at birth and causing the hepatocytes to slow their rate of proliferation and begin the maturation process. β -catenin expression is reestablished at postnatal day 10 (P10) in mice and participates in cell-cell adhesion [66] and maintaining stem cell niches [67, 68]. During liver damage, β -catenin is rapidly activated to assist in hepatocyte proliferation [69]. With partial hepatectomy, a loss of β -catenin results in a delayed response of proliferation [70, 71].

Studies of HCC as well as other cancers has revealed a number of mutations in the Wnt signaling pathway, including loss-of-function mutations in the inhibitory complex, mainly in APC, and gain-of-function mutations in β -catenin [72, 73]. If β -catenin is not degraded due to these mutations, it becomes constitutively active and causes uncontrolled proliferation through activation of cyclin D [74]. Cadoret et al, investigating β -catenin mutations in murine HCC, found a correlation with pericentral gene overexpression in different tumor and cell lines using subtractive hybridization. These results were corroborated by injecting mice with an adenovirus containing a mutant β -catenin that remains constitutively active (S37A); this results in increased expression of the pericentral genes GS, OAT and GLT-1. Correlation between GS expression and active β -catenin was found in ~11% of human HCC samples [75]. This

study provided the first association between β -catenin and zonal regulation. A later study found that the negative regulator APC had a zonal pattern in adult liver with the highest APC expression in periportal hepatocytes and a gradual decrease towards the central vein. β -catenin staining was mainly membranous; however, cytosolic accumulation of β -catenin was observed in pericentral hepatocytes. Conditional deletion of APC increased β -catenin activity and increased expression of GS in non-pericentral hepatocytes along with a loss in periportal gene expression. In contrast, blocking β -catenin through adenoviral-mediated introduction of the Wnt antagonist, dickkopf-1, had the opposite effect: a loss of pericentral gene expression and increased expression of periportal genes in pericentral regions [36]. This elegant study provided strong evidence for the role of β -catenin in liver zonation.

Alpha-Fetoprotein

Alpha-fetoprotein (AFP) is a 65kDa serum glycoprotein that is produced during fetal development. AFP is predicted to have a number of functions, including a regulator of osmolarity and transporter of a variety of molecules [76]. During gestation, AFP is highly expressed in the yolk sac, fetal liver and to a lesser extent in the intestine [77]. Elevated maternal serum AFP levels, monitored during pregnancy, can indicate fetal neural tube defects [78] as well as chromosome 21 trisomy [79]. AFP is abundantly expressed in the fetal liver and repressed to nearly undetectable levels during the first few weeks after birth [80]. AFP is often found reactivated in cancers [81] and during liver regeneration from expansion and differentiation of the oval cells [82]. HCC tumors also express AFP that can be detected in serum and therefore, is used as a diagnostic marker [83].

AFP belongs to the gene family on mouse chromosome 5 that includes albumin, vitamin D binding protein and alpha-albumin [84-86] (Figure 3A). The AFP regulatory elements consists of 250 bp minimal promoter as well as three enhancers located between albumin and AFP [87] at positions 2.5 (E1), 5.0 (E2) and 6.5 (E3) kb upstream of the AFP transcription start site [87, 88] (Figure 3B). To understand their contribution to AFP expression, each AFP enhancer was fused individually to a reporter gene

containing the human β -globin promoter linked to the histocompatibility class I gene H-2 D^d (β gl-D^d) and analyzed in transgenic mice. The enhancerless β gl-D^d transgene had no activity in any tissue. In contrast, each enhancer could activate β gl-D^d to high levels in the fetal liver and adult liver. Surprisingly, immunohistochemical analysis with anti-D^d antibodies revealed distinct patterns of expression in the adult liver. E1- and E2-regulated transgenes were expressed in all hepatocytes with a gradual decrease in a pericentral-periportal direction. E3 activity became highly restricted in one to two hepatocyte layers around the central vein, the same pattern of endogenous GS expression [89]. Transgenes with E2 and E3 together exhibited a highly restricted zonal pattern that was identical to transgenes with E3 alone, demonstrating an active repression of transgenes in non-pericentral hepatocytes mediated by E3 [90].

Based on these data, I hypothesize that β -catenin signaling controls zonal E3 activity in the adult liver. The first part of my dissertation will explore this model (Figure 4) in which Wnt/ β -catenin signaling is active in pericentral hepatocytes activating the pericentral genes including E3- β gl-D^d. On the other hand, periportal hepatocytes degrade any cytosolic β -catenin and target genes are actively repressed. In addition, I hypothesize that TCF4 binding sites in E3 and the GS upstream enhancers are required for the activation. Since both AFP and β -catenin have similar developmental profiles and can be reactivated in HCC, I also hypothesize that β -catenin has a role in AFP regulation during development.

Zhx2

Although the AFP gene is dramatically repressed after birth, the basis for this silencing is not fully understood. Clues regarding AFP repression have come from humans with Hereditary Persistence of AFP (HPAFP) and BALB/cJ mice. Several human pedigrees have found an association between HPAFP mutations in hepatocyte nuclear factor 1 (HNF1) binding sites in the AFP promoter [91]. The explanation for HPAFP in BALB/cJ mice is more complex. In 1979, analysis of serum from numerous inbred mouse strains found that one strain, BALB/cJ, had significantly elevated serum AFP levels [92]. The locus controlling this trait was called Alpha-fetoprotein regulator 1

(Afr1). Furthermore, the Tilghman lab showed that BALB/cJ adult liver AFP mRNA levels were 10- to 20-fold higher than adult liver AFP levels in other strains (Figure 5A) [93]. These and other studies suggested that Afr1 was a negative regulator responsible for postnatal AFP repression and that Afr1 was mutated in BALB/cJ mice. Afr1-mediated postnatal repression does not occur through the enhancers [94], but through the 250 bp AFP promoter [95]. Linkage analysis performed in the Spear lab identified a region on mouse chromosome 15 associated with high levels of AFP as well as high H19, another target of Afr1. Examination of this region identified a gene encoding zinc-finger and homeoboxes 2 (Zhx2), a protein predicted to contain two zinc-fingers and four homeodomains. Further investigation revealed that the BALB/cJ Zhx2 gene contained a hypomorphic mutation due to insertion of mouse endogenous retroviral (MERV) element into the first Zhx2 intron, causing aberrant splicing to the element, although correct splicing occurs approximately 5% of the time [96, 97]. Northern blots show absence of Zhx2 elevates H19 mRNA while presence of Zhx2 lowers H19 mRNA to undetectable levels (Figure 5B). A hepatocyte specific transthyretin (TTR) promotes Zhx2 transgene on a BALB/cJ background corrected the persistent AFP and H19 phenotype, indicating that Zhx2 is responsible for the Afr1 phenotype and demonstrating the role of Zhx2 in AFP postnatal repression (Perincheri, Peyton et al. 2008).

Zhx2 was originally identified in yeast 2-hybrid studies to interact with either transcription factor NF-Y and other closely-related family members Zhx1 and Zhx3 [98, 99]. Despite the prediction that Zhx2 encodes a transcription factor and data indicating that it controls the AFP promoter, there is no direct evidence that it binds the AFP promoter (unpublished data Morford L., Peterson, M., Spear B.T.). Nuclear run-on studies indicate that there is no difference in the transcription of AFP and H19 genes between BALB/cJ and other mouse strains, despite the differences in steady-state AFP and H19 mRNA levels (Morford L., Peterson, M., Spear, B.T.). This suggests that Zhx2 might function at the posttranscriptional level. Interestingly, cytoplasmic accumulation of unspliced AFP mRNA is found in wild-type mice, suggesting that Zhx2 might be involved in pre-mRNA splicing and/or export (Turcios and Peterson, unpublished). BALB/cJ mice, on the other hand, maintain correct splicing of AFP which could account for higher AFP mRNA levels in these mice. Since AFP is also frequently reactivated in

HCC, there is interest in whether Zhx2 is also dysregulated in liver cancer. To date, the data are not clear. One study suggested that Zhx2 was repressed in liver cancer, whereas another study saw increased Zhx2 in HCC. [100, 101]. Studies from our collaborator in China, Chunhong Ma (Shandong University), suggested that nuclear localization of Zhx2 might be reduced in HCC. It should also be noted that Zhx2 is not a liver-specific factor but is ubiquitously expressed [96]. An increasing number of studies have implicated Zhx2 in erythrocyte development [102], kidney disease [103, 104], B-cell development [105] and multiple myeloma progression [106].

Liver cholesterol homeostasis

Cholesterol, a hydrophobic molecule, is required for membrane stability, establishing membrane microdomains for signaling [107] and is a substrate for producing steroid hormones and bile acids [108, 109]. Cholesterol can be ingested or can be synthesized *de novo*. Complex regulatory mechanisms have evolved to maintain appropriate physiological cholesterol levels. While all cells are capable of synthesizing cholesterol, the liver is the main site for cholesterol homeostasis by balancing biosynthesis, export, uptake and catabolism of cholesterol. Once ingested, cholesterol is packaged in chylomicrons and transported to the liver via the lymphatic system [110]. Cholesterol is then either metabolized or incorporated into low density lipoproteins (LDL), which also contain triglycerides and transport cholesterol to peripheral sites. Reverse cholesterol transport occurs through efflux using ATP-binding cassette transporter A1 (ABCA1); high density lipoproteins (HDL) transport excess cholesterol from the periphery back to the liver [111]. Balanced levels of cholesterol need to be maintained; an excess of LDL in the circulation can lead to atherosclerosis and arterial cholesterol plaque buildup. This results in cardiovascular disease (CVD), one of the leading causes of morbidity and mortality in developed countries.

Atherosclerosis is a complex disease with genetic and environmental contributions. Based on its clinical significance, there is considerable interest in understanding the biochemical basis for elevated cholesterol and developing improved strategies to reduce serum cholesterol. Sterol regulatory element binding-proteins

(SREBP) are critical regulators of genes that control lipogenesis [112]. Under non-stimulated conditions, SREBP proteins are sequestered in the endoreticulum (ER). Upon stimulation, Sterol sensing SREBP cleavage protein (SCAP) escorts SREBP to the golgi where it becomes biologically active through cleavage events [113, 114]. This leads to SREBP accumulation in the nucleus, where it up-regulates genes required to synthesize triglycerides and/or cholesterol [115]. Importantly, insulin resistance, which often occurs in obese patients, can lead to de-repression of the de novo synthesis genes resulting in an overproduction of cholesterol and triglycerides [116]. In response to excess cholesterol, the rate-limiting bile acid enzyme Cyp7a1 is up-regulated to synthesize bile acids as is the LDL receptor to remove cholesterol from circulation [117]. Production of bile acids using cholesterol as the substrate is seen as the most effective way for the removal of excess cholesterol and one major target for treatment of high cholesterol. Mouse studies have identified apolipoprotein E (ApoE) as an important regulator of cholesterol homeostasis; ApoE knockout mice develop atherosclerosis even when they are fed a normal non-atherogenic diet [118]. Treatments to treat elevated cholesterol in humans have been developed based on these studies and include statins, to inhibit de novo cholesterol synthesis [119], and bile acid sequestrants, to reduce cholesterol recycling from the intestine and increase bile acid synthesis [120].

Various genetic studies, including Quantitative Trait Locus (QTL) mapping, have been used to identify genes involved in cholesterol-mediated atherosclerosis. The lab of Dr. Jake Lusis (UCLA) performed QTL mapping using the mouse strains BALB/cJ, which is resistant to atherosclerosis, and MRL, which is susceptible to atherosclerosis, to identify loci that govern this trait. One locus identified from this study, located on chromosome 15, was called hyperlipidemia 2 (Hyplip2) [121]. Higher resolution mapping localized Hyplip2 to the same interval that contained *Zhx2*. In collaboration with the Lusis lab, we have found that *Zhx2* is the gene responsible for the Hyplip2 phenotype [122]. These data indicate that *Zhx2* also controls genes involved in cholesterol and triglyceride homeostasis.

Microarray analysis performed by the Lusis lab identified genes that might be potential targets of *Zhx2* [121]. The second part of my thesis involves diet studies using the strains BALB/cJ, BALB/c, and BALB/cJ mice containing the hepatocyte specific

Zhx2 transgene. I hypothesize that Zhx2 regulates cholesterol-homeostasis genes, identified from the microarray study, such that cholesterol is unable to get processed and cleared, leading to accumulation in the serum. Unexpectedly, mice lacking wildtype Zhx2 had an increase in serum alanine aminotransferase (ALT) and liver AFP mRNA levels suggesting Zhx2 protects the liver from lipid-induced damage. This part of my dissertation will analyze the liver phenotype in BALB/cJ mice maintained on a high fat diet.

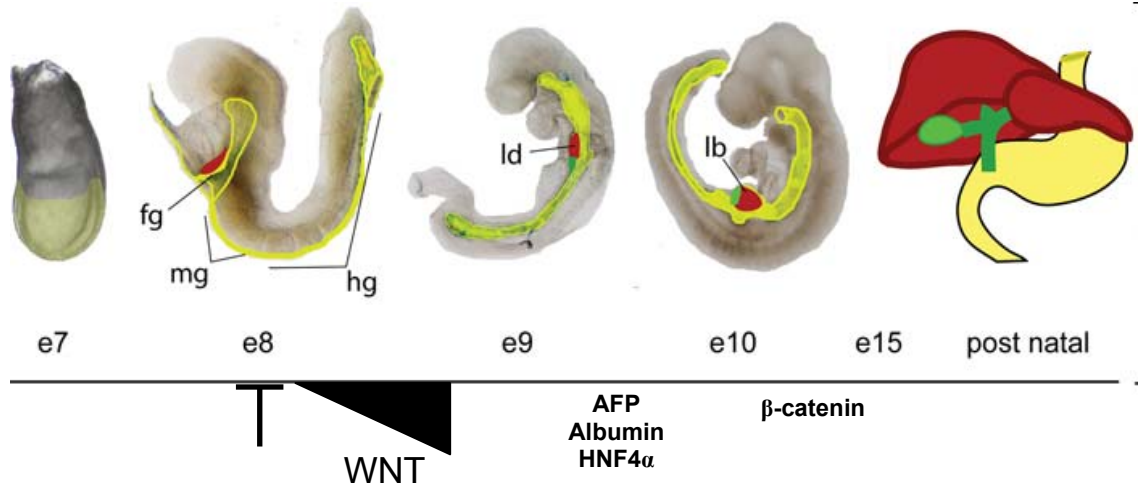


Figure 1. Embryonic liver development modified from [123]. Endoderm formation (in yellow) begins at e7 in the mouse embryo. By e8 the patterning of the endoderm is determined by the gradient of Wnt signaling. Foregut (fg; in red) ultimately developing into the liver, expresses wnt antagonists to prevent signaling in these cells. The midgut (mg) and hindgut (hg) have increasing levels of wnt signaling, the highest found within the hindgut. By e9 the developing liver bud has invaded the STM and begins to express hepatocyte marker genes including AFP, Albumin and HNF4 α . Between e10 and e15 the liver bud undergoes massive expansion mediated in part by β -catenin as well as other growth signals. At e13 the expanded hepatoblasts begin differentiating into hepatocytes or bile epithelial cells. Prior to birth, β -catenin expression has decreased and the architecture develops as cells mature. After birth, the postnatal liver (in red) continues maturing while gradually decreasing AFP expression.

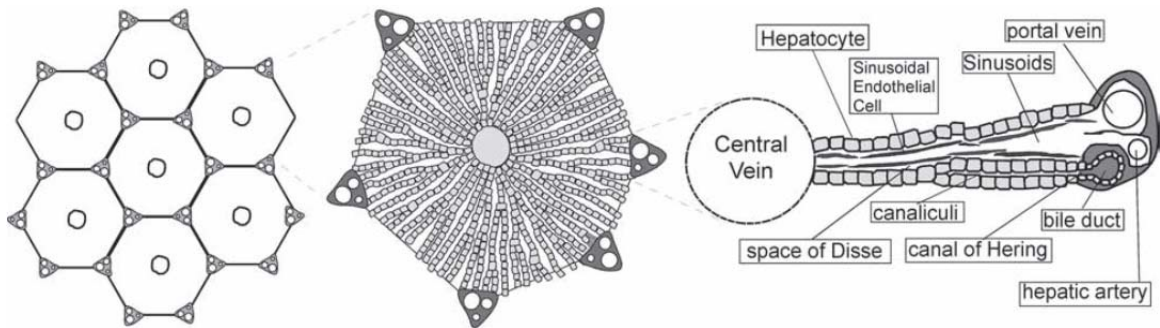


Figure 2. Liver architecture cartoon used with permission from [2]. Hexagonal in shape, the liver lobule is the basic unit of the liver. A central vein (CV) lies within the center of the lobule surrounded by hepatocytes radiating outward to the portal triad. Bile duct, the portal vein and the hepatic artery makes up the portal triad. Blood enters into the lobule at the portal triad via the HA rich in oxygen and in nutrients from the portal vein flows toward the central vein. Bile produced by hepatocytes flows opposite of blood towards the bile duct for collection. Two main zones exist in the liver; periportal hepatocytes surround the portal triad, alternatively, pericentral hepatocytes surround the central vein. One well accepted hypothesis for establishing zonation is that blood flow creates a gradient of oxygen or hormones and other signaling molecules. This gradient therefore creates differential signaling in periportal hepatocytes that receive more of the molecule versus pericentral hepatocytes that receive less.

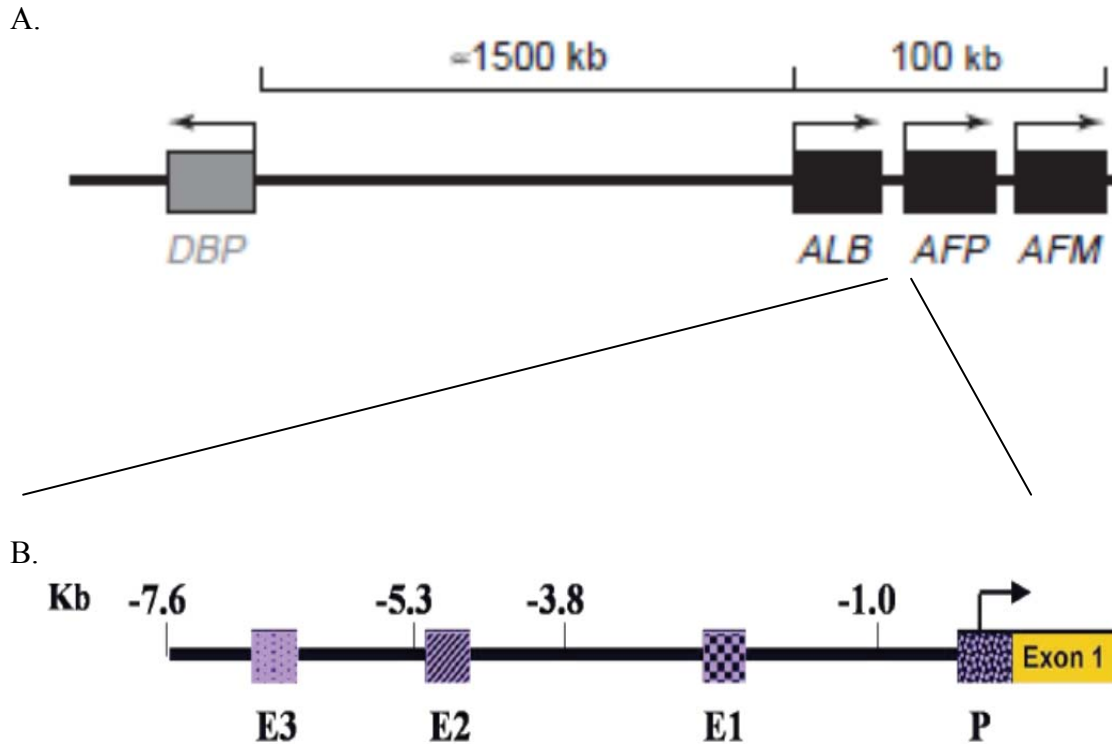


Figure 3. Gene family and regulatory elements of AFP. **A.** Chromosomal representation modified from [124] shows AFP is linked to a gene family on mouse chromosome 5. Albumin (ALB), AFP and alpha-albumin (AFM) are in close proximity to each other while vitamin D binding protein (DBP) lies far upstream separated by three unrelated genes. Duplication events gave rise to these different genes which is reflected by their similar gene and protein structures. While they all are expressed in the liver, the timing of each is slightly different. **B.** In the 14kb intergenic region between AFP and ALB three enhancers E1, E2 and E3 are located at -2.5, -5.0 and -6.5kb, respectively. This enhancer region contributes to early fetal liver expression of AFP and ALB but not expression of the other family members.

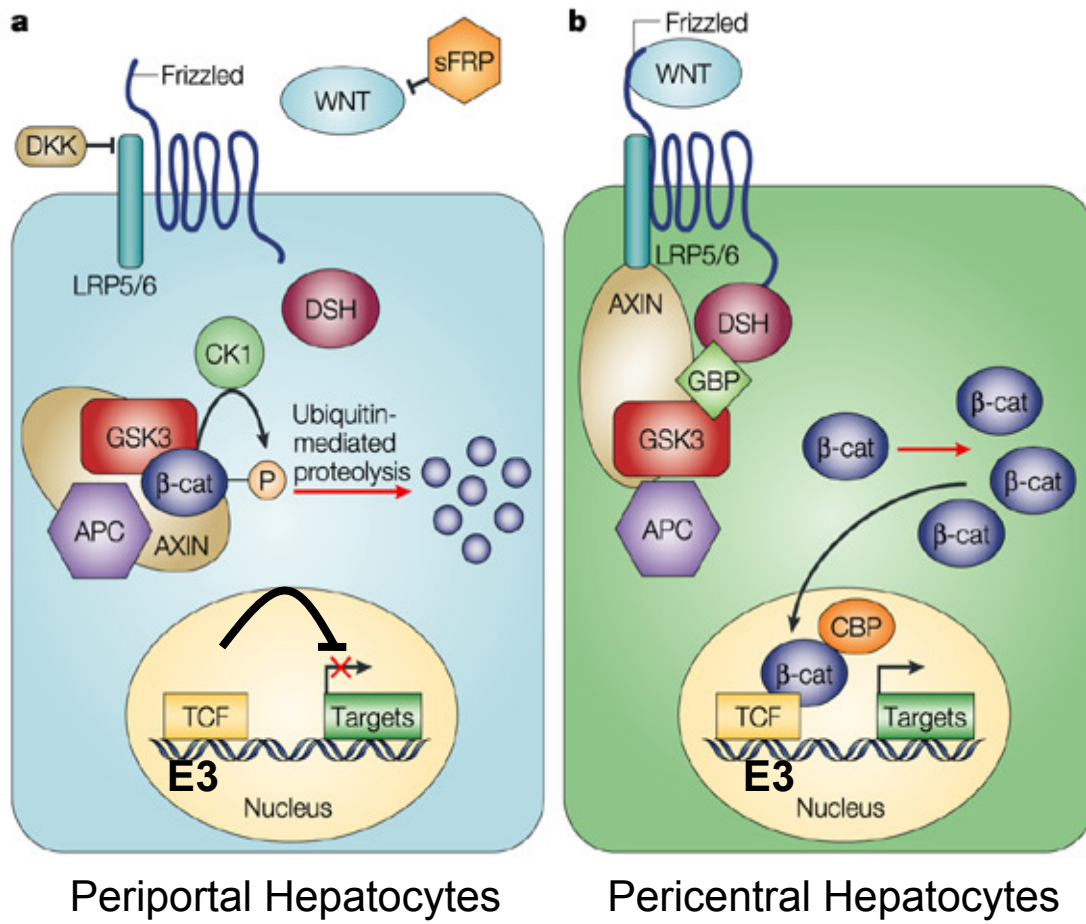
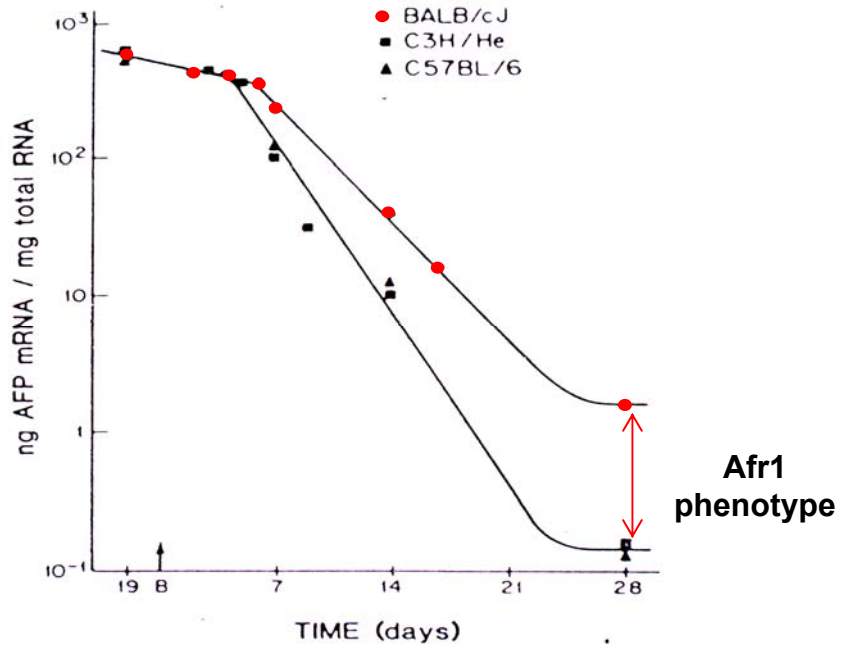


Figure 4. Model for E3 regulation by wtn/ β -catenin used with permission from [125]. Based on previous work showing wnt/ β -catenin signaling pathway is involved in zonation, we have developed a working model for β -catenin regulation of E3 in pericentral hepatocytes. **A.** This cartoon figure depicts events in periportal hepatocytes. In these cells the Frizzled receptor is not active therefore any cytosolic β -catenin is complexed with Axin, APC, GSK3 β and CK1. These proteins phosphorylate β -catenin which is then targeted for degradation by proteosomes. In the nucleus of periportal hepatocytes proteins are bound to E3 actively repressing expression of the reporter gene including TCF4, the traditional β -catenin binding partner. **B.** Pericentral genes, in the right cartoon, have activated wnt signaling although, the wnt isoform responsible for this activation is not known. Activated Frizzled receptor results in Disheveled (DSH) disassembling β -catenin from the inhibitory complex causing cytosolic accumulation. Translocation of β -catenin into the nucleus occurs through unknown mechanisms where it activates target genes by associating with bound TCF4 and recruiting co-activators such as CBP.

A.



B.

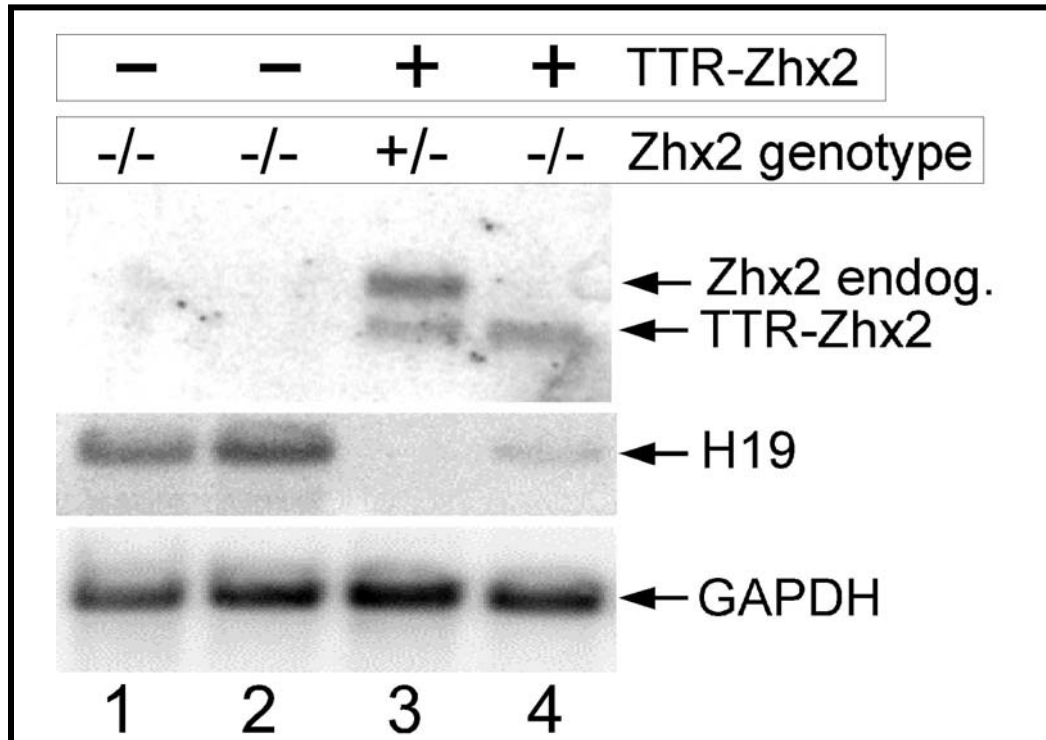


Figure 5. Regulation of AFP by Zhx2. **A.** Liver RNA was extracted and analyzed for AFP expression. This was normalized to total mRNA and values were plotted on a log scale from [93] with permission. Mouse livers at e18.5 express high levels of AFP which is repressed gradually after birth (B). In C3H and C57BL/6 mice this repression continues until it is barely detectable by postnatal day 28 (P28). BALB/cJ mice, in red, reduce AFP after birth although not completely thereby remaining 10 to 20-fold higher compared to wild-type strains C3H and C57BL6. **B.** Zhx2 is responsible for the Afr1 phenotype identified through linkage map analysis. Adult BALB/cJ livers (first two lanes) have very low endogenous Zhx2 expression observed through northern blot. This is coordinated with high expression of H19, another known target of the Afr1. When endogenous Zhx2 is present in the third lane, H19 mRNA levels decrease. Hepatic specific expression of a Transthyretin (TTR)-Zhx2 transgene on a BALB/cJ background decreases liver H19 expression [96]. AFP mRNA expression exhibits the same patterns of H19 with or without presence of Zhx2 from an endogenous gene or hepatic transgene (not shown). This data demonstrates Zhx2 is involved in postnatal repression of AFP and TTR-Zhx2 corrects the Afr1 phenotype. Copyright 2005 National Academy of Sciences, U.S.A.

CHAPTER 2

Materials and Methods

Mice. All transgenic mice generated by the University of Kentucky Transgenic Facility were housed in the Division of Laboratory Animal Research (DLAR) facility and kept according to [Institutional Animal Care and Use Committee](#) (IACUC) approved protocol. All mice had access to food and water and were kept on a 12-12hr light-dark cycle. Mice containing the E3- β gl-D^d transgene or ROR α mutant allele were bred with B16/C3H mice (Jackson Lab). Mice containing TTR-Zhx2-Flag transgene were bred with BALB/cJ mice (Jackson Lab). Hepatocyte β -catenin knockout transgene positive mice were generated through crosses between β cat^{fl/fl}, Alb-Cre and either E3- β gl-D^d or RGS^e- β gl-D^d mice. Offspring from intercrosses were monitored for presence of Alb-Cre and β gl-D^d.

Genotyping. At approximately 14-days old each mouse pup was given an ear tag for numbering and 1mm tail snips were taken. To extract the DNA, the tail pieces were incubated with a lysis solution (100mM Tris-HCl pH 8.5, 5mM EDTA, 200mM NaCl) with added 2.5 units proteinase K (Sigma). After overnight incubation in a 52°C waterbath the remaining material was pelleted through centrifugation at 14,000xg for 12 min. The supernatant containing DNA was decanted into isopropanol to precipitate the DNA. After another centrifugation for 1 min the DNA was washed with 95% ethanol and dried for 5 min. Resulting DNA was resuspended in 250 uL water with vortexing. 2.5 uL of DNA was added to 12.5 uL ThermoStart Master Mix (ThermoScientific), 5 uL water and 5 uL of primer mix using primers in Table 1. 0.2 mL tubes were placed in the thermocycler for the indicated number of cycles. All samples underwent the same protocol: 95°C for 15 min, cycles with 95°C 30 sec, annealing temperature for 30 sec, 72°C for 20 sec and then a final 72°C for 5 min. When complete, 5 uL of 6X loading dye (Fermentas) was added to each sample and mixed by pipetting. 10 uL of sample was loaded into the wells of a 1.5% w/v agarose (SeaKem) gel along with a 100 bp ladder (Fermentas). Electrophoresis separation was done at 140V for 45 min in gel box using 1X TBE. Bands were visualized with UV light and captured using software.

Cloning. Megaprimer PCR was used to generate TCF4 mutations in E3. Platinum PCR Supermix High fidelity (Invitrogen) was added to 1uL of 1:10 diluted E3-pGL3 plasmid along with 400 uM primer mix of pGL3R and E3TCF4mutF (Table 2). After amplification in the thermocycler, the whole sample was separated with electrophoresis in a 1.5% agarose gel. 250 bp amplicon was cut out from the gel using a scalpel, weighed and gel extracted (Gel extraction kit, Qiagen) according to the manufacturer's protocol. 10 uL of the eluted megaprimer was added to 1 uL of 1:100 diluted E3-pGL3, 12.5 uL of ThermoStart Taq and 200 uM of pGL3F primer. PCR was run for 35 cycles with annealing temperature of 45°C and increasing the times during the PCR reaction to 1 min. Once complete, the 450 bp amplicon was gel extracted and ligated into pGEMTeasy (Promega) vector according to the kit protocol. After JM109 transformation and miniprep plasmid DNA extraction (Qiagen), positive colonies, selected through restriction enzyme digestion, were sent off for sequencing (ACGT). Correct plasmids and pGL3 promoter were digested with SacI in 50 uL reaction for two hours in 37°C water bath. Afterwards the plasmids were precipitated by adding 5uL 5M NaCl and 110uL 100% ethanol. Pelleted DNA was resuspended in 43 uL water and immediately digested with BglIII in the 37°C waterbath for 2 hours. The 400 bp E3TCF4mut fragment and digested pGL3 promoter vector were gel extracted on a 0.75% agarose gel and processed with the gel extraction kit. After checking eluted DNA on a 1.5% gel 1uL pGL3 promoter was ligated to 8uL E3TCF4mut fragment with 10 uL 2X buffer and 1 uL ligase. Positive plasmids in JM109 were maxi prepped as described below.

RGS^e was first cloned into pGL3 promoter. RGS^e-βgl-D^d in pUC9 was digested with BamHI while pGL3 promoter was digested with BglIII. pGL3 after digestion was incubated with 5 units of Antarctic Phosphatase in the supplied buffer (New England BioLabs) for 15min at 37°C to remove the phosphate groups and then at 65°C for 10 min to heat inactivate the enzyme. The 350 bp RGS^e fragment and 5 kb pGL3 promoter were gel extracted as described above from a 0.75% gel with the Qiagen gel extraction kit. The two purified fragments were ligated with 2X buffer and ligase from the pGEMT Promega kit. Positive clones in JM109 cells monitored with HindIII for orientation were grown up in maxi prep as described below. For TCF4 mutation in RGS^e two PCR reactions were

performed using 25 uL ThermoStart Taq, 1 uL 1:10 diluted RGS^e-βgl-D^d and a combination of either pUC9F and RGSTCF4mutR (RGS1) and RGSTCF4mutF and βglR (RGS2). RGS1 115 bp and RGS2 300 bp fragments were gel purified from a 1.5% agarose gel. Both fragments were ligated into pGEMTeasy according to the kit protocol. Once positive clones in JM109 were identified both were grown up for maxi prep and extracted using the Qiagen Maxi Prep kit according to the manufacturer's protocol. 5 ug of RGS2 was digested with XmaI, dephosphorylated as described above and purified on a 1.5% gel. 20 ug of RGS1 was digested with XmaI and the 87 bp fragment was purified from a 12% acrylamide gel through electroelution as described in Maniatis Molecular Biology. The DNA was extracted through addition of an equal volume of Phenol:Chloroform:Isoamyl Alcohol (PCI), was ethanol precipitated and the resulting pellet resuspended in 20 uL water. 10 uL of RGS1 was ligated into RGS2-pGEMT XmaI digested vector using 2x buffer and ligase from the pGEMTeasy kit. Minipreps of transformed JM109 cells were digested with BamH1 to determine correct orientation. Positive colonies were digested with BamH1 to excise the RGSTCF4mut fragment. After gel purification RGSTCF4mut was ligated into pGL3 promoter BglII digested and dephosphorylated as described above. Minipreps of RGSmut-pGL3 were digested with HindIII to find positive inserts and determine correct orientation. The final positive plasmid was grown up in maxi prep as described below.

Transformations. Plasmids were transformed into JM109 E coli bacteria by incubating 10uL of plasmid DNA with 100uL JM109 cells on ice for 30 min. Cells were heat shocked for 45 seconds at 45°C and then incubated on ice for 2 minutes. S.O.C. media was added and the cells were shaken at 37°C for one hour. Cells were then pelleted in a one minute centrifugation. S.O.C. was decanted and the cells resuspended in the remaining liquid. Cells were then plated out on LB plates containing ampicillin (Sigma) and incubated overnight at 37°C. If plasmid was from a low stock of an already maxi prepped sample, after shaking, 100 uL of sample was placed on the LB plate without pelleting the cells.

Miniprep. Individual plated colonies were picked and placed in 1.5mL of LB plus ampicillin. Cells grew overnight in a shaker at 37C. Cells were pelleted by one minute centrifugation and the LB media was aspirated. Cells were resuspended in 100 uL of Plasmid Prep Solution 1 (50mM sucrose, 25mM Tris pH8, 10mM EDTA) through vortexing. Fresh 200 uL plasmid prep solution 2 (0.2N NaOH, 1%SDS) was added to the cell mixture and mixed by inversion. 150 uL of 3M potassium acetate was added to each sample and mixed by inversion. 150 uL Chloroform:Isoamyl Alcohol at 24:1 was added to each sample and vigorously mixed. Samples were centrifuged for three minutes at 14,000 x g. Interface precipitated was removed with a toothpick and the upper layer was removed. 900 uL of 100% Ethanol precipitated the plasmid DNA/RNA which was then pelleted through centrifugation for 3 min at 14,000 x g. All liquid was aspirated and pellet was resuspended in 100 uL of water. After vortexing 100uL of 3M Ammonium Acetate (Sigma) was added and mixed by vortexing. 500 uL of 100% ethanol was mixed by vortexing and spun for 3 min at 14,000 x g. All liquid was removed and the pellet washed in 95% ethanol. After drying on the bench for 5 minutes the pellet was resuspended in 50 uL of water.

Maxi Prep. Transformed cells were grown in 450 mL LB with 450 uL amp. After overnight growth at 37°C bacteria was pelleted by centrifugation in Jouan for 20 min. Cells were resuspended first in 10 mL of plasmid prep solution 1 then shaken with 20 mL of plasmid prep solution 2. 10mL KoAc was added and the precipitate was pelleted by centrifugation in the Jouan. The supernatant was passed through gauze and precipitated with addition of isopropanol. Pelleted DNA was resuspended with 3 mL (TE) then cesium chloride (CsCl) and ethidium bromide. Samples were placed in a centrifuge tube, sealed and spun overnight in the ultra centrifuge at 55,000 x g. Bands isolated were transferred to a new centrifuge tube with TE-CsCl. After a 6 hour spin isolated bands had ethidium bromide extracted using isoamyl alcohol. Samples were then placed in dialysis tubing and dialyzed overnight with 0.5M EDTA and 1M Tris-EDTA. After 6 hours of a second round of dialysis DNA was ethanol precipitated. DNA concentrations were measured in the spectrophotometer and diluted to 1ug/uL.

Cell Culture. Cryopreserved Hep3B, HepG2 and HEK293 cells were removed from liquid nitrogen and placed in the appropriate media in T75 Flasks. Hep3B and HEK293 cells grow in Dulbecco's minimal eagle's media (DMEM, Cellgro) supplemented with 10% fetal bovine serum (FBS, Cellgro), 1% L-glutamine and 1% Pen-Strep (Gibco). HepG2 cells are grown in media containing 1:1 mix of DMEM: Ham's F-12(Gibco) supplemented with 10% FBS, 1% L-glutamine, 1% penstrep and 0.1% insulin (Gibco). All cells were cultured in the Napco incubator set at 37C and 5% CO₂.

Transient transfections. Hep3B cells were seeded onto 12 well plates. The following day cells were transfected using the calcium phosphate protocol. 43.5 uL water, 5 uL calcium chloride (CaCl₂) and plasmids: 500 ng of reporter, 1 ug of the expression plasmid along with 12.5 ng of normalizing vector Renilla were mixed together in a microfuge tube. While the samples were bubbled with air, 50 uL 2X HEPES-buffered saline (HBS; 280mM NaCl, 1.5mM Na₂HPO₄, 50mM HEPES, pH 7.1) was added dropwise and bubbling continued for 30 sec. After 30 min incubation at room temperature the mixture was added dropwise into the wells. Each sample was made for duplicate wells. 6 hours later the media was changed by washing the cells with 1X PBS and at 48 hours the cells were harvested.

Dual luciferase assay. Cells were harvested 48 hours after transfection by washing with sterile 1X phosphate buffered saline (PBS) and 10 min incubation with 200 uL Glo Lysis Buffer. Glo lysis collected samples were pelleted with 30 sec 13,000 x g centrifugation. 25 uL of Hep3B or 20 uL of HepG2 supernatant were placed in duplicate into 96-well luciferase plates (CoStar). Analysis was performed on luminometer that injected 25 uL of both dual luciferase substrates (Promega) into the designated wells. Luciferase values were normalized to renilla values.

Nuclear extractions. HEK293 cells were seeded onto 10 cm plates. The following day cells were transfected using the calcium phosphate protocol containing 15 ug of TCF4 expression plasmid. Six hours later the media was changed and 48 hours the cells were scrapped from the plate using 1 mL of PBS. Cells were pelleted for 1 min at 13,000 x g

and supernatant removed. Cell fractions were collected from the cells using the NE-PER nuclear and cytoplasmic extraction kit (Thermo-scientific). Protein concentrations from nuclear fractions were analyzed from each sample in duplicate using the BCA protein concentration assay kit (Peirce) at 562 nm in the spectrophotometer.

Electromobility shift assay (EMSA). Oligos (IDT) in Table 3 were generated based on sequences from E3 and RGS^c enhancers containing the TCF4 site as well as a consensus TCF4 which were resuspended to a final stock concentration of 1 ug/uL. Oligos were annealed using 10 ug of each plus 5M NaCl and water and by heating to 75°C for 3 minutes and cooling to room temperature. The products were checked on a 12% acrylamide gel using a 29:1 bis:acrylamide mixture. 10 uL of wildtype annealed products were radiolabeled with 32P using T4 kinase (Lucigen) according to manufacturer's protocol. After quenching the reaction with 0.5M EDTA the sample was passed through a centri-spin20 column (Princeton Separations) to collect only the kinased sample. Radioactive counts of 1 uL of sample in 2 mL of scintillation fluid (Research Products Int) was measured in scintillation counter. Labeled probes were diluted with water to 15,000 cpm/uL. 5X Binding buffer, dl:dC, nuclear extract and possible cold double stranded unlabeled probe were incubated on ice for 10 min before 1 uL of probe was added and incubated for an additional 30 min at room temperature. Samples were then loaded on a 5% acrylamide gel using a 75:1 bis:acryl mixture and run at 230V for about 1.5 hours at room temperature. After the gel was dried it was placed onto a phosphorus screen overnight in a cassette in the dark. Screens were analyzed with Storm 860 phosphoimager (ThermoScientific) with ImageQuant software.

Diet Study. 6 week old BALB/cJ females (Jackson Lab) and age matched BALB/c female mice (Harlan) along with age matched female Zhx2 transgene positive and negative mice were placed on either normal low fat chow with 6.8% fat or “western style” chow (Harlan Teklad) with 15.8% of fat from cocoa butter for eight weeks. Contents of these diets can be found in Table 3 Mice had access to water at all times during the study. Every week weight measurements for each mouse were recorded and every other week the mice were fasted for four hours for serum collection.

ALT measurement. Every two weeks each mouse was fasted for four hours but still had access to water. Mice were warmed under a heat lamp for increased blood flow and after 5 minutes restrained in a plastic tube. A #20 scalpel blade was used to make a small incision near the tip of the tail across a vein. Approximately 200 uL of blood was collected in a serum separator tube (Becton, Dickson Co.). Centrifugation at 13,000 x g for 1 min separated the serum from red blood cells. 1 uL of serum and 9 uL 0.9% Saline were incubated with 1 mL prepared ALT solution from the ALT kit (Pointe Scientific). Kinetic measurements were taken on the Biomate 3 spectrophotometer (Thermo Scientific) every minute after incubation at 37°C for a total of 3 readings. Differences in the absorbance readings were calculated and multiplied by the dilution factor 1768 to determine concentration (IU/L) of ALT in the serum.

RNA extraction and Real-Time PCR. Approximately 100 mg of liver was placed into 1 mL of Trizol (Invitrogen) in 2063 tube and homogenized at highest speed for 30 sec. All liquid was transferred to 1.5 mL microfuge tube. 200 uL of chloroform was added to each sample and vigorously mixed for 15 sec. Samples were spun down in 4°C cold room at 10,000 x g for 15 min. The top clear aqueous layer was carefully removed and placed into a new tube which was then filled with 500 uL of isopropanol. After incubation at room temperature for 10 min the samples were spun down for 10 min at 10,000 x g at 4°C. Liquid was decanted off and the RNA pellet washed with 1 mL 95% ethanol. Samples were spun again for 5 min and the wash fluid removed. Pellets were dried at the bench for 10 min and resuspended in 100 uL water. Complete resuspension was accomplished by placing the samples in the 52°C waterbath for 10 min. After another round of Trizol extraction the RNA was quantified at 260 nm using a Biomate 3 spectrophotometer (Thermo Scientific) by placing 2 uL of RNA in 500 uL of water. Concentration was determined by applying the dilution factor to the calculation. 1 ug of RNA was processed into cDNA using qscript kit (Quanta Biosciences) according to the manufacturer's instructions and run in the reverse transcriptase protocol in iCycler (BioRad). Each sample was then diluted 1:5 with water. Quantitative PCR was carried out using 2.5 uL of diluted cDNA with 10 uL of Sybr Green (Quanta Biosciences), 5 uL of primer mix and

2.5uL of water. Samples were placed in duplicate in a 96 well plate and covered with film and analyzed with Bio-rad iCycler. All CT (MyIQ) levels were normalized in $\Delta\Delta\text{CT}$ method to ribosomal gene L30.

Immunohistochemistry. Livers frozen in OCT (Richard-Allen Scientific) were allowed to acclimate to -20°C 5 minutes prior to sectioning in the Microm HM505 N cryostat. Slices at 10 μm thickness were placed onto glass slides. For transgene activity, surface staining analysis, the sections were fixed for 10 min in 100% ethanol. After two 10 minute PBS washes the slides were placed in a humidifier chamber at 4°C . Slides were incubated overnight with a 1:75 mix of H2-D^d FITC antibody in PBS. Slides were then washed for 3 min and coverslipped using VECTAshield (Invitrogen). For intracellular/nuclear analysis slides were fixed in 4% paraformaldehyde plus 0.5% Triton-X for 10 min then washed twice for 10 minutes in PBS plus 0.5% Triton-X. Blocking occurred for one hour at room temperature with 10% normal sheep serum or 10% normal goat serum. Primary antibody Rabbit anti-GS (Sigma), were incubated overnight at 4°C in a 1:75 dilution in PBS plus 0.5% Triton-X. After a 3 minute wash the slides were incubated with respective secondary antibody at 1:100 dilution in PBS plus 0.5% Triton-X for 1.5 hours at 4°C . Slides were coverslipped using VECTAshield. Pictures were taken on Zeiss Upright Microscope with AxioVision Software.

Oil Red O. Frozen OCT livers were cryosectioned at 10 μm thick and placed on slides. Sections were first fixed in ice cold formalin (Fisher). Sections were then rinsed in water and equilibrated in propylene glycol (Sigma). Slides were then placed in 60°C 0.5% oil red o (Sigma) in propylene glycol for 8 min. Sections were first placed in 85% propylene glycol for 5 min then rinse. Slides were incubated in haematoxylin for 1 min to counterstain the nuclei blue. After a 3 min rinse in tap water slides were coverslipped using VECTAshield. Pictures were captured using Ziess Upright Microscope and AxioVision software.

Statistical analysis. All values within a group were averaged and plotted as mean \pm standard deviation. p-values were calculated between two groups using student's t-test.

Diet study groups were analyzed with ANOVA followed by Tukey's test. To determine significant interaction between strain and type of diet two-way ANOVA analysis was performed. A p-value less than 0.05 was considered significant.

Table 1. List of Plasmids

Plasmid Name	Description
pcDNA 3.1	Empty expression vector
pGL3 promoter	Promega SV40 promoter driving luciferase
pGL3 control	Promega SV40 promoter and enhancer luciferase vector
WT-pGL3	wildtype AFP E3 linked to E2 in pGL3 promoter generated by J. Butler
SS-pGL3	NR mutant E3 linked to E2 in pGL3 promoter generated by J. Butler
mE3-pGL3	Mouse E3 cloned into expanded MCS in pGL3 promoter generated by J. Butler
RGS ^e -pGL3	PCR amplicon from RGS ^e -Bgl-Dd in pGL3 promoter
mE3mut-pGL3	E3 megaprimer mutation of TCF4 site in pGL3 promoter
RGS ^e mut-pGL3	RGS ^e mutation of TCF4 site in pGL3 promoter
TOP-Flash	Tandem TCF4 sites - minimal promoter - luciferase
FOP-Flash	Tandem mutant TCF4 sites - minimal promoter - luciferase
Renilla	Promega CMV driven luciferase vector
β -catenin	Wild-type β -catenin expression plasmid obtained from C. Mao University of Kentucky
β catS37A	Constitutively active β -catenin expression plasmid obtained from C. Mao University of Kentucky
ROR α	ROR α expression plasmid obtained from J.L.Danan CNRS
Rev-erb α	Rev-erb α expression plasmid obtained from J.L.Danan CNRS
Rev-erb β	Rev-erb β expression plasmid obtained from J.L. Danan CNRS
TCF4	TCF4 expression plasmid obtained from Chunming Liu University of Kentucky

Table 2. Oligos**Genotyping**

βgl-D ^d transgene	F	5' CACTCACCAGCGCGGGTCTGAGTC
	R	5' ACACCCTAGGGTTGGCCAATCTACT
Cre recombinase	F	5' ACCTGAAGATGTTTCGCGATTATCT
	R	5' ACCGTCAGTACGTGAGATATCTT
ROR wildtype allele	F	5' TCTCCCTTCTCAGTCCTGACA
	R	5' TATATTCCACCACACGGCAA
ROR mutant allele	F	5' GATTGAAAGCTGACTCGTTCC
	R	5' CGTTTGGCAAACCTCCACC
TTR-Zhx2 transgene	F	5' TGTCCAGAGTCTATCACCG
	R	5' CCCATAGATTTACCTCAACC

Real-time

AFP	F	5' CCGGAAGCCACCGAGGAGGA
	R	5' TGGGACAGAGGCCGGAGCAG
β-catenin	F	5' CTCTTCAGGACAGAGCCAATG
	R	5' ATGCTCCATCATAGGGTCCA
Rev-erba	F	5' CAAGGCAACACCAAGAATGTTC
	R	5' TTCCAGATCTCCTGCACAGT
PEPCK	F	5' TGGCTACGTCCCTAAGGAA
	R	5' GGCCTCCAGATACTTGTCGA
OAT	F	5' AGGACACTGCCACCCAAAGA
	R	5' GACGAGCGAGCTTACATGCA
RhBG	F	5' GTGTGGGCTTTACCTTCCTCG
	R	5' CGCAGAAGTCAGCGTTGAT
Rnase4	F	5' GAACGGCCAGATGAACTGTCA
	R	5' CTGGTTCTTGCCCTGTATCTA
GLUT1	F	5' ATGTCCACGACCATCATTGC
	R	5' ACCTCGTCGTTCTTCTTCCC
Glutaminase2	F	5' AACCCAGTGGTCTGCGCTAT
	R	5' ACAATGGCACCAGCATTGAC
Glutamine Synthetase	F	5' TTTATCTTGCATCGGGTGTG
	R	5' TTGATGTTGGAGGTTTCGTG
Glut2	F	5' GAAGACAAGATCACCGAACCTTGG
	R	5' CACACCGACGTCATAGCCGAACCTGG
Cyp8B1	F	5' CAGAGAAAGCGCTGGACTTC
	R	5' GGCCCCAGTAGGGAGTAGAC
Lipoprotein Lipase	F	5' TGGCTACACCAAGCTGGTGGGA
	R	5' GGTGAACGTTGTCTAGGGGGTAGT

Oligos continued on next page

Elovl3	F	5' CCTCTGGTCCTTCCTGGCA
	R	5' CGGCGTCATCCGTGTAGATGGC
TNF α	F	5' CATCTTCTCAAATTCGAGTGACAA
	R	5' TGGGAGTAGACAAGGTACAACCC
L30	F	5' ATGGTGGCCGCAAAGAAGACGAA
	R	5' CCTCAAAGCTGGACAGTTGTTGGCA

Probes

mE3 TCF4	F	5' AGATAAAATTCCTTTGATGAAGGAAAA
	R	5' TTTTCCTTCATCAAAGGAATTTTATCT
mE3 TCF4 mut	F	5' AGATAAAATTCCTTTGATGAAGGAAAA
	R	5' TTTTCCTTCATCCCAGGGAATTTTATCT
RGS ^e TCF4	F	5' CATGGAAGGATCAAAGCAAGCCTGC
	R	5' GCAGGCTTGCTTTGATCCTTCCATG
RGS ^e TCF4 mut	F	5' CATGGAAGGACCCGGGCAAGCCTGC
	R	5' GCAGGCTTGCCCAGGTCCTTCCATG
TCF4 consensus	F	5' GGTACTGGCCCTTTGATCTTTCTGG
	R	5' CCAGAAAGATCAAAGGGCCAGTACC
TCF4 consensus mut	F	5' GGTACTGGCCCGGGGATCTTTCTGG
	R	5' CCAGAAAGATCCCAGGTCCTTCCATG

Cloning

pGL3	F	5' CAGTGCAAGTGCAGGTGCCAGAAC
	R	5' GGGACTATGGTTGCTGACTAATTG
E3 TCF4 mut	F	5' AGATAAAATTCCTTTGATGAAGGAAAA
	R	5' TTTTCCTTCATCCCAGGGAATTTTATCT
RGS ^e TCF4 mut	F	5' CATGGAAGGACCCGGGCAAGCCTGC
	R	5' GCAGGCTTGCCCAGGTCCTTCCATG

CHAPTER 3

β -catenin Positively Regulates AFP E3 and RGS^e Pericentral Activity

Introduction

Zonality of enzyme expression compartmentalizes opposing metabolic pathways in different regions of the adult liver. As described in Chapter 1, numerous liver enzymes have been shown to be expressed only in hepatocytes around the central vein (pericentral) or surrounding the portal triad (periportal). While several hypotheses have been proposed to explain this special expression, the regulatory mechanism has yet to be fully elucidated. During gestation, many zonal genes are expressed throughout the fetal liver [34] since architecture has not yet been fully developed. After birth, nutritional signals from the diet and energy demands that begin during the perinatal period help to establish the zonal pattern of gene expression observed in the adult liver. Regardless of the stimulus, intracellular signaling pathways are required to link extracellular events to the nucleus to govern zonal gene regulation.

An elegant study by Benhamouche et al [36] demonstrated that β -catenin signaling governs pericentral gene regulation in the adult liver. β -catenin is the downstream activator of the wnt signaling pathway. In the absence of Wnt signaling, cytosolic β -catenin is complexed with adenomatous polyposis coli (APC), axin and the kinases GSK-3 β and CK1. This inhibitory complex phosphorylates β -catenin at specific serine residues that signal it for ubiquitin-mediated proteolysis. In the absence of this inhibitory complex (i.e., when blocked by wnt signaling via the frizzled receptor), β -catenin is stabilized and can enter the nucleus and regulate target gene expression [126]. In regards to zonal gene regulation, activated β -catenin (through the expression of a non-degradable form of β -catenin or loss of APC) is associated with increased expression of pericentral genes including GS and ornithine aminotransferase (OAT), and decreased expression of phosphoenolpyruvate carboxykinase (PEPCK) and other periportal enzymes [36, 75]. In contrast, blocking β -catenin signaling results in a loss of pericentral enzymes and increased expression of periportal enzymes [127, 128].

β -catenin does not bind DNA directly, but instead regulates target genes through several pathways. In the canonical pathway, β -catenin controls target genes via interactions with T cell factor/lymphoid enhancer factor (TCF/LEF) family of factors [60]. In the absence of β -catenin, TCF/LEF proteins are bound to consensus motifs and silence target genes by recruitment of co-repressors such as Groucho. Upon entry into the nucleus, β -catenin interacts with TCF/LEF proteins, resulting in the dissociation of repressors and recruitment of the co-activators CBP/p300, leading to target gene activation [129]. While β -catenin is clearly involved in zonal gene regulation, the *cis*-acting elements and *trans*-acting factors involved in this control have not been identified.

Alpha-fetoprotein (AFP) is expressed abundantly in the fetal liver and is repressed after birth. While AFP itself is not considered a zonal gene, there are parallels between AFP silencing and zonal gene control. Perinatal repression of AFP begins near the portal triad and continues towards the central vein, with pericentral hepatocytes being the last cells to express AFP until the gene is completely silenced. The AFP gene remains inactive in the adult liver but can be transiently reactivated during liver regeneration and is frequently activated in hepatocellular carcinoma (HCC) and other cancers. Previous transgenic studies have shown that three distinct upstream enhancers, each roughly 300 bp in length, contribute to high AFP activity during fetal development. We showed previously that AFP enhancer 3 (E3) activity in the adult liver is highly restricted to a single layer of pericentral hepatocytes whereas enhancers E1 and E2 are active in a broader pattern across the liver lobule [90]. Immunohistochemical staining shows that hepatic E3 transgene expression overlaps with endogenous GS (Figure 6) suggesting similar regulation mechanisms. Regulation through E3 is dominant; when E3 and E2 are linked together on the same transgene, expression in the adult liver is highly restricted to pericentral hepatocytes as seen with transgenes regulated by E3 alone [90].

Based on previous studies on β -catenin and zonal control, we hypothesized that it was also a key regulator for AFP E3 and enhancers of other pericentral genes. Our lab previously analyzed the upstream enhancer from the rat GS gene (RGS^e) using the β gl-D^d reporter transgene and found that it is also zonally active similarly to endogenous GS. We also showed by immunohistochemistry that E3 activity overlaps with unphosphorylated (nuclear) β -catenin around the central vein. Hydrodynamic tail vein

injections of a constitutively active form of β -catenin (β catS37A) activated E3 transgenes in non-pericentral hepatocytes. In addition, conditional deletion of β -catenin in the livers of adult mice containing either E3- or RGS^e-regulated transgenes resulted in a loss of transgene expression, along with endogenous GS (Butler, J., Spear, B.T. unpublished data). Taken together, these data demonstrate that β -catenin is required for E3 and RGS^e activity in pericentral hepatocytes.

The work described in this chapter builds on our current model of zonal gene regulation by β -catenin. As mentioned above, β -catenin lacks the ability to bind to DNA itself. Therefore, I hypothesized that TCF4 binding sites would be present in E3 and RGS^e and are required for β -catenin-mediated control. Binding site analysis, sequence alignment and electromobility shift assays all provide data to support this hypothesis. Furthermore, binding site mutations and cotransfections in cell culture demonstrate an inability of β -catenin to activate the enhancers. Both β -catenin and AFP have a somewhat similar developmental pattern and are both often found reactivated in liver cancer so I hypothesize that β -catenin activates endogenous AFP early in development through its control of E3. Immunohistochemistry and RNA analysis of postnatal d1 livers provides evidence in support of this hypothesis.

Results

TCF4 binding sites identified in E3 and RGS^e enhancers show high conservation. AFP E3 is a 340 bp element found 6.5 kb upstream from the AFP transcription start site (TSS) and RGS^e is a 400 bp element isolated from 2.5 kb upstream of the rat GS TSS. Both were cloned into the β gl-D^d reporter cassette. Immunohistochemistry, using a fluorescent antibody against the membrane-bound D^d protein, showed that both E3- β gl-D^d and RGS^e- β gl-D^d transgenes were expressed in the same pericentral region as active β -catenin (Figure 7).

The role of β -catenin in zonal gene control raises the possibility that TCF/LEF factors are also involved in this regulation. In several zonal genes, including GS, TCF sites have been identified and ChIP analysis has shown binding of TCF4 proteins. TCF4

is a traditional binding partner for β -catenin. Further analysis revealed that the TCF site in RGS^e is highly conserved across many species, providing further evidence of the importance of this site (Table 3A). My analysis of E3 using the transcription element software search (TESS, University of Pennsylvania) binding site program predicted a strong candidate TCF binding site near the 3' end of E3. While deletion analysis of E3 many years ago suggested a role for the 3' end of E3 in HepG2 cells, the factor(s) that bind this region of E3 were not identified [88]. This putative TCF site in E3 is also highly conserved, further suggesting an important role in E3 activity (Table 3B).

TCF4 proteins bind to putative TCF binding sites in both E3 and RGS^e enhancers. Based on the putative site identification, I investigated whether TCF4 is capable of binding to these sites using electromobility shift assays (EMSA). Nuclear extracts were prepared from HEK293 cells transfected with a FLAG-tagged TCF4 expression vector. Oligonucleotides, 23 bp in length, were generated to contain either the TCF sites from E3 (E3-TCF), RGS^e (RGS^e-TCF), and a consensus TCF sequence (TCFc); double-stranded versions of these 23-mers with mutated TCF sites were also prepared. All wildtype double-stranded products were used as radiolabeled probes or as cold competitors. These data indicated that all three wild-type TCF sites could bind TCF4 and were effective as cold competitors at a 100X molar excess. None of the mutant probes could compete. Further evidence that TCF4 was bound to these sites came from supershifts using anti-FLAG antibodies (Figure 8A-C). These data provide direct evidence that TCF4 can bind the predicted TCF sites in E3 and RGS^e.

β -catenin activates E3 and RGS^e enhancers through the predicted TCF4 sites. To provide further evidence of β -catenin regulation of E3 and RGS^e through the predicted TCF sites, wild-type and TCF-mutant versions of these enhancers were linked to the pGL3 luciferase vector. The TCF mutations were generated by PCR-based megaprimer mutagenesis to incorporate the same mutations as those used in EMSA as cold competitors. These mutations converted three T nucleotides, which are essential for TCF4 binding, to G nucleotides; these changes also create a *Sma*I restriction site. The

full-length mutant amplicons were purified, cloned into pGEMT-easy and sequenced before being subcloned into pGL3.

To determine responsiveness to β -catenin, wild-type and mutant enhancer-pGL3 reporter constructs were transiently co-transfected into Hep3B cells with wild-type and constitutively active β -catenin (β catS37A) expression vectors. TOP-Flash, which contains tandem repeats of consensus TCF4 binding sites linked to a minimal promoter driving a luciferase gene and FOP-Flash, identical to TOP-Flash except the TCF sites are mutated, were used as positive and negative controls, respectively. Hep3B cells were used since they contain less endogenous β -catenin than do HepG2 cells, which are also frequently used in our lab for transfections. Forty-eight hours after transfections, cell lysates were prepared. Luciferase levels from the reporter and renilla were measured using a dual-luciferase assay. TOP-Flash, E3-pGL3 and RGS^c-pGL3 exhibited a dose-dependent increase in activity in response to β catS37A compared to the empty vector control. In contrast, FOP-flash and TCF4 mutant constructs, E3(tcf⁻) -pGL3 and RGS^c(mut⁻)-pGL3, did not respond to increasing β catS37A levels. Similar results were observed using a wild-type β -catenin expression construct although these results were not as dramatic (data not shown). Hep3B cells harbor intact inhibitory β -catenin proteins; therefore a wildtype β -catenin construct can undergo degradation while β catS37A construct does not. These data demonstrate that β -catenin activation occurs through TCF4 at these sites in the enhancers. Interestingly, E3(tcf⁻)-pGL3 and RGS^c(tcf⁻)-pGL3 had increased activity than their corresponding wild-type constructs in the absence of co-transfected β -catenin (Figure 9). This result provides further evidence of β -catenin responsiveness. In the absence of β -catenin, TCF4 recruits Groucho-family co-repressors to their cognate sites. Through the action of other positive-acting factors that bind E3 and RGS^c, these enhancers have greater activity when co-repressors cannot be recruited by the mutated TCF sites are mutated.

β -catenin influences perinatal E3 activity and AFP expression during the perinatal period. In contrast to the pericentral expression seen in the adult liver, E3- β gl-D^d transgenes are expressed in all hepatocytes in the fetal liver [89]. This loss of E3

activity in periportal hepatocytes occurs during the first few weeks after birth. This gradual loss of E3 activity after birth led me to consider whether E3 activity in the perinatal period, when the enhancer is still active in non-pericentral hepatocytes, is also dependent on β -catenin. To test this, I monitored E3- β gl-D^d expression by immunofluorescence with anti-D^d antibodies at postnatal day 1 (p1) in β Cat^{Aliv} mice. I chose p1 rather than prenatal timepoints since hepatic Alb-Cre expression begins late during fetal development [130]. Similarly to what was seen in the adult liver, E3- β gl-D^d transgenes were expressed at low levels in p1 β Cat^{Aliv} livers compared to β Cat^{fl/fl} livers (Figure 10A). Transgenes were still expressed sporadically in hepatocytes throughout the liver; continued expression is likely due to incomplete Cre-mediated deletion of β -catenin in this small population of cells.

Since endogenous AFP expression in the developing liver requires the AFP enhancer region, I predicted that β -catenin is important for AFP expression in early liver development. I analyzed hepatic AFP mRNA levels in expression through real-time PCR in p1 β Cat^{Aliv} and wild-type livers. While the reduction of β -catenin and AFP levels varied between mice, we found a significant reduction in AFP mRNA levels when β -catenin levels were low (Figure 10B). This data suggests that β -catenin contributes to AFP expression in the developing liver.

Discussion

The compartmentalization of function in the adult liver, resulting from the expression of certain genes in pericentral or periportal regions, enables this organ to carry out a variety of different, and in some cases, opposing functions. Previous studies have shown that the β -catenin signaling pathway has an important role in regulating a number of zonally-regulated genes in the adult liver. However, the mechanism by which β -catenin regulates target genes in the adult liver is not fully understood. Here, we have shown that two defined enhancer elements that exhibit pericentral activity in the adult liver, AFP enhancer E3 and the -2.5 kb rat GS enhancer RGS^c, are regulated by β -catenin. Furthermore, we have identified highly conserved TCF/LEF sites in these

enhancers that are required for β -catenin responsiveness. These studies also indicate that expression of AFP is reduced in the perinatal liver in the absence of β -catenin, indicating that this pathway may also be involved in developmental AFP regulation. Taken together, these data support a model in which postnatal AFP repression and zonal regulation are mechanistically related.

While β -catenin can control target genes through several pathways, my data indicate that the canonical pathway involving interactions between β -catenin and TCF/LEF family of DNA binding proteins contributes to E3 and RGS^e enhancer activity *in vivo*. The ~350 bp E3 and RGS^e enhancers were found to contain a single strong consensus TCF/LEF site, both of which are highly conserved across numerous mammals. These sites could bind TCF4 *in vitro*, and both enhancers were activated by wild-type and the constitutively active S37A variant of β -catenin in transient co-transfections. These data provide strong evidence that the highly conserved TCF/LEF sites in E3 and RGS^e are important for β -catenin-mediated regulation. In the absence of co-transfected β -catenin, the activities of both E3 and RGS^e were increased when their respective TCF/LEF sites were mutated. Since TFC/LEF factors can bind the Groucho family of co-repressors in the absence of β -catenin, it is not surprising that we saw a de-repression of enhancer activity when TCF/LEF proteins could no longer bind their cognate sites. While I did not directly test the role of Groucho-related proteins in repressing E3 or RGS^e, these results do raise the question whether these co-repressors also contribute to zonal gene regulation in the adult liver. In this regard, it is interesting that overexpression of the Groucho-related co-repressor Grg3 in the H2.35 liver cell line reduces endogenous AFP activity (Sekiya and Zaret, *Molec. Cell*, 28:291-303), and at least one groucho-related protein, Grg5, is expressed in the adult mouse liver (Miyasaka et al, *Eur. J. Biochem*, 216:343; Mallo & Gridley, *Mech of Develop*, 42:67)

β -catenin and AFP are both expressed during liver development and reactivated in HCC which led us to examine a potential relationship. At postnatal d1, mouse livers lacking β -catenin have greatly reduced E3 activity and a significant decrease in AFP expression. While this suggested β -catenin regulates endogenous AFP, no linkage

between these two is found in HCC tumor samples. These data suggest that AFP reactivation in HCC is independent of β -catenin. It should also be considered that E3 is one of four AFP regulatory elements that govern AFP expression. Regulation occurring at the other elements may override β -catenin activation at E3. E1 and E2 are robustly active in adult liver for which the mechanism and trans-acting factors are not known. It is possible activation through E1, E2 or the AFP promoter are the primary sites for AFP induction in HCC.

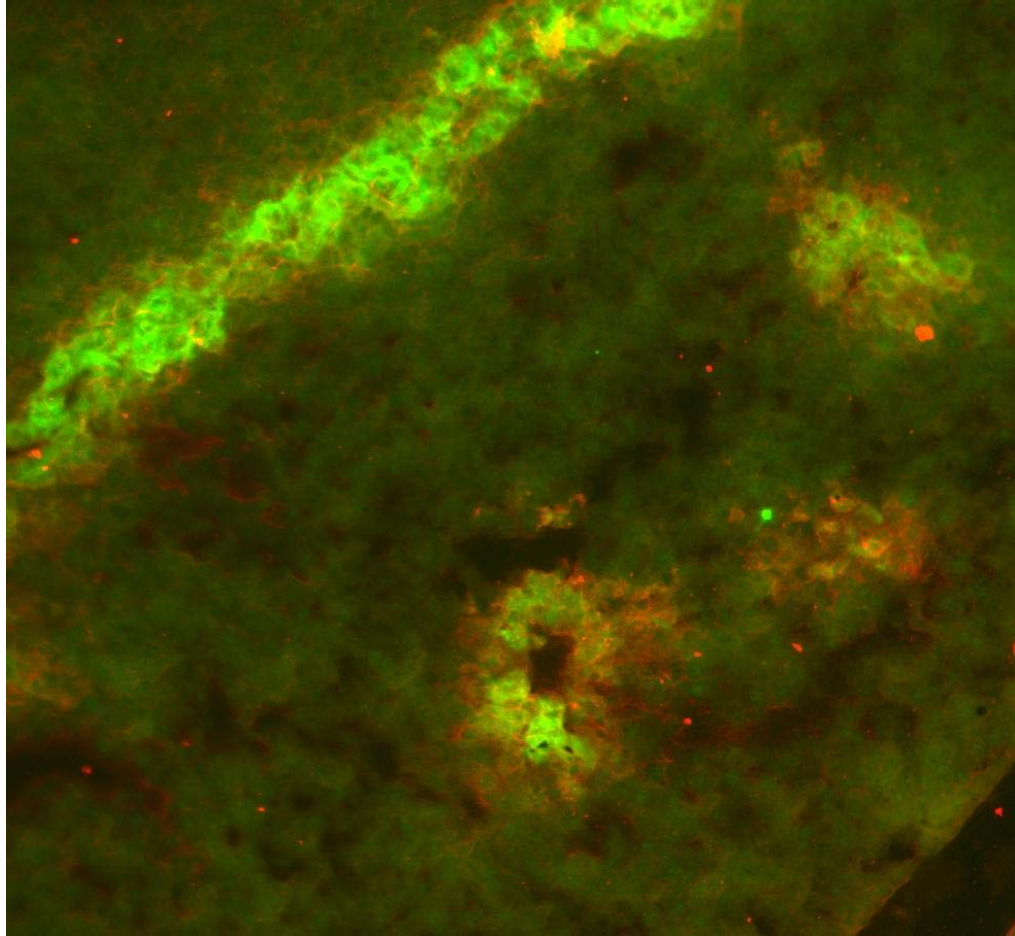


Figure 6. E3 shows pericentral activity in adult liver correlating with endogenous GS expression. E3 was cloned from the mouse genome and placed into the reporter gene construct with human β -globin promoter driving MHC class I D^d gene (E3- β gl- D^d). When the reporter gene is active the protein is detected on the cell surface membrane through the use of a fluorescent conjugated anti- D^d antibody. Adult livers from E3- β gl- D^d mice stained for D^d show activity only in hepatocytes directly surrounding the central vein (green) which coincides with endogenous pericentral GS staining (red).

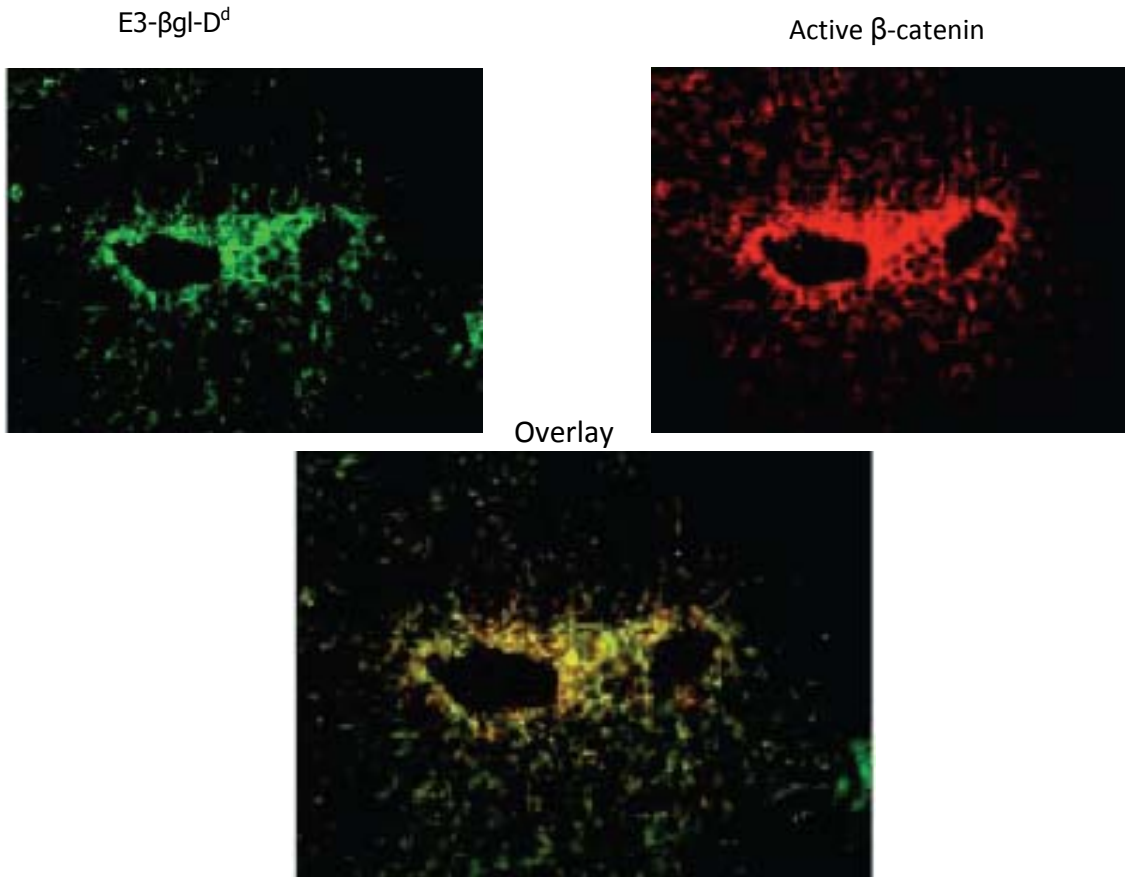


Figure 7. Active β -catenin and E3 overlap in pericentral hepatocytes. Adult livers were dissected and cryosectioned at 10 μ m thick. Liver sections underwent double staining for E3, using the anti-D^d antibody conjugated to FITC (green) and unphosphorylated active β -catenin TRITC antibody (red). While there is robust background for β -catenin, cytoplasmic accumulation is only observed in pericentral hepatocytes. Overlay of these two antibodies produce a yellow color indicative of co-localization demonstrating a relationship between E3 activity and β -catenin signaling.

Table 3. TCF4 sites from E3 and RGS^e are highly conserved. Binding site analysis with TESS revealed putative TCF4 sites at the 3' end in both E3 and RGS^e. DNA sequence for **A)** RGS^e and **B)** E3 were assembled from multiple species including Human (hum), Chimpanzee (Chi), Rhesus monkey (Rhe), Lemur (Lem), Horse (Hor), Dog, Cat, Guinea pig (Gui), Mouse (Mou) and Rat and aligned. TCF4 binding site centered the alignment flanked by sequences 5' and 3' to the site. Based on this analysis the TCF4 binding site sequence is highly conserved in E3 and RGS^e from mouse to human with single nucleotide polymorphisms observed in a few species in red. Such a high degree of conservation suggests an important function.

A. RGS^e TCF4 site alignment

TCF4 site			
Hum	acatgaatgg	atcaaagc	aaatccattt
Chi	aacatgaatgg	atcaaagc	aaatccattt
Rhe	acatgaatgg	atcaaagc	aaatccattt
Lem	acatgaacgg	atcaaagc	gaatctcttt
Dog	acatgaatgg	atcaaagc	aaatccattt
Cat	acatgaatgg	atcaaagc	aaatccattt
Gui	acatgaaagg	atcaaagc	aaatccattt
Rat	acatgaaagg	atcaaagc	aagcctgctt
Mous	acatgaaagg	atcaaagc	aaatccgctt

a/ta/tcaaag(g)

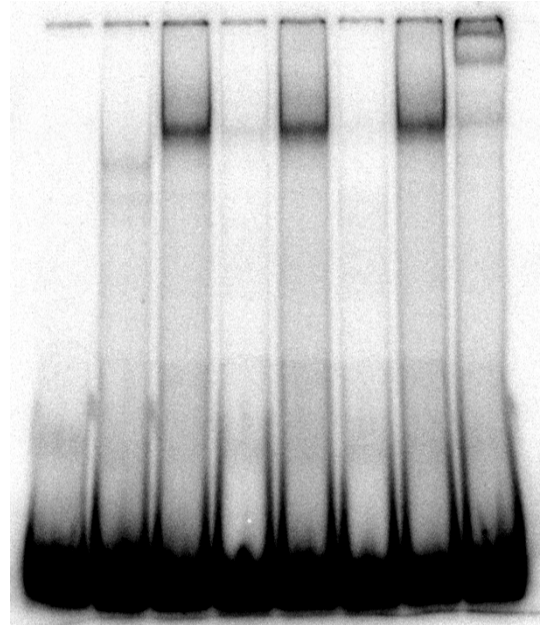
B. E3 TCF4 site alignment

TCF4 site			
Hum	atctcacttc	atcaaagg	gatcttctcc
Chi	atctcacttt	atcaaagg	gactccgccc
Rhe	gtttcccttt	atcaaagg	gatcttgtcc
Lem	gttttccttc	atcaaagg	gattttatct
Dog	tcattccttc	atcaaagg	gattttgctt
Cat	tcattccttt	atcaaaga	gattttgctt
Gui	catttcctca	atcaaaag	gactttactt
Rat	ttttccctcc	atcaaagg	aattttatct
Mous	gttttccttc	atcaaagg	aattttatct

a/ta/tcaaag(g)

A.

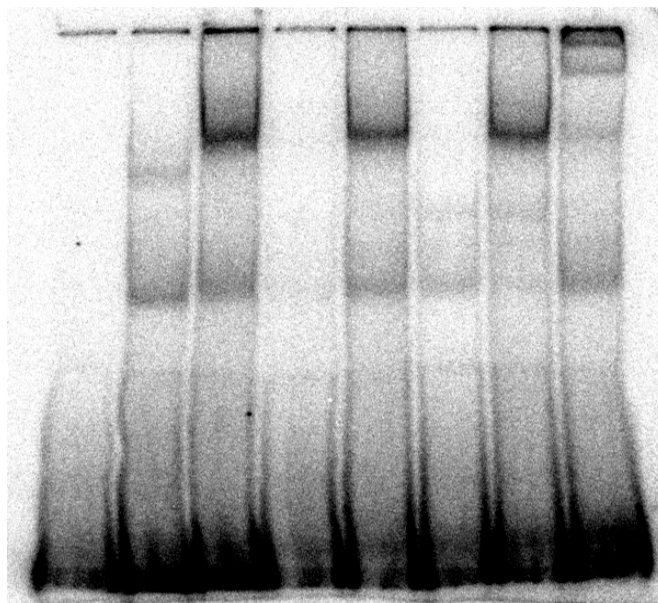
Nuclear extract: - Mock TCF4 TCF4 TCF4 TCF4 TCF4 TCF4
100x Competitor: - - - Self E3mut 4c 4cmut -
Antibody: - - - - - - - Flag



Mouse AFP E3
- TCF4 site

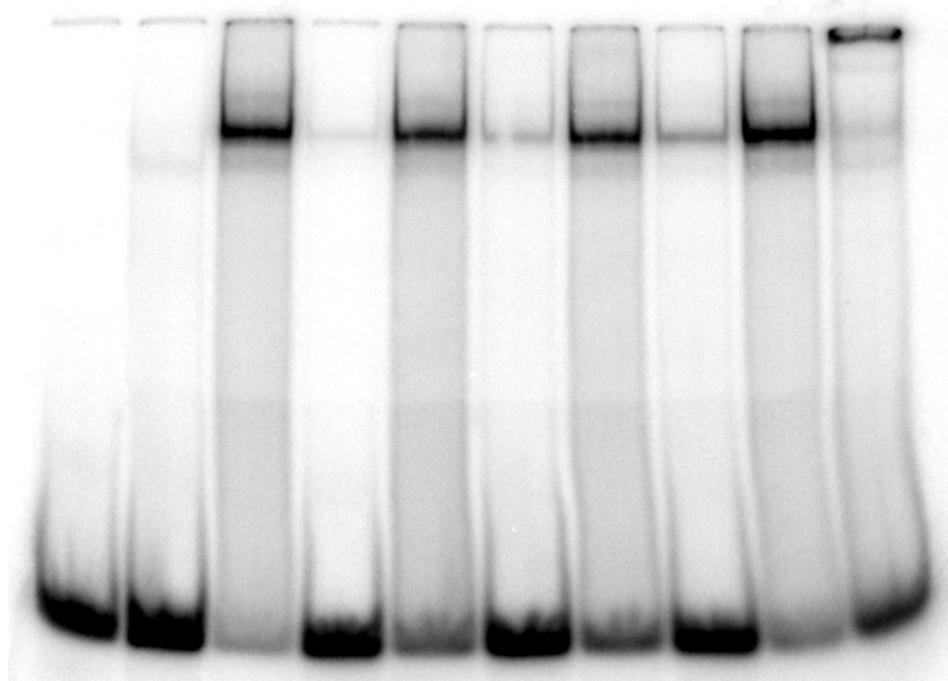
B.

Nuclear extract: - Mock TCF4 TCF4 TCF4 TCF4 TCF4 TCF4
100x Competitor: - - - Self RGSmut 4c 4cmut -
Antibody: - - - - - - - Flag



Rat Glutamine
Synthetase enhancer
- TCF4 site

C. NE (5ug): - Mock TCF4 TCF4 TCF4 TCF4 TCF4 TCF4 TCF4 TCF4 TCF4
 100x competitor: - - - Self Selfmut E3 E3mut RGS RGSmut
 Antibody: - - - - - - - - - Flag



TCF4
 Consensus
 site probe

Figure 8. The TCF4 protein binds to putative sites within E3 and RGS^e. **A)** Wild-type E3 (E3Tcf4) probe was incubated with 5ug nuclear extracts from HEK293 cells either mock transfected or expressing TCF4-flag protein. 100X cold competitors were used to assess specificity of the shifted band. These include cold E3Tcf4, mutant TCF4 site (E3Tcf4mut), TCF4c and mutant consensus (TCF4cmut). A supershifted band appears when incubated with the anti-Flag antibody. **B)** RGS^e (RGS^eTcf4) probe was incubated with 5 ug nuclear extracts from HEK293 cells either mock transfected or expressing TCF4-flag protein. 100X cold competitors were used to assess specificity of the shifted band. These include cold RGS^eTcf4, mutant TCF4 site (RGS^eTcf4mut), TCF4c and mutant consensus (TCF4cmut). A supershifted band appears when incubated with the anti-flag antibody. **C)** A synthetic consensus TCF4 (TCF4c) probe was incubated with 5 ug nuclear extracts from HEK293 cells either mock transfected or expressing TCF4-flag protein. TCF4c was also incubated with 100X cold self, TCF4cmut, E3Tcf4, E3Tcf4mut, RGS^eTcf4 and RGS^eTCF4cmut. A supershifted band appears when incubated with the anti-Flag antibody. TCF4 has the ability to bind to each probe which can be competed away with all other wild-type probes and not mutant probes. Binding is specific to TCF4 for a supershifted band appears when using the flag antibody.

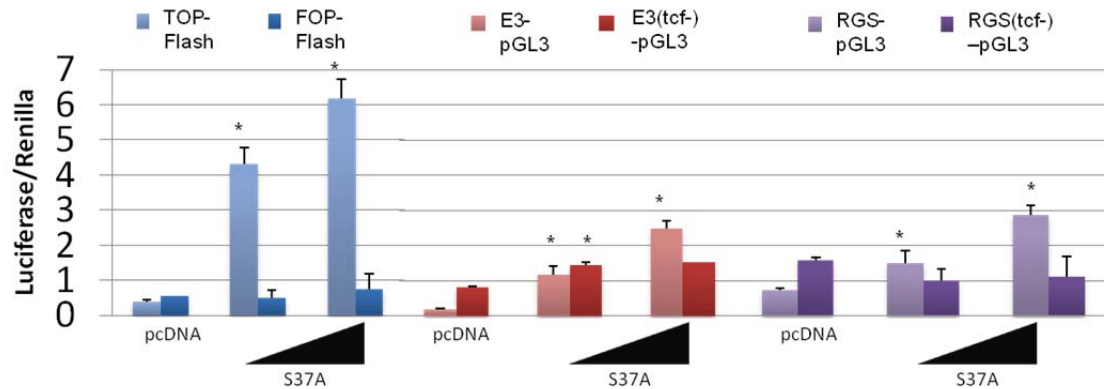
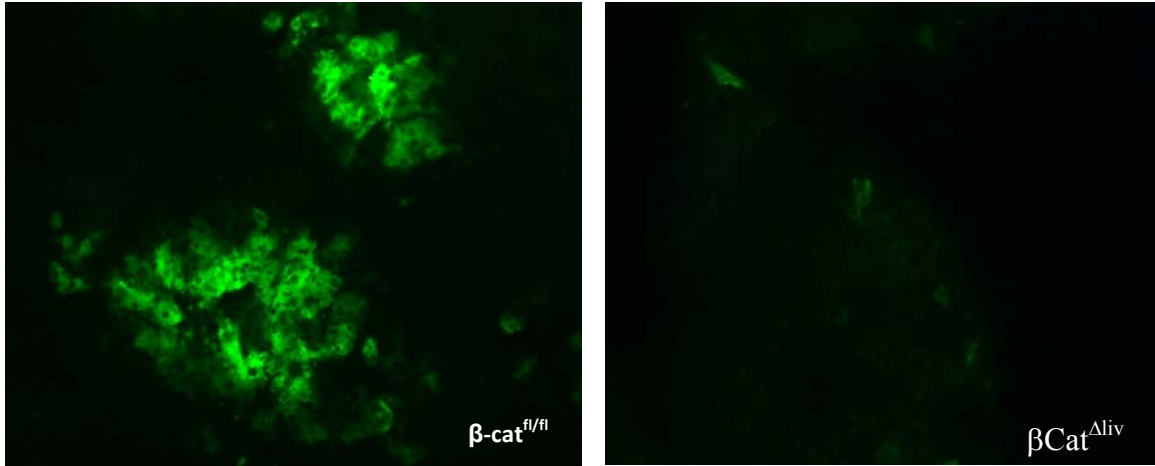


Figure 9. β -catenin activates enhancer constructs through TCF4 sites. Hep3B cells were transfected with 0.5ug luciferase reporter, 1.0ug expression construct and 12.5ng Renilla. Cells were harvested 48 hours post-transfection using Glo Lysis buffer. Supernatants were measured for luciferase and normalized to Renilla levels to account for transfection efficiency (Y-axis). All reporter constructs were co-transfected with an empty vector pcDNA to determine basal luciferase activity. Positive control TOP-Flash (light blue) as well as wildtype TCF4 constructs E3-pGL3 (light red) and RGS^c-pGL3 (light purple) increased luciferase activity with constitutively active β -catenin (β catS37A) compared to empty vector. When the TCF4 sites were mutated in E3(tcf)-pGL3 (dark red) and RGS^c(tcf)-pGL3 (dark purple) basal luciferase activity with pcDNA increases compared to the respective wild-type enhancer construct. The negative control FOP-Flash (dark blue), E3(tcf)-pGL3 and RGS^c(tcf)-pGL3 are not more active with increasing β catS37A demonstrating β -catenin activation is abolished with mutation of the TCF4 binding site. Therefore β -catenin activates these enhancers via TCF4. TCF4 binding is also important for repression in the absence of activated β -catenin signaling.

A.



B.

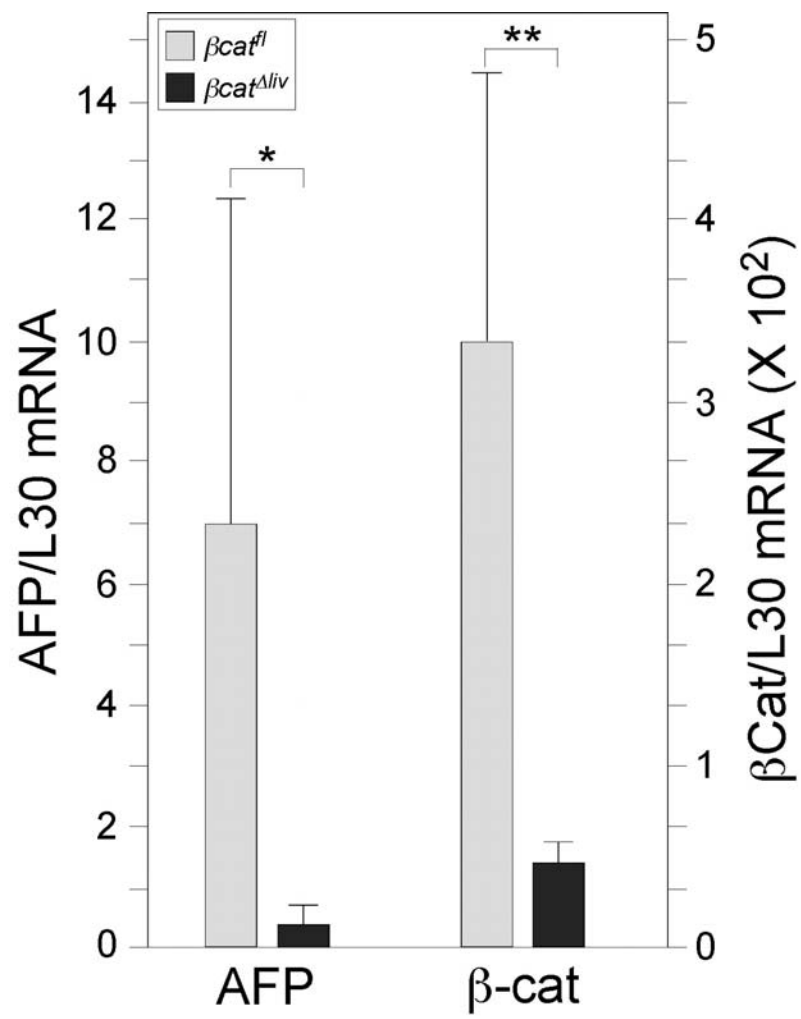


Figure 10. β -catenin regulates E3 and AFP expression after birth. To determine if β -catenin impacts postnatal developmental expression livers from hepatocyte β -catenin knockout (β Cat ^{Δ liv}) mice were analyzed. **A.** Sections from d3 livers from β -catenin wildtype (β cat^{fl}) or β Cat ^{Δ liv} both containing E3- β gl-D^d were cryosectioned and immunohistochemically stained with anti- D^d. β cat^{fl} livers have robust E3 activity around the central vein, but in more hepatocytes than adult livers. β Cat ^{Δ liv} d3 livers have very little E3 activity. Sporadic cells that still express the transgene may not have undergone the cre-recombinase reaction to remove β -catenin. (Magnification, 20X) **B.** RNA was extracted from two d1 litters and analyzed for AFP (left two bars) and β -catenin (right bars) mRNA from β cat^{fl} (black, n=5) and β Cat ^{Δ liv} (shaded, n=7) livers. Real-time RT-PCR Ct values from each primer set were normalized to the ribosomal gene L30. β -catenin (β Cat) mRNA levels are significantly decreased in knockout livers compared to β cat^{fl} livers which correlated with a significant decrease of AFP mRNA. β -catenin expression is crucial during fetal development and also plays a role in regulating AFP expression in fetal liver development through E3. * and ** p<0.05

CHAPTER 4

Orphan nuclear receptors ROR α and Rev-erb α / β regulate AFP enhancer 3

Introduction

The adult liver has a defined architecture consisting of multiple lobules that is essential for hepatic function. The lobule is a hexagonal structure with the central vein at the center and portal triads (each consisting of a hepatic artery, portal vein, bile duct) at each of the six corners of the hexagon (Figure 2). Blood enters the liver through the hepatic arteries and portal veins, flows through sinusoids along anastomosing plates of hepatocytes, and exits through the central veins. The distance between each portal triad and central vein is roughly 15-20 hepatocytes. An active exchange of compounds occurs between the basolateral surface of hepatocytes and blood. The apical sides of hepatocytes contain the bile caniculi; bile salts and other compounds are trafficked from hepatocytes to the caniculi and flow in a retrograde manner to the bile ducts for transport to the gall bladder.

In 1983, Gebhardt and colleagues discovered that the Glutamine Synthetase (GS) gene is expressed in a layer of 1-2 hepatocytes surrounding the central veins[32]. Since then, numerous other hepatic enzymes have been found to be zonally regulated, with many genes expressed similarly to GS in pericentral regions and other genes expressed in the region around the portal triad (periportal region). This phenomenon of zonal gene regulation enables the liver to carry out specific functions in different populations of hepatocytes. Elegant studies by Perret and colleagues have shown that β -catenin is an important regulator of zonal gene regulation [36]. However, the molecular basis of zonal gene regulation is still not fully understood.

Our lab has been studying alpha-fetoprotein (AFP) regulation for many years. The AFP gene is abundantly expressed in the fetal liver but dramatically repressed after birth. This postnatal silencing can be reversed; the AFP gene is transiently induced in regenerating liver and frequently reactivated in liver cancer. AFP expression is controlled by a promoter region and three upstream enhancers, E1, E2 and E3, located

2.5, 5.0 and 6.5 kb upstream of AFP exon 1, respectively. Each enhancer is roughly 300 bp in length. In our studies of the AFP enhancers in transgenic mice, we found that all three enhancers continued to be active in the adult liver. E1 and E2 were active in all hepatocytes, with a gradual reduction in activity in a pericentral-periportal direction. E3, however, was active only in a layer of 1-2 hepatocytes surrounding the central veins in a pattern that was identical to the GS gene. This was the first example of a defined regulatory element that exhibited zonal activity [89, 90]. We have continued to investigate E3 activity as a model of zonal gene regulation. Our studies support a model in which the absence of E3 activity in non-pericentral hepatocytes is due to active repression.

Once E3 was found to have pericentral activity, we began to focus on factors that might regulate this pattern. As described in chapter 3, we have identified a TCF4 site at the 3' end of E3. However, most studies have focused on the 5' end of this enhancer. Three major binding sites have been identified in this region, including a C/EBP site, FoxA site and a nuclear receptor (NR) site (Fig 11) [131-133]. Nuclear receptors comprise a large class of transcription factors involved in regulating growth and homeostasis [134-136]. Many NRs are ligand-inducible transcription factors. Generally, the NR response element in target genes is tandem or inverted repeats of AGGTCA separated by two or three nucleotides. However, slight variations in the sequence allow for gene-specific actions of these receptors [137, 138]

One class of NRs is the orphan nuclear receptor family, appropriately named since their activating ligand remains unknown or does not exist. Rev-erb α was the first orphan nuclear receptor identified [139]. Interestingly, while Rev-erb α maintains the domain to bind a ligand and has recently been suggested to bind to heme [140, 141], it cannot undergo a conformational change upon ligand binding. Therefore, Rev-erb α acts as a constitutive transcriptional repressor [142]. Rev-erb α can homodimerize, but it can also bind as a monomer to a half-site or single repeat of the response element, where it recruits nuclear receptor corepressor (NcoR) [140, 142] and histone deacetylase 3 (HDAC3) to repress target genes [143, 144]. Rev-erb α is expressed in metabolically active tissues including liver, adipose, where it is involved in adipocyte differentiation [145], and muscle, where its function remains unknown. Loss of Rev-erb α in mice results

in a mild phenotype, but these mice exhibit a disruption in circadian pattern. Rev-erb α and its closely related family member Rev-erb β , both regulate the major circadian rhythm regulator Bmal1 [146]. Monomeric response element binding is also a feature of orphan nuclear receptor ROR α (retinoic-acid orphan receptor α), which binds to a half-site that is similar to that of Rev-erb α preceded by an A/T rich region [147]. Unlike Rev-erb α , ROR α was observed to bind to the co-activators glucocorticoid receptor-interacting protein 1 (GRIP-1) and peroxisome proliferator-activated receptor binding protein (PBP) [148]. Competition between ROR α and Rev-erb α to the same DNA element was observed when both proteins were co-expressed, with both Rev-erbs opposing gene activation by ROR α [149].

Having found that β -catenin was important in zonal control of E3 activity, we wanted to determine whether any of the factor binding sites on the 5' end of E3 contributed to zonal E3 activity. In particular, we were interested in whether any of these sites contributed to the repression of E3 activity in non-pericentral hepatocytes. As mentioned previously, our working model is that the absence of E3 activity in this population of cells is due to active repression. This was based on the fact that a transgene with E3 and E2 combined had the same pericentral activity as transgenes with E3 alone, whereas E2-regulated transgenes were active throughout the liver [90]. We generated a series of E3-E2 containing transgenes in which the NR, C/EBP and FoxA sites were individually mutated. Transgenes with the FoxA and C/EBP mutations exhibited the same pericentral activity as wild-type transgenes. However, the mutation in the NR site had a dramatic effect, resulting in transgene activity throughout the adult liver similarly to those transgenes with E2 alone (J. Butler, ELC, BTS, in preparation). This indicated that the NR site is required for repression of E3 activity, in non-pericentral hepatocytes. Previous EMSA studies from our lab showed that ROR α , Rev-erb α and Rev-erb β were able to bind to this NR site [133]. Transient transfections also showed that ROR α enhanced E3 activity while both Rev-erb α and Rev-erb β repressed E3 activity (Figure 12). My work in this chapter continues from these observations. Since there is strong evidence that ROR α is the activator, I predicted E3 activity would be lost in mice lacking ROR α . ROR α and the Rev-erb proteins are both involved in circadian rhythm regulation

and in fact have a circadian pattern themselves. Therefore, I also performed a study to test if E3 activity has a circadian pattern.

Results

ROR α is an important positive regulator of E3 activity. Studies have shown that the orphan nuclear receptor ROR α is normally a positive transcription factor of its target genes. Transfections and EMSAs performed by a previous graduate student in the lab, James Butler, indicated that the E3 NR site is regulated by ROR α . This result led me to consider whether ROR α controls E3 activity in the adult liver. Unlike other orphan receptors, such as HNF4 α , ROR α has not been shown to have any zonal pattern of expression in the adult liver. The Staggerer mutant mouse contains a natural mutation in the ROR α gene, a 6.8kb deletion that results in a complete null allele that cannot express any of the four ROR α isoforms. These mice have altered cerebellular development resulting in an impaired gait, hence, the name [150]. Staggerer mice are viable for only 3-4 weeks after birth. Heterozygous female mice (ROR $\alpha^{wt/-}$) purchased from the Jackson laboratory were crossed with E3- β gl-D^d mice. Transgene positive ROR $\alpha^{wt/-}$ mice were then intercrossed to generate E3-positive mice that were ROR $\alpha^{wt/wt}$ or ROR $\alpha^{-/-}$. Livers were removed as close to four weeks of age as health of the animal would allow. Immunohistochemistry of frozen liver sections with anti-D^d antibody showed a dramatic reduction in E3 activity in the ROR α null mice. Even though there is not a complete loss of E3 activity, it is still only found in hepatocytes directly adjacent to the central vein (Figure 13).

ROR α regulates other pericentral enzymes. Since E3 and GS are similarly regulated by β -catenin (chapter 3) we hypothesized that ROR α may also regulate other pericentral genes. RNA isolated from ROR $\alpha^{-/-}$ and littermate ROR $\alpha^{wt/wt}$ livers were analyzed by real-time RT-PCR for the expression of pericentral genes GS, OAT, and Rhesus Monkey Glycoprotein B (RhBG) (Figure 14A) as well as periportal genes PEPCK, Glutaminase2 and GLUT2 (Figure 14B). All pericentral genes examined showed

reduced expression in $ROR^{-/-}$ mice compared to the wild-type littermates. Periportal gene expression was not changed regardless of $ROR\alpha$ status.

E3- β gl-D^d does not exhibit a circadian rhythm pattern of expression. $ROR\alpha$ and the Rev-erb proteins have opposing effects on E3 activity in cultured cells, and my data indicates that $ROR\alpha$ is a positive regulator of E3 in the adult liver. Assessing the contribution of the Rev-erb proteins became difficult mainly due to the redundancy of the two proteins and the requirement for double knockout mice that, at this point, do not exist. Both Rev-erb proteins and $ROR\alpha$ are involved in circadian regulation [151] and exhibit circadian rhythm changes in expression [152]. We hypothesized that the circadian pattern changes in these orphan receptors might lead to a circadian change in E3 in vivo. Adult E3 livers, three in each group, were removed at 4 hour intervals during a 24 hour time period. RNA analysis using reverse transcriptase PCR (RT-PCR) for Rev-erb α showed expression peaks at 4-8 hours after the light cycle begins. However, RT-PCR shows no change in E3- β gl-D^d transgene expression was detected at any of the time points (Figure 15).

E3 with a mutant NR site is still activated by $ROR\alpha$ but not repressed by Rev-erb proteins in transient transfections. To examine further the role of the NR proteins in E3 regulation, co-transfections were performed in Hep3B cells using E3/E2-regulated pGL3-luciferase reporter plasmids [with E3 wild-type (WT) or a mutant NR site (SS)] and expression vectors for $ROR\alpha$, Rev-erb α , and Rev-erb β (Figure 16A-B). Luciferase activity of WT and SS plasmids are very similar when co-transfected with the empty vector pcDNA 3.1. Addition of $ROR\alpha$ increased luciferase levels from both WT and SS vectors. In contrast, increasing concentrations of Rev-erb proteins (especially Rev-erb β) dramatically decreased luciferase levels from the WT vector (Figure 16A). However, the highest levels of the Rev-erb proteins did not affect SS luciferase levels (Figure 16B). These data demonstrate SS is still activated by $ROR\alpha$, but the NR mutation prevents repression by Rev-erb α and Rev-erb β .

Discussion

Nuclear receptors are gaining attention due to their global role in gene regulation that can be tissue-specific and involved in diseases [153, 154]. The structure of these proteins lends themselves to being good pharmacological targets since complete activity requires binding a ligand. The orphan nuclear class of receptors are important gene regulators even though their ligands have not yet been identified. In particular Rev-erb α and ROR α are known to regulate circadian rhythm through competitive regulation of Bmal1 [151]. Both proteins were also recently shown to regulate energy metabolism. ROR deficient mice develop severe atherosclerosis on a high fat diet [155] and Rev-erb^{-/-} mice exhibit dislipidemia [156]. One of the main metabolic tissues is the liver, which compartmentalizes metabolic pathways. This study shows that ROR α and Rev-erb α and Rev-erb β play a role in this regulation.

E3 offers a model system for studying this mechanism of zonation. Initial binding site analysis elucidated a nuclear receptor site at the 5' end of E3. Classification of the NR binding site found it was a half-site, thereby limiting potential binding proteins to orphan nuclear receptors. Previous studies by our lab and others show all of these candidate orphan nuclear receptors ROR α and Rev-erb α and Rev-erb β bind to the E3 NR site through EMSA analysis [133]. Regulation by these proteins, observed with in vitro transfections, is consistent with what is observed at other target genes; ROR α increases E3 activity while Rev-erb α and Rev-erb β repress E3 activity. My studies build upon these observations to better understand regulation of E3 via these proteins.

Consistent with ROR α activating E3 *in vitro*, ROR^{-/-} liver lost the majority of E3 activity, although it was not completely absent. Nuclear receptors bind very similar sites and can occasionally be promiscuous in binding. In particular, chicken ovalbumin upstream promoter-transcription factor (COUP-TFII) is known to bind to ROR α response elements [157]. This is supported by observations from J. Butler in which COUP-TF proteins bind to the NR site of E3 by EMSA. Surprisingly, β -catenin, another potent activator of E3 discussed previously, does not seem to compensate and activate E3 in ROR α deficient livers. Competition between ROR α and Rev-erb proteins is known to

occur since they bind the same half site in E3 [149, 151]. It is possible that without ROR α present there is no competition, thus Rev-erb α or Rev-erb β bind and repress E3 in pericentral hepatocytes. I also found many pericentral genes have reduced expression in ROR deficient livers compared to wild-type littermates suggesting ROR α plays a positive role in zonation. Many of these same enzymes are also decreased in β -catenin knockout liver suggesting a potential relationship between β -catenin and ROR α . β -catenin has recently been found to interact with nuclear receptors [158]. Moreover, a direct interaction between these two proteins was found in colon cancer; however, in this case, ROR α inhibited β -catenin activity [159]. I do not believe this occurs in pericentral hepatocytes since both proteins are required for full E3 activity. Therefore, it is possible that cooperation occurs in pericentral hepatocytes between ROR α and β -catenin to activate pericentral genes as shown in the model (Figure 17).

This coordination may also not be limited to ROR α . HNF4 α , another orphan nuclear receptor, was found to have a zonal pattern in the adult liver [48]. If HNF α is disrupted, zonal genes GS and PEPCK lose their normal compartmentalization [50]. Studies examining GS found HNF4 α binding sites in close proximity to TCF/LEF binding sites that coordinated to regulate expression of GS [51]. RGS^e, the GS enhancer which recapitulates zonal activity in vivo, was not examined in these studies. While we have not identified any ROR α binding site in RGS^e, we have found a putative HNF4 α binding site near the 5' end.

The NR site in E3 is clearly involved in zonal regulation because when it was mutated (SS), E3 repression is lost and was expressed in all hepatocytes (Butler J, unpublished data). Using in vitro transient transfections I found that NR mutant E3 (SS) still responds to the activator but becomes non-responsive to the repressor. The NR within E3 is considered a half-site; mutation of the NR should abolish site recognition and binding capability of both Rev-erb proteins and ROR α . SS is linked to E2 for added activity, an enhancer which has not been well characterized. Therefore it is possible E2 may contain a ROR α nuclear receptor site. In an attempt to elucidate the role of Rev-erb proteins, I performed a circadian rhythm study. Rev-erb α and Rev-erb β peak in expression in the liver approximately 4-6 hours after the light cycle begins. Alternatively,

ROR α expression peaks a bit sooner around 2-3 hours after beginning of the light cycle and begins to decrease while Rev-erb proteins peak [160]. However, E3 transgenes showed no change in expression levels during the 24 hour period. This is consistent with the fact that expression of pericentral enzymes, for the most part, is static. While ROR α and Rev-erb α participate in zonal regulation, other trans-acting factors could potentially overcome circadian rhythm pattern changes of these proteins.

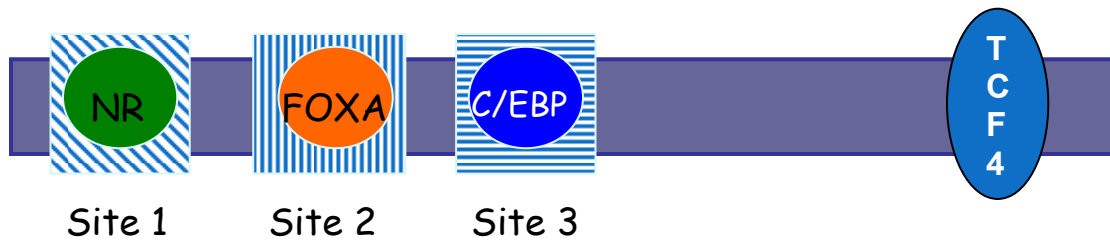


Figure 11. Binding sites identified within AFP E3. Early studies with E3 identified three important binding sites at the 5' end of the enhancer depicted in this cartoon. These sites include binding sequences for a nuclear receptor (NR), C/EBP and FoxA. From chapter 3 we also identified a TCF4 binding site at the 3' end. E3 transgenes mutated at either the C/EBP or FoxA binding sites did not alter the zonal activity in adult livers. However, the E3 transgene with a mutated NR site (SS) in the adult liver gained non-pericentral activity demonstrating the importance of the NR site for compartmentalization of E3 activity.

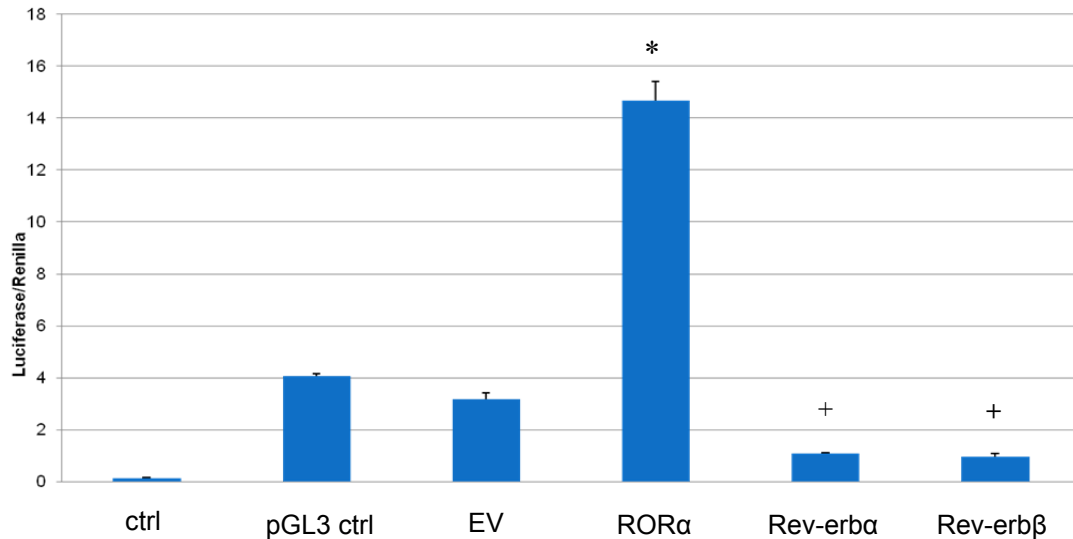


Figure 12. E3 is activated by ROR α and repressed by Rev-erba/ β in HepG2 cells. Cells were transfected with 0.5 ug of reporter and 1.0 ug of an expression vector and 12.5 ng of Renilla. 48 hours after transfection cells were harvested with Glo Lysis buffer. Luciferase was measured from the supernatant and normalized to Renilla levels to account for transfection efficiency (y-axis). pGL3 promoter (ctrl) and pGL3 control (pGL3) are luciferase constructs were used as negative and positive controls for the assay in the first two lanes. The next four lanes are co-transfections of the E3-pGL3 luciferase reporter with expression vectors as labeled. A pcDNA empty vector (EV) assesses basal activity from E3-pGL3. Addition of ROR α to E3 increased luciferase compared to the empty vector control. Both Rev-erba and Rev-erb β reduced luciferase values as compared to the empty vector control. This demonstrates the ability of these orphan nuclear receptors to regulate activity of E3. * $p < 0.00001$, + $p < 0.0005$

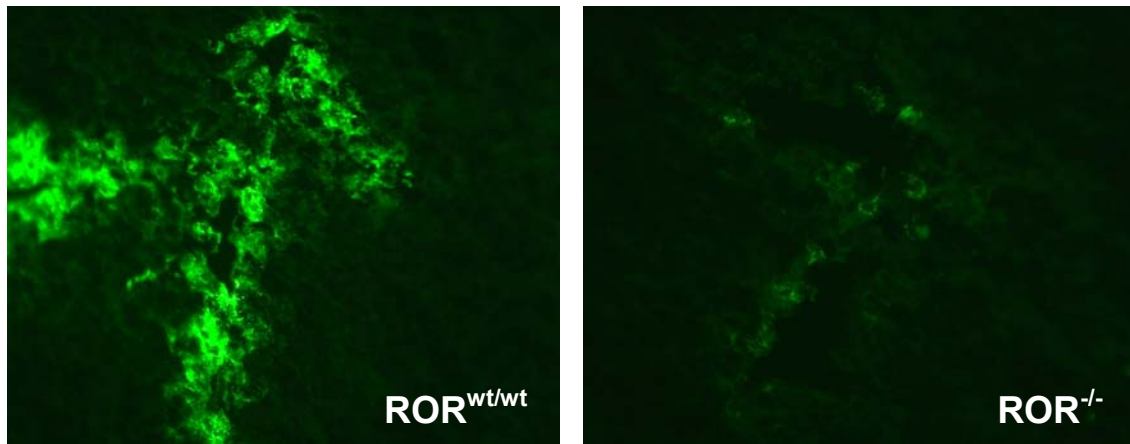


Figure 13. E3 activity is regulated by ROR α in vivo. E3- β gl-D^d transgenic mice were crossed to ROR^{-/-} mice to assess hepatic E3 regulation. Livers from four week old ROR^{wt/wt} or ROR^{-/-} mice were sectioned and immunohistochemically stained for E3 activity using the anti- D^d antibody. E3 activity in the ROR^{-/-} liver was dramatically reduced compared to ROR^{wt/wt} liver (Magnification, 20X). Sporadic punctate staining was observed in ROR^{-/-} livers but it was strictly in pericentral hepatocytes. These observations show ROR α is an important positive regulator of E3 in vivo.

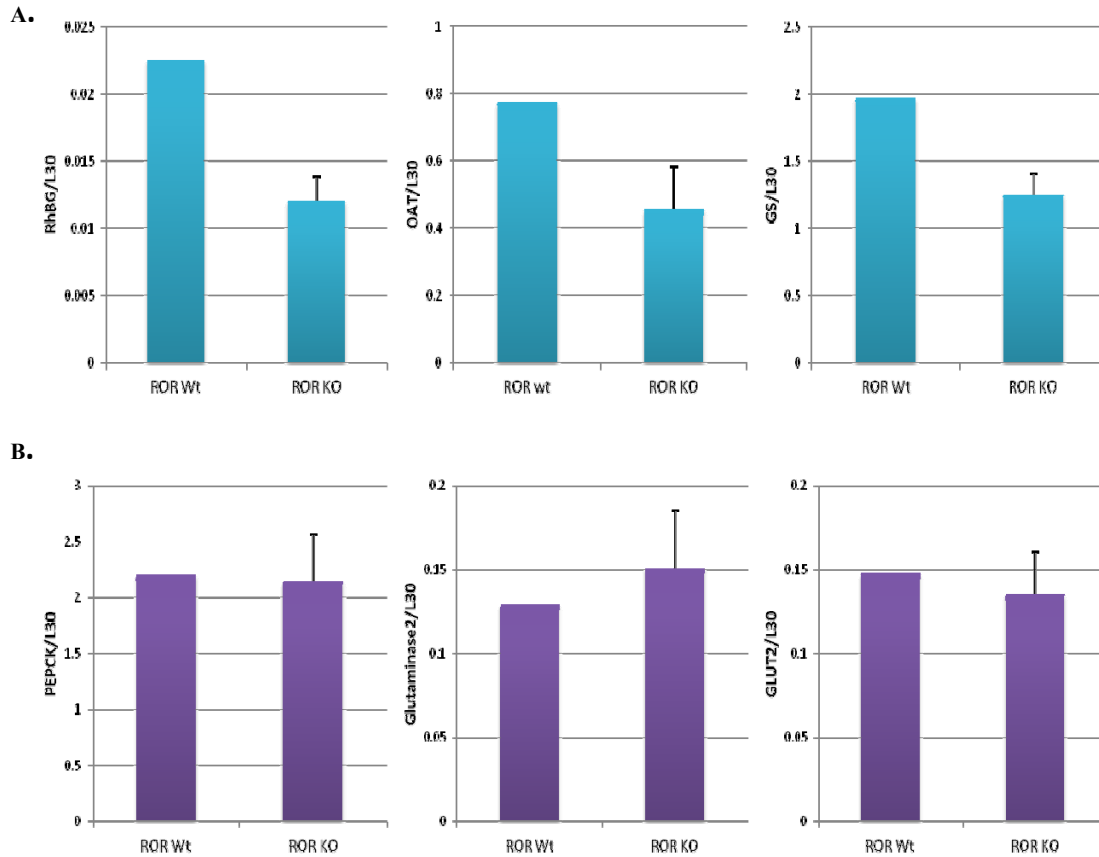


Figure 14. ROR α regulates expression of pericentral but not periportal genes. RNA was isolated from livers that were ROR^{wt} (n=1) or ROR^{-/-} (n=6) genotypes. **A.** RNA was analyzed by real-time RT-PCR for expression of pericentral genes including glutamine synthetase (GS), ornithine aminotransferase (OAT) and Rhesus monkey glycoprotein B (RhBG). Gene expression was normalized to the ribosomal gene L30. For these pericentral genes, lacking ROR α decreases expression. **B.** RNA was analyzed by real-time RT-PCR for expression of periportal genes including PEPCK, Glutaminase2 and GLUT2. Expression levels were normalized to L30. No change in periportal expression is observed in ROR^{-/-} livers compared to wildtype livers. This suggests that ROR α plays a role in regulating zonation in of pericentral gene expression.

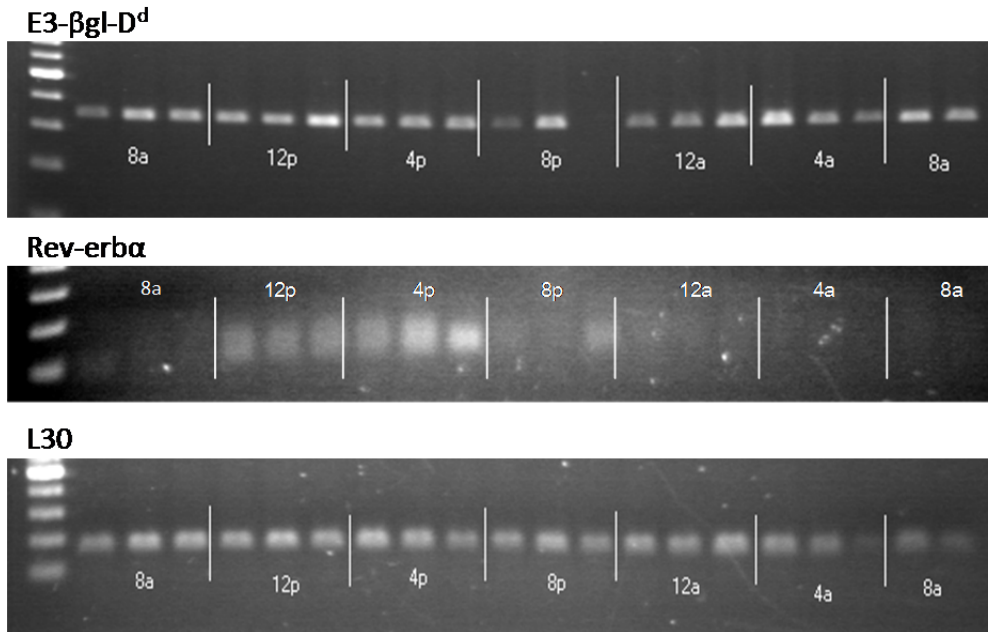
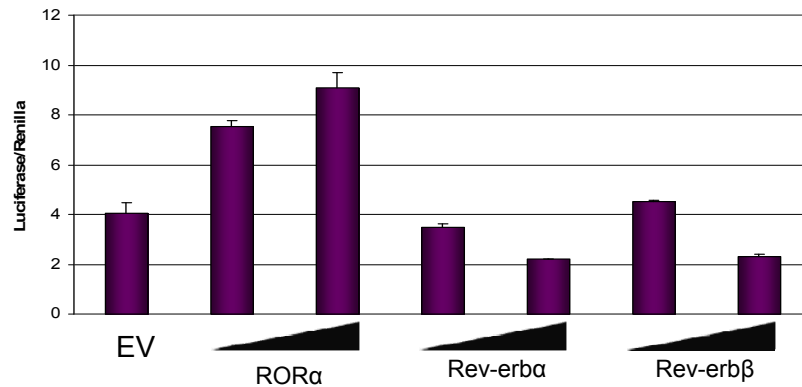


Figure 15. Circadian rhythm of Rev-erb α does not affect expression of E3- β gl-D^d. Livers were dissected from eight week old age matched E3- β gl-D^d transgenic mice at 4 hour intervals, at the indicated times, over a 24 hour time period. From these, RNA was analyzed by RT-PCR for β gl-D^d (top panel), Rev-erb α (middle panel) expression and L30 (bottom panel) used as a normalizing control. Rev-erb α peaks at the 12p and 4p time points. No reduction in β gl-D^d expression is observed at these time points. At 8p the third animal was a negative transgenic mouse used as a control for genomic contamination. This suggests that activity of E3 is static in adult pericentral hepatocytes and does not respond to circadian pattern changes.

A.



B.

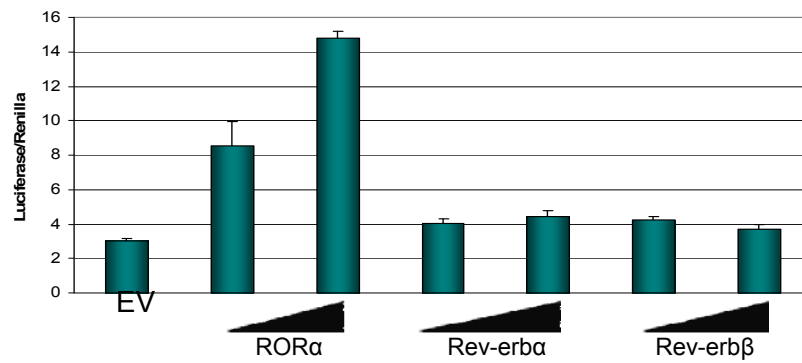


Figure 16. E3 enhancer containing the NR mutation SS is activated by ROR α but not repressed by Rev-erb α or Rev-erb β . To see if SS is able to respond to the nuclear receptors, Hep3B cells were transfected using the calcium phosphate method. **A.** WT (E3/E2-pGL3) and **B.** SS (E3NRmut/E2-pGL3) were co-transfected with either an empty vector (EV) or increasing concentration of the nuclear receptor expression vectors. 48 hours post transfection cells were collected in Glo Lysis buffer. Supernatants were analyzed for luciferase levels which then normalized to Renilla to account for transfection efficiency. ROR α activates both WT and SS constructs in a dose dependent manner compared to the empty vector control. Rev-erb α and Rev-erb β decreases luciferase levels from the WT construct in a dose dependent manner. Despite the presence of the repressor at the highest concentration, SS luciferase levels do not decrease, demonstrating repression through the NR site is lost when mutated.

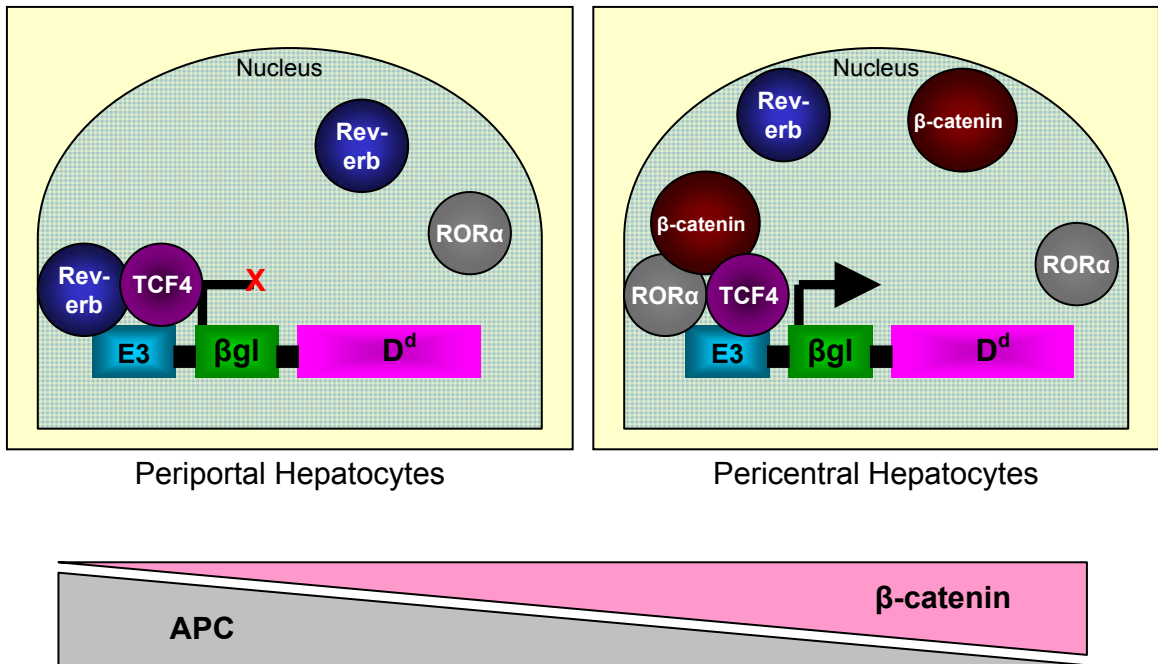


Figure 17. Model for zonal E3 regulation by β -catenin and nuclear receptors. In the liver lobule a gradient exists along the porto-centro axis where APC expression is highest in periportal hepatocytes and gradually decreases towards the pericentral hepatocytes. Low APC allows for β -catenin accumulation and translocation into the nucleus of pericentral hepatocytes. β -catenin binds to target genes through interaction with TCF4 and ROR α to activate, in this case, E3. Alternatively, high APC in periportal hepatocytes degrades any cytosolic β -catenin. Within the nucleus, TCF4, its co-repressors as well as Rev-erb α/β bind to the TCF4 site within E3. While ROR α is still present in these cells, Rev-erb proteins out-compete ROR α for binding and contribute to E3 repression.

CHAPTER 5

Zhx2 protects against liver damage during a high fat diet

Introduction

High fat and high cholesterol diets found in developed countries are primarily responsible for the epidemic rise in obesity rates as well as increased incidence of atherosclerosis [161, 162]. Cholesterol, an essential biological compound, is used in cell membranes for stability and signaling [163] and as a precursor for steroid hormones, bile acids and vitamin D [164-166]. Cholesterol homeostasis is achieved by balancing de novo synthesis, ingestion, catabolism and excretion through the feces [167]. Imbalance of cholesterol and other lipids such as triglycerides can cause diseases such as atherosclerosis, the leading cause of death in the United States [168], and nonalcoholic fatty liver disease (NAFLD) [169].

Despite the development of various drugs, current treatments for high cholesterol are not completely effective. This in part is due to the fact that cholesterol-initiated disease progression is not fully understood. Due to its essential role, all cells have the ability to synthesize cholesterol de novo. However, dietary cholesterol can be distributed to peripheral sites through low density lipoprotein (LDL) packaged in the liver [170]. Unfortunately, LDL has the ability to deposit cholesterol on arterial walls, increasing the likelihood of atherosclerotic plaque formation and giving LDL the reputation of “bad cholesterol” [171]. In times of cholesterol excess, high density lipoprotein (HDL) can transport cholesterol back to the liver to either be converted to bile acids or incorporated in the emulsified material to be excreted in the feces [172]. HDL has the ability to remove arterial cholesterol and is therefore thought of as “good cholesterol”. Patients with highly elevated LDL, carrying high levels of cholesterol and triglycerides, and low HDL, are at high risk of developing atherosclerosis [173]. Drug treatments, including statins, which inhibit de novo cholesterol synthesis [174, 175], and bile acid sequestrants [175], which increase fecal excretion and processing of cholesterol into bile acids, are

effective in lowering cholesterol levels but they are not able to completely prevent atherosclerosis.

Atherosclerosis is a complex disorder influenced by multiple environmental and genetics factors. Historically, such complex traits have been more difficult to study than monogenic traits. However, the advent of genomic methods have provided new approaches to study the genetic basis of complex traits. One of these strategies is Quantitative Trait Locus (QTL) mapping, an unbiased method to identify multiple loci that contribute to a particular phenotype [176], including serum hyperlipidemia and atherosclerosis. Several years ago, Jake Lusis (UCLA) carried out a QTL study using BALB/cJ and MRL inbred strains of mice that are resistant and susceptible, respectively, to diet-induced atherosclerosis. This study identified several QTLs, including one on chromosome 15 that was associated with hypertriglyceridemia and was called hyperlipidemia 2 (Hyplip2). The presence of MRL DNA from this region on a BALB/cJ background (Congenic15 or Con15) increased incidence of atherosclerotic plaques and hyperlipidemia compared to a BALB/cJ mouse when mice were on a high fat diet [121].

The chromosome 15 interval containing Hyplip2 includes the gene zinc-finger and homeobox2 (*Zhx2*). *Zhx2* was originally identified in this region by our lab based on its ability to control hepatic alpha-fetoprotein (AFP) mRNA levels in the adult liver [96]. In BALB/cJ mice, an endogenous mouse retroviral (MERV) element inserted in the first *Zhx2* intron, causing aberrant splicing and dramatically reducing *Zhx2* mRNA and protein levels [97]. Adult liver AFP levels are elevated in BALB/cJ mice, indicating that *Zhx2* is a repressor of AFP expression. Expression of a *Zhx2* transgene in the liver of BALB/cJ mice results in normal AFP repression, confirming a role for *Zhx2* in AFP silencing [96]. Based on the physical linkage of *Zhx2* and *Hyplip2*, we collaborated with the Lusis lab to determine whether *Zhx2* is responsible for the Hyplip2 phenotype. Expression of the *Zhx2* transgene in BALB/cJ mice led to increased serum triglyceride and cholesterol levels, indicating that *Zhx2* is Hyplip2 [122]. This data also indicates that *Zhx2* regulates liver genes involved in lipid homeostasis.

To better understand the role of *Zhx2* in hepatic control of lipids, I have analyzed the expression of genes in the livers of BALB/cJ, BALB/c and BALB/cJ +/- *Zhx2*

transgenic mice on a high fat diet. At the beginning of this study, the only known targets of *Zhx2* were AFP, H19 and Glypican3. Using microarray data provided by the Lusis lab performed between CON15 and BALB/cJ mice, I have used real-time RT-PCR to confirm additional *Zhx2* targets. Interestingly, these studies also identified a *Zhx2*-dependent hepatic phenotype. When placed on a high fat diet, mice without *Zhx2* (BALB/cJ) exhibit increased lipid accumulation, AFP mRNA levels and serum ALT when compared to mice with *Zhx2* (BALB/c and BALB/cJ + *Zhx2* transgene). These data indicate that *Zhx2* is hepatoprotective in mice on a high fat diet. I examined potential inflammatory mediators and stress pathways to explore further the basis of liver damage in the absence of *Zhx2*.

Results

To investigate the role of *Zhx2* in diet-induced changes in liver gene expression, two comparisons were made. First, studies were performed in BALB/cJ and BALB/c mice. These two substrains are highly related, having separated roughly 60 years ago. However, BALB/c mice have the wild-type *Zhx2* allele since the MERV element is found only in the BALB/cJ substrain. I also carried out experiments in BALB/cJ mice that do or do not contain the TTR-*Zhx2* transgene; this construct contains the liver-specific transthyretin promoter/enhancer linked to a full-length *Zhx2* cDNA. These mice will be referred to as BALB/cJ+*Zhx2*; *Zhx2* levels in the livers of these transgenic mice are similar to wild-type *Zhx2* levels (Morford, L., Turcios, L., Peterson, M., unpubl obs). As mentioned earlier, BALB/cJ+*Zhx2* exhibited increased serum triglyceride levels compared to BALB/cJ mice when placed on a high fat diet. Age matched female mice from each strain (n=5) were placed on either normal chow (LF, 6.8% fat) or a high fat (HF) Harlan Teklad chow (15% fat) for eight weeks; previous studies showed that atherosclerotic plaque formation occurs during this time period. Weekly measurements indicated weight gain in all cohorts (Figure 18). By week 8, BALB/cJ mice in both cohorts on the high fat diet weighed significantly more than their counterparts on normal chow. A significant interaction of strain and diet on weight gain (p=0.046) was observed in the BALB/cJ and BALB/c cohort. At the end of 8 weeks, all mice were sacrificed and

the dissected livers were weighed prior to processing. Livers of all mice on the high fat chow became enlarged and white in color due to lipid accumulation (Figure 19A). Liver weight normalized to body weight showed that all cohorts on high fat chow had significantly increased liver: body weight ratios compared to littermates on normal chow. A significant interaction was found between strain and diet type for both BALB/cJ and BALB/cJ+Zhx2 ($p=0.018$) and BALB/cJ and BALB/c ($p=0.012$) cohorts.

Zhx2 regulates Cyp8B1, Lpl and Elovl3 in the liver. Zhx2 is predicted to contain two zinc fingers and four homeodomains [99], hallmarks of nucleic acid binding proteins. While other data suggest that Zhx2 functions as a transcription factor, data from our lab in collaboration with Dr. Peterson suggest that Zhx2 might also function at the posttranscriptional level. Until recently, the known targets of Zhx2 regulation were AFP, H19 and glypican 3 (Gpc3) [177, 178]. Interestingly, all three of these genes are low in normal adult liver but are frequently reactivated in liver cancer. I have analyzed other candidate targets of Zhx2 based on microarray data between CON15 and BALB/cJ mice provided by the Lusis lab.

Sterol 12 α -hydroxylase (Cyp8B1) was my first gene of interest due to its important role in the synthesis of bile acids, which requires cholesterol as a precursor. Cyp8B1 is the key enzyme for cholic acid production, which determines the hydrophobicity of bile acid salts [179]. RNA extracted from the livers of each mouse was analyzed for Cyp8B1 using standard RT-PCR. There was no difference observed between cohorts on the normal chow (Figure 20A-B). BALB/cJ mice maintain slightly higher levels of Cyp8B1 compared to BALB/c mice on the high fat chow even though there is a dramatic reduction in levels compared to the mice on the normal chow (Figure 20B). This difference was not observed with BALB/cJ +/- Zhx2, while there is a reduction in Cyp8B1 in response to the high fat chow there is no strain specific difference (Figure 20A). Cyp8B1 is mainly produced in hepatocytes. However, based on immunohistochemical studies, it is also found in cholangiocytes and Kupffer cells [180]. Since the TTR-Zhx2 transgene is expressed only in hepatocytes, the differences in Cyp8B1 between BALB/c and BALB/cJ+Zhx2 might be due to Zhx2 expression in non-parenchymal liver cells.

Another gene of interest from the microarray data was Lipoprotein Lipase (Lpl). Lpl is known to clear triglycerides from the serum [181], and previous Hyplip2 studies suggested that mice with normal *Zhx2* had reduced triglyceride clearance [182]. Lpl also has a similar developmental profile to AFP in the liver [183], making Lpl a reasonable target of *Zhx2*. Real-time RT-PCR analysis of Lpl showed a 2-fold decrease in steady-state mRNA levels in mice containing wild-type *Zhx2* on a normal chow diet. Lpl levels increased in all mice on the high fat chow with only a significant difference observed between BALB/c and BALB/cJ cohorts (Figure 21) which was also found to have a significant interaction of strain and diet type ($p=0.033$).

Another interesting target gene based on the microarray was Elongation of very long-chain fatty acid-like 3 (*Elovl3*). However, whereas other targets are high when *Zhx2* is low, *Elovl3* appeared to be regulated in an opposite manner in that its levels were low when *Zhx2* was low. Analysis of *Elovl3* mRNA levels by real-time RT-PCR confirmed that mice on normal chow with wild-type *Zhx2* (BALB/c and BALB/cJ+*Zhx2*) had significantly higher *Elovl3* levels compared to BALB/cJ mice. The high fat diet significantly reduced *Elovl3* to similar levels in all cohorts (Figure 22A-B). Both BALB/cJ and BALB/cJ+*Zhx2* as well as BALB/cJ and BALB/c cohorts were found to have a significant interaction between strain and diet for *Elovl3* expression ($p=0.0002$ and $p=0.00035$, respectively)

Reduced hepatic *Zhx2* results in increased liver damage in mice on a high-fat diet. Over the past several years, we have begun to appreciate the implication of hyperlipidemia on liver function. Nonalcoholic fatty liver disease (NAFLD), which occurs when lipids accumulate in the liver and is frequently found in obese patients, was originally considered a benign side effect [184]. However, continued fat accumulation can lead to increasing damage and inflammation and progress to chronic hepatitis and, ultimately, HCC [185, 186]. To assess liver damage in my diet study, serum was collected over the 8-week period and analyzed for Alanine Aminotransferase (ALT). ALT is abundantly produced by the liver; increased serum ALT levels reflect leakage from damaged hepatocytes [187]. ALT levels were found to be increased in all groups during this eight week study (Figure 23A-B). As expected, mice on the high fat chow

showed a greater ALT increase than those on the normal chow. However, BALB/cJ exhibited the greatest increased ALT on the high fat diet, suggesting that the absence of *Zhx2* led to increased liver damage.

AFP, a known target of *Zhx2*, is induced during the regeneration period and can also be used as a marker of liver damage [188]. Real-time RT-PCR analysis of liver AFP mRNA from mice on normal chow indicated ~10-fold higher AFP mRNA levels in BALB/cJ mice compared to mice expressing *Zhx2*, as expected. In addition, BALB/cJ on the high fat diet showed a dramatic rise in AFP mRNA levels while BALB/c and BALB/cJ+*Zhx2* AFP levels remained the same (Figure 24). A significant interaction between diet and strain was observed for the BALB/cJ and BALB/cJ+*Zhx2* cohort ($p=0.005$) The AFP induction in BALB/cJ on high fat chow is consistent with the ALT data and provides further evidence of increased liver damage in the absence of *Zhx2*.

Chronic inflammation, under any circumstance, is detrimental to liver function and can cause cell death and fibrosis from collagen deposition. Inflammation is found in NAFLD and is often seen as the “second hit” in disease progression [189]. One pro-inflammatory cytokine known to be increased in NAFLD patients is Tumor Necrosis Factor α (TNF α) [190]. Therefore, TNF α mRNA levels were analyzed as a measure of inflammation between the different groups of mice. In all liver samples from mice on a normal chow diet, TNF α expression was very low (Figure 25). All mice on a high fat diet showed a significant increase in TNF α levels. However, these levels were the same regardless of *Zhx2* levels, suggesting that TNF α cannot account for the increased liver damage found in BALB/cJ mice on the high fat chow.

Lipid accumulation and histological alterations are greater in BALB/cJ mice than other groups on high fat chow. At the time of tissue removal all groups on the high fat diet exhibited gross liver phenotypic differences compared to those on normal chow. To explore further the liver phenotype, formalin-fixed, paraffin-embedded liver sections were stained with H&E and analyzed in a “blinded” manner by Dr. Eun Lee, the liver pathologist at UK. Livers from the different groups on the normal chow had no differences identifiable by the pathologist. All livers from mice on the high fat diet showed hepatocyte ballooning, hepatic unrest and neutrophil abscesses (Figure 26A-B).

However, BALB/c mice exhibited less severe changes than the other groups (Figure 26B). BALB/cJ mice on high fat chow was the only group that showed macrovesicular lipid droplets, but these were low (<1%). Oil Red O staining was performed on frozen liver sections to evaluate triglycerides and lipid accumulation. As expected, livers from all groups on a normal chow had very little staining. In contrast, significant Oil Red O staining was seen throughout the livers of all mice on the high fat diet. BALB/cJ mice had substantially more staining than BALB/c and BALB/cJ+Zhx2, indicating that reduced Zhx2 resulted in greater lipid accumulation (Figure 27A-B).

Discussion

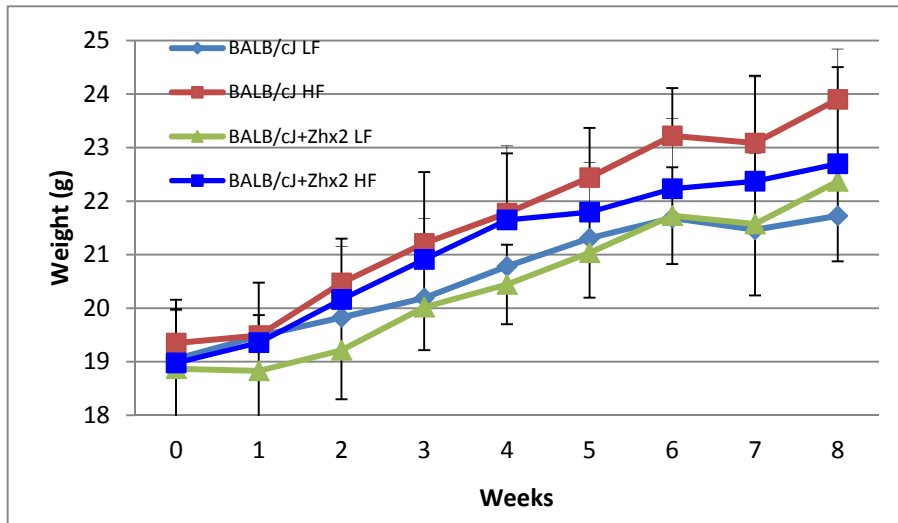
High fat diets in developed countries have resulted in an epidemic rise in obesity rates as well as associated complications including atherosclerosis and NAFLD. Currently, drug treatments for atherosclerosis target a reduction in cholesterol, but are ineffective at a complete reversal of the disease. NAFLD remains under investigation since pathophysiology and clinical manifestations are not completely understood. There is no known effective drug treatment for NAFLD although changing diet can reverse some of the histology of the disease in the liver [191]. These metabolic diseases are complex due to multiple parameters that contribute to disease progression. A better understanding of the genetic basis of diet-associated cardiac and liver disease will lead to better treatment strategies. In collaboration with Jake Lusis, our lab previously showed that Zhx2 can regulate serum lipid levels and atherosclerosis.

My studies described in this chapter have confirmed several hepatic targets of Zhx2 predicted by microarray analysis, including Cyp8B1, Lpl, and Elovl3. All of these genes could contribute to the control of serum cholesterol levels. The bile acid synthesis gene Cyp8B1 is still expressed in BALB/cJ on high fat chow compared to BALB/c. However, hepatic Zhx2 transgene expression is unable to completely repress Cyp8B1, suggesting that other liver cells such as Kupffer and cholangiocytes (also known as bile duct epithelial cells) may contribute to Cyp8B1 expression. Another target of Zhx2 is Lpl; on normal chow, BALB/cJ have higher Lpl mRNA levels compared to BALB/c and BALB/cJ+Zhx2. However, Lpl mRNA levels were similar in all strains on the high fat

diet. A study found an upregulation of Lpl in obese patients and suggest a role in hepatic steatosis [192]. It is possible that the upregulation of Lpl during high fat diet is an attempt to clear excess serum triglycerides. However, it unfortunately results in hepatic lipid accumulation to the detriment of the liver. The final target of Zhx2 described here, Elov13, is particularly interesting in that it appears to be positively regulated by Zhx2. Elov13 is one of seven family members involved elongation of fatty acids up to C24 [193]. Knockout Elov13 mice were recently found resistant to diet-induced obesity. Moreover, reduction in Elov13 decreased hepatic lipid droplets and also reduced VLDL-TG levels in mice fed high fat chow [194]. A combination of elevated Lpl and lower Elov13 is potentially why BALB/cJ mice have lower serum triglyceride levels compared to BALB/c mice on normal chow. More detailed analysis of Elov13 is being carried out by another graduate student in the lab (Ren, H. unpublished data).

Genetic changes and altered lipid levels are the “first hit” in NAFLD. It is inflammation and subsequent liver damage that is the “second hit” to progress the disease [184]. Without intervention, inflammation and fibrosis can lead to cirrhosis and possible HCC development. Interestingly, this study shows presence of Zhx2 protects from diet-induced liver damage. Mice expressing Zhx2 had lower serum ALT and no AFP induction when on the high fat chow. Inflammation is one possible mechanism for liver damage; pro-inflammatory cytokines are elevated in NAFLD patients [195]. Through monitoring TNF α mRNA in this study, it increased to similar levels amongst all cohorts on a high fat chow which could be produced by the Kupffer cells. Accumulated lipids cause inflammation, but can also put stress on cells, ultimately causing death [196]. Histology and Oil Red O show that BALB/c mice with wild-type Zhx2 have less severe steatosis and decreased lipid accumulation suggesting less lipid related stress. There are multiple avenues in which excess lipids cause cell death that will be explored in future studies to determine the mechanism of damage occurring in BALB/cJ mice.

A.



B.

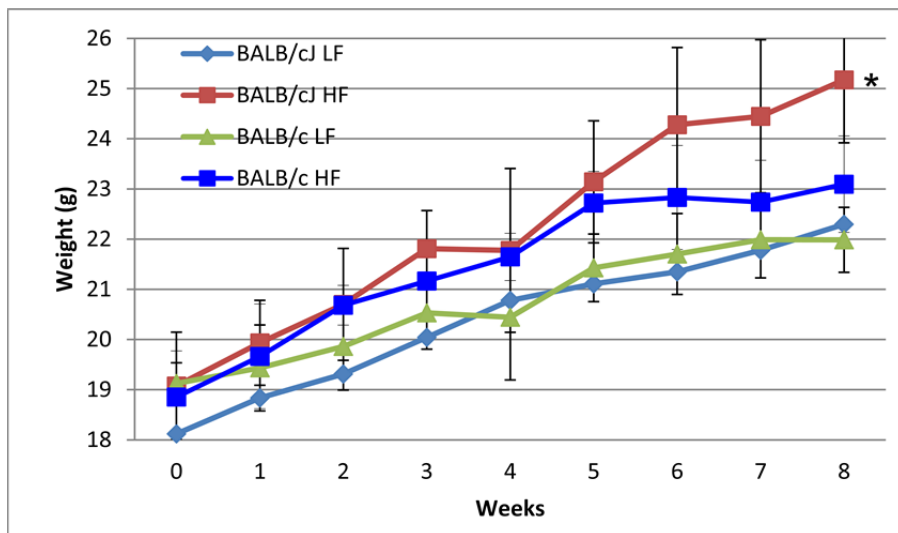
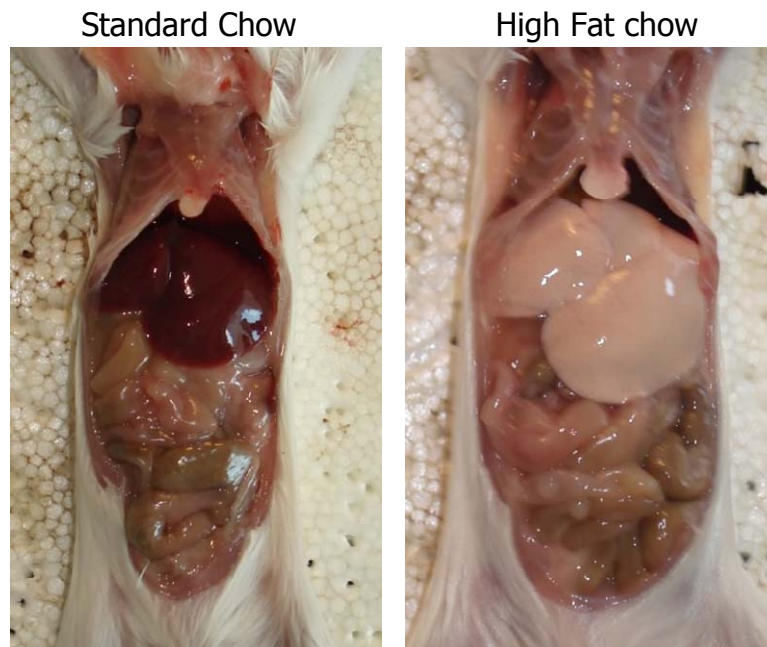


Figure 18. All cohorts gain body weight on an eight week high fat chow diet study. Female mice from the strains **A.** BALB/cJ and BALB/cJ+Zhx2 or **B.** BALB/cJ and BALB/c were placed on either normal chow (LF) or a high fat chow (HF) for 8 weeks. Weekly weight measurements were averaged and are plotted for each cohort as mean +/- standard deviation. * $p < 0.05$

A.



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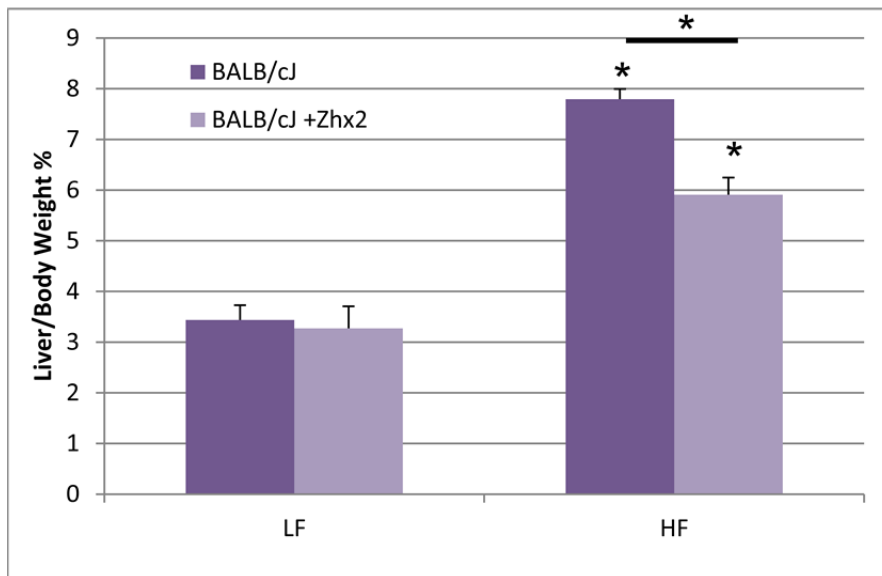


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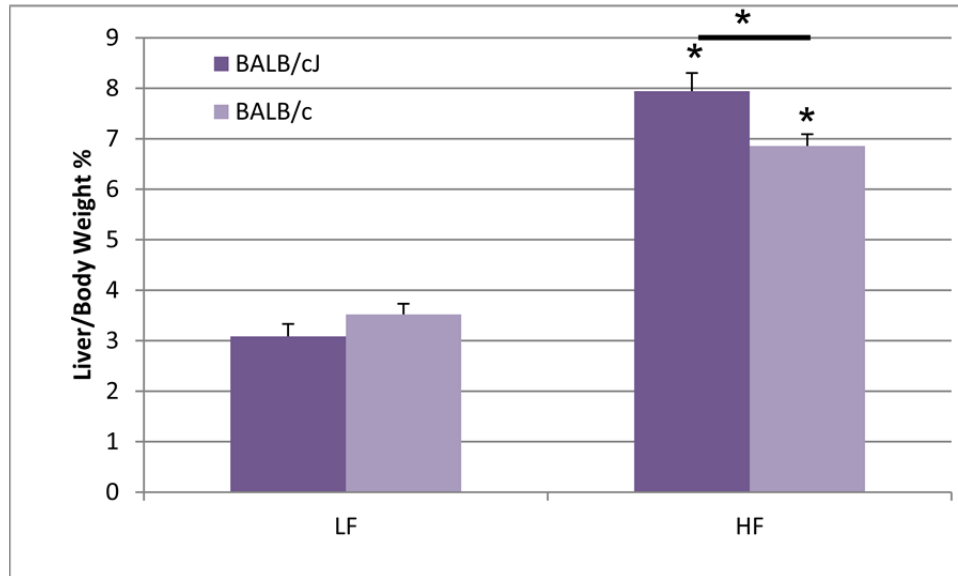
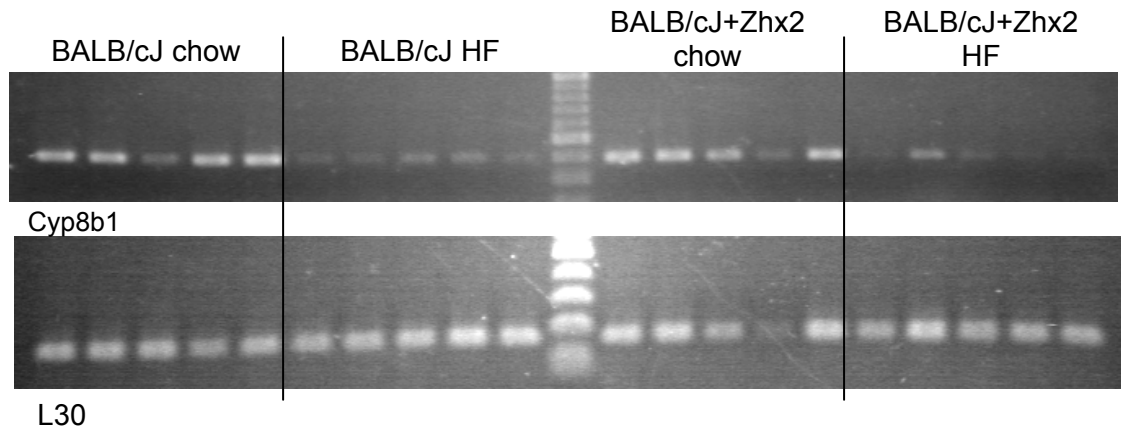


Figure 19. Liver weight increases with high fat chow feeding. At completion of the eight week diet study the livers were collected. **A.** These are representative pictures for the liver phenotype seen in mice of the study. All strains on chow exhibited a normal liver phenotype while mice on HF chow had enlarged livers with a white color indicative of lipid accumulation. **B.** All dissected livers were weighed and normalized to body weight and calculated as percent. The liver to body weight ratio was averaged for the cohort and plotted as mean+standard deviation. All cohorts on HF chow significantly increase liver:body weight ratios compared to LF cohort. BALB/cJ on HF in both panels had significantly higher ratios as compared to BALB/cJ+Zhx2 (upper panel) and BALB/c (lower panel). * $p < 0.05$

A.



B.

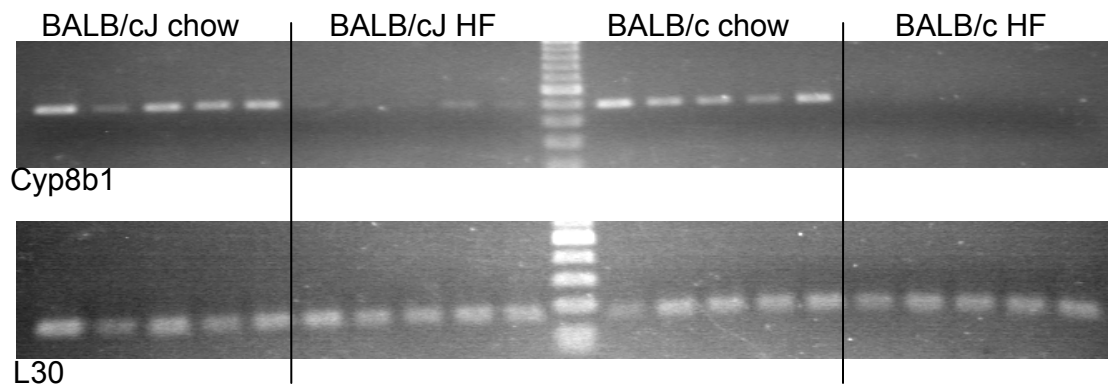
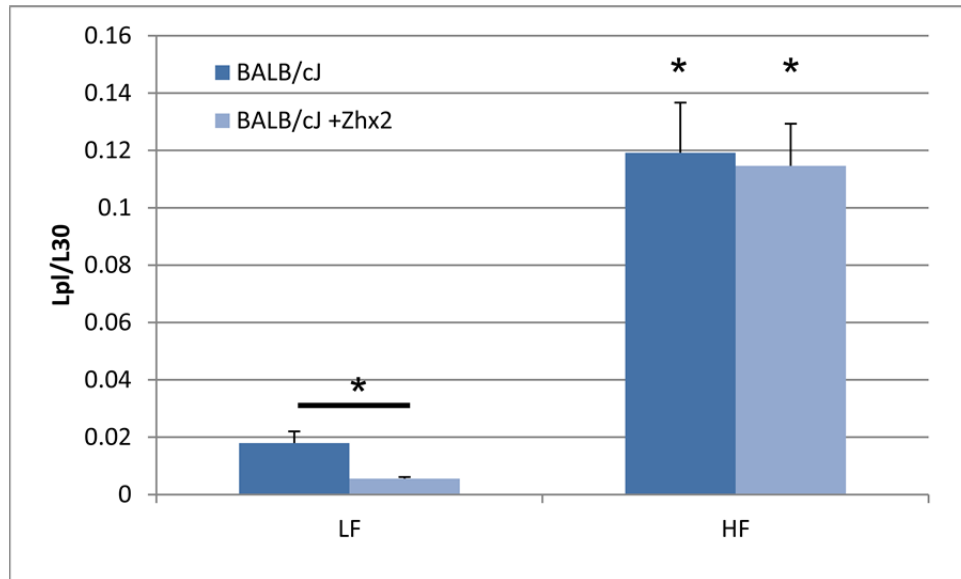


Figure 20. CYP8B1 levels decrease in mice on HF chow. RNA was extracted from each liver and analyzed by RT-PCR for Cyp8B1 expression and L30 as a normalizing gene. **A.** BALB/cJ livers were compared to BALB/cJ+Zhx2 livers on chow or HF. No differences are observed in cohorts on chow. While there is a decrease in expression on HF there is no obvious difference between these cohorts. **B.** BALB/cJ livers were compared to BALB/c livers on normal chow (chow) or high fat chow (HF). No difference is observed between the cohorts on chow. Cyp8B1 levels decrease on HF; for BALB/c it is completely shut off, but still remains detectable in BALB/cJ.

A.



B.

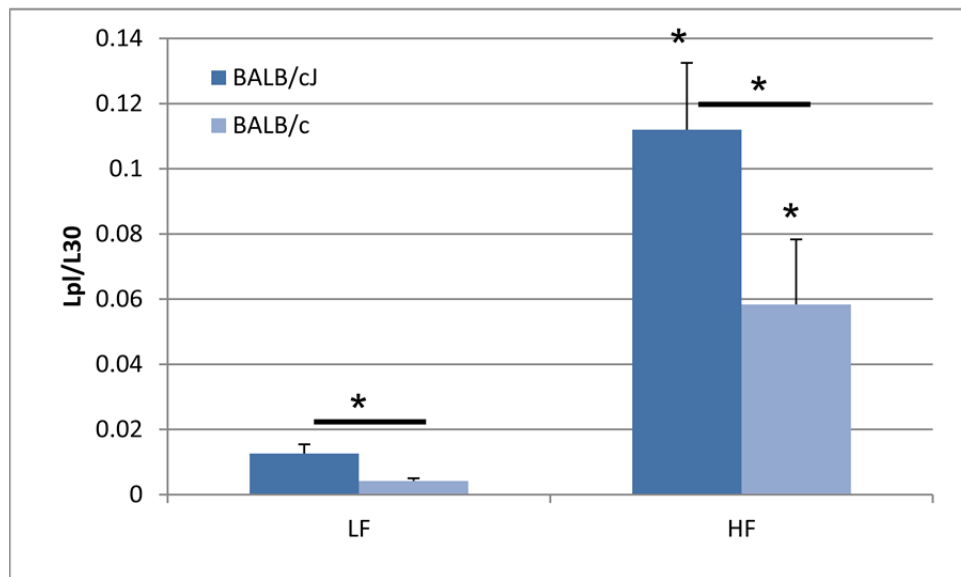
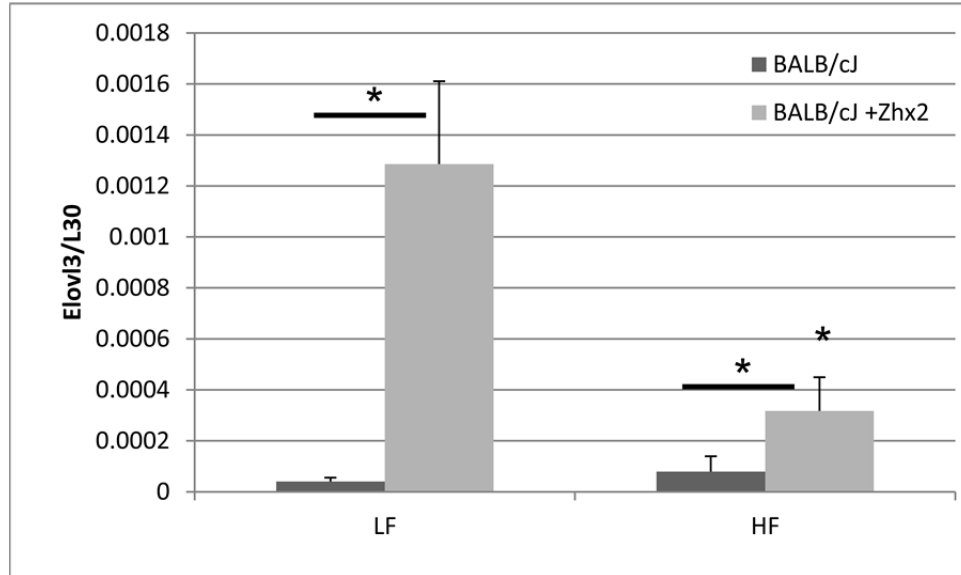


Figure 21. Lpl increases on a high fat chow. **A.** RNA collected from BALB/cJ or BALB/cJ+Zhx2 livers on chow (LF) or high fat chow (HF) were analyzed through Real-time RT-PCR for expression of Lipoprotein Lipase (Lpl). Ribosomal gene L30 was used as a normalizing control for all samples. Average from n=5 for each group was calculated and plotted as mean+standard deviation. Lpl is significantly higher in BALB/cJ compared to BALB/cJ+Zhx2 LF. HF significantly induces Lpl expression; no difference is observed between the two strains. **B.** RNA collected from BALB/cJ or BALB/c livers on LF or HF were analyzed similarly for Lpl. BALB/cJ have significantly higher expression of Lpl compared to LF BALB/c. While a significant induction of Lpl occurs with HF groups, BALB/cJ HF livers have significantly higher expression. This data demonstrates Lpl is a target of Zhx2 regulation. * p<0.05

A.



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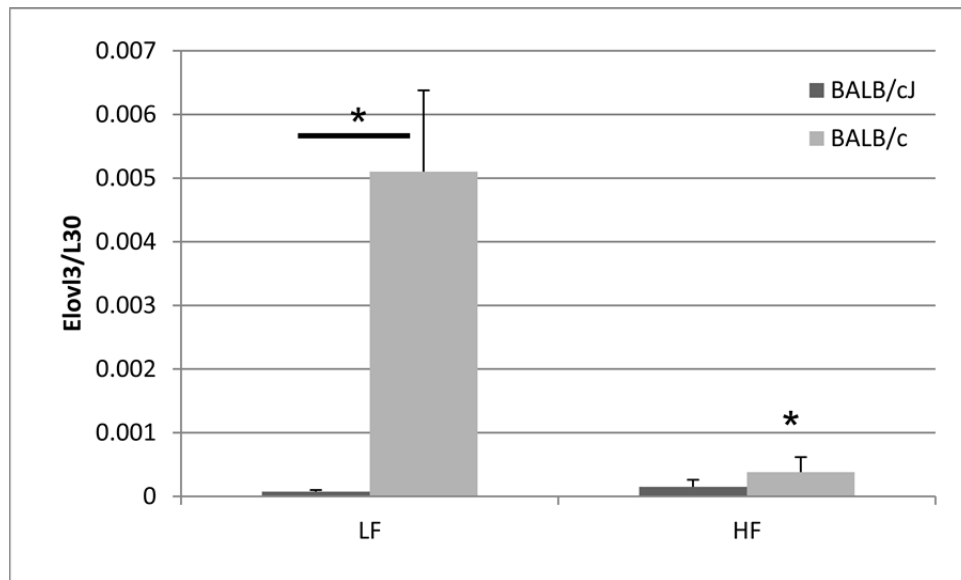
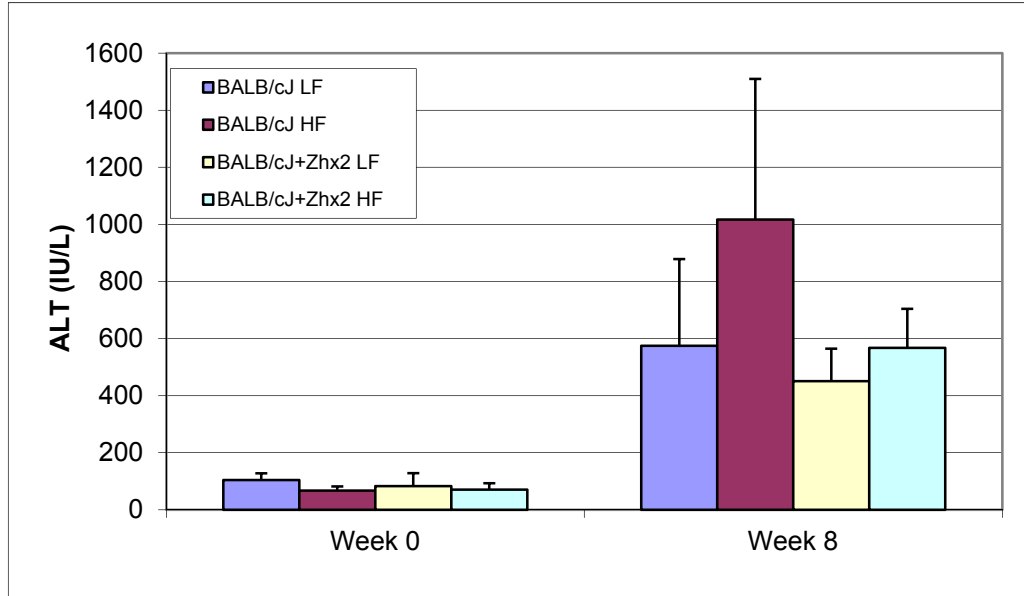


Figure 22. Hepatic Elov13 is positively regulated by Zhx2 in mice on normal chow but decreases with high fat chow. **A.** RNA collected from BALB/cJ or BALB/cJ+Zhx2 livers on chow (LF) or high fat chow (HF) were analyzed through Real-time RT-PCR for expression of elongation of very long chain fatty acids 3 (Elov13). Ribosomal gene L30 was used as a normalizing control for all samples. Average from n=5 for each group was calculated and plotted as mean+standard deviation. Presence of Zhx2 in BALB/cJ+Zhx2 LF results in significantly higher Elov13 expression. While HF chow causes a reduction in Elov13 expression in BALB/cJ+Zhx2, the resultant levels are significantly more than BALB/cJ. No reduction of Elov13 is observed in BALB/cJ on HF chow. **B.** RNA collected from BALB/cJ or BALB/c livers on chow or HF were analyzed similarly for Elov13. Again, LF BALB/c mice had significantly higher expression of Elov13 compared to BALB/cJ. In BALB/c mice the HF chow dramatically reduced Elov13 expression however this was not different from BALB/cJ levels. No reduction in Elov13 expression is observed in BALB/cJ on HF chow. * p<0.05

A.



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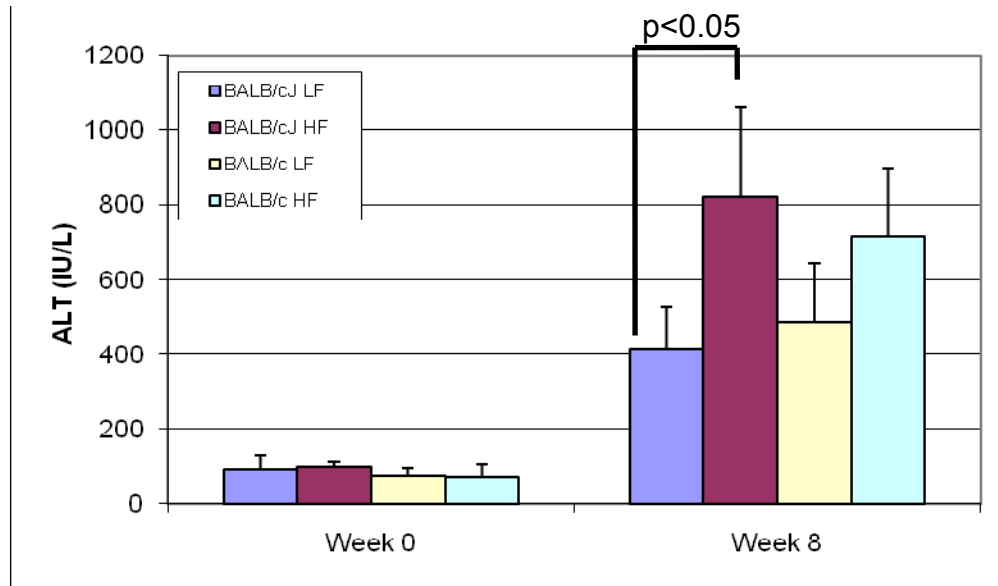
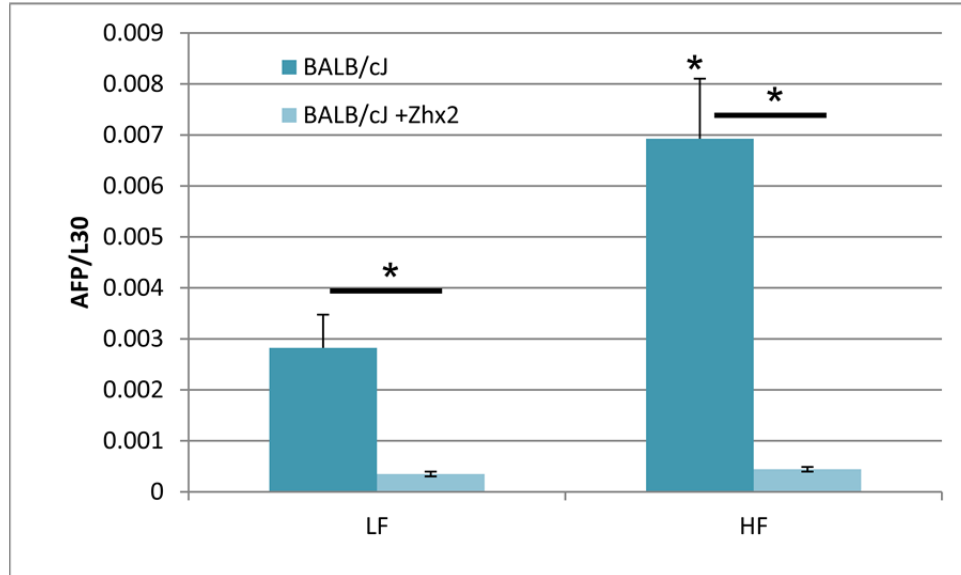


Figure 23. Serum ALT levels are induced on a high fat diet. Tail vein blood was collected from the cohorts **A.** BALB/cJ and BALB/cJ on either chow (LF) or HF chow and **B.** BALB/cJ and BALB/c on either chow or HF chow at week 0 and at the end of the study, week 8. Serum separate from red blood cells was analyzed from each mouse for ALT which is an indicator of liver damage. Results were averaged from each mouse in the group (n=5) and graphed as mean+standard deviation. Mice on the diet study all had ALT increases. BALB/cJ mice on HF had the highest induction by week 8. Although levels are higher in BALB/cJ HF they did not reach statistical significance over Zhx2 expressing mice (BALB/cJ+Zhx2 and BALB/c) on HF.

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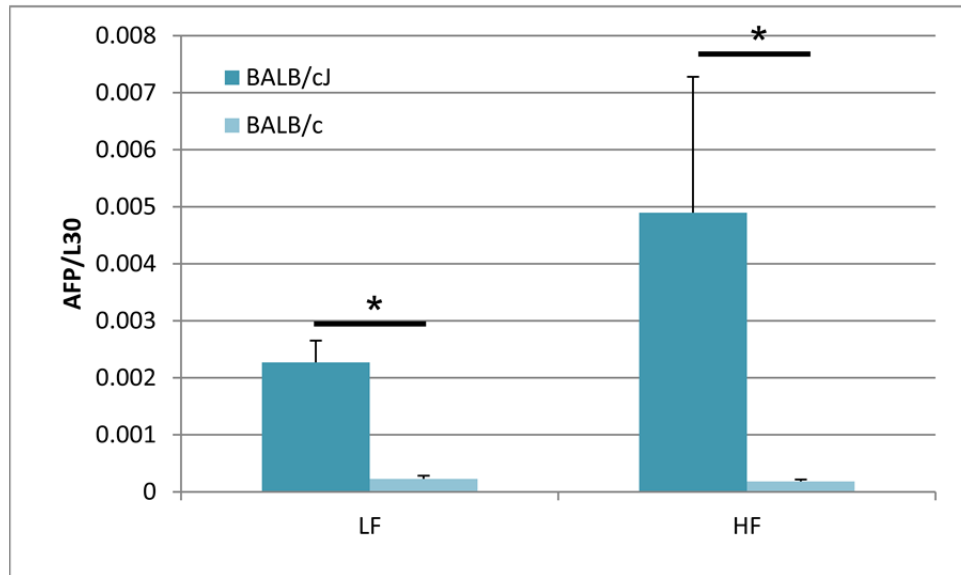
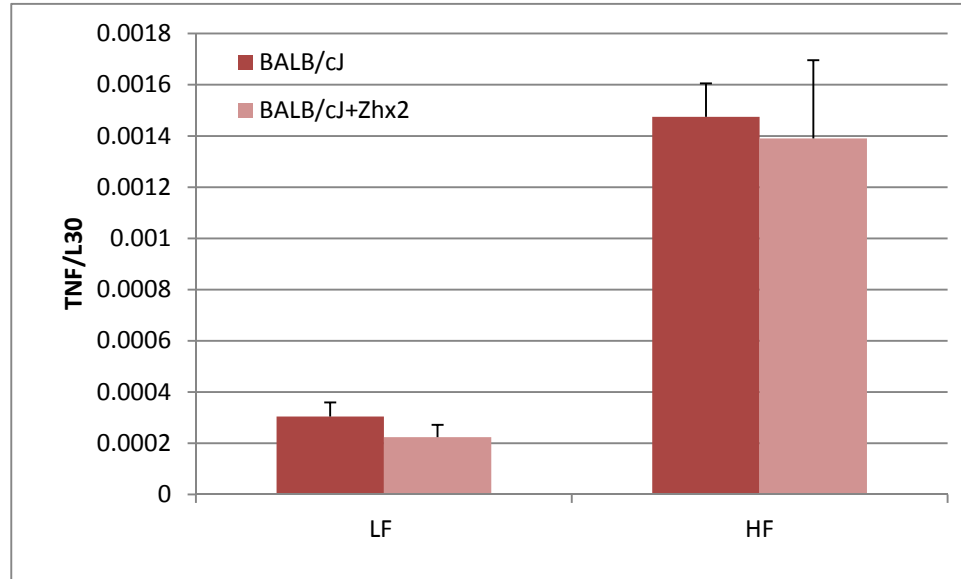


Figure 24. AFP expression is induced in BALB/cJ mice on HF chow. **A.** RNA collected from BALB/cJ or BALB/cJ+Zhx2 livers on chow (LF) or high fat chow (HF) were analyzed through Real-time RT-PCR for expression of alpha-fetoprotein (AFP). Ribosomal gene L30 was used as a normalizing control for all samples. Average from n=5 for each group was calculated and plotted as mean+standard deviation. BALB/cJ have significantly higher AFP levels compared to LF BALB/cJ +Zhx2. AFP is dramatically induced in BALB/cJ livers on HF which is not observed in BALB/cJ+Zhx2. **B.** RNA was analyzed from BALB/cJ and BALB/c on chow or HF chow similarly for AFP. Again, BALB/cJ have significantly higher AFP mRNA levels compared to LF BALB/c. AFP is induced in BALB/cJ on HF chow which does not occur in BALB/c. This suggests damage is occurring in BALB/cJ livers on HF chow for there to be this AFP induction. Significance levels * $p < 0.05$

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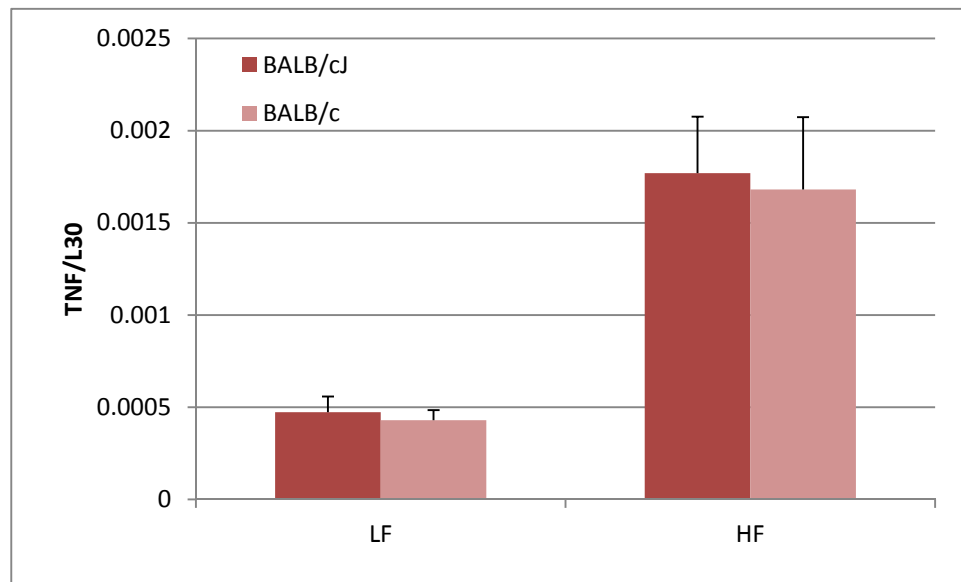
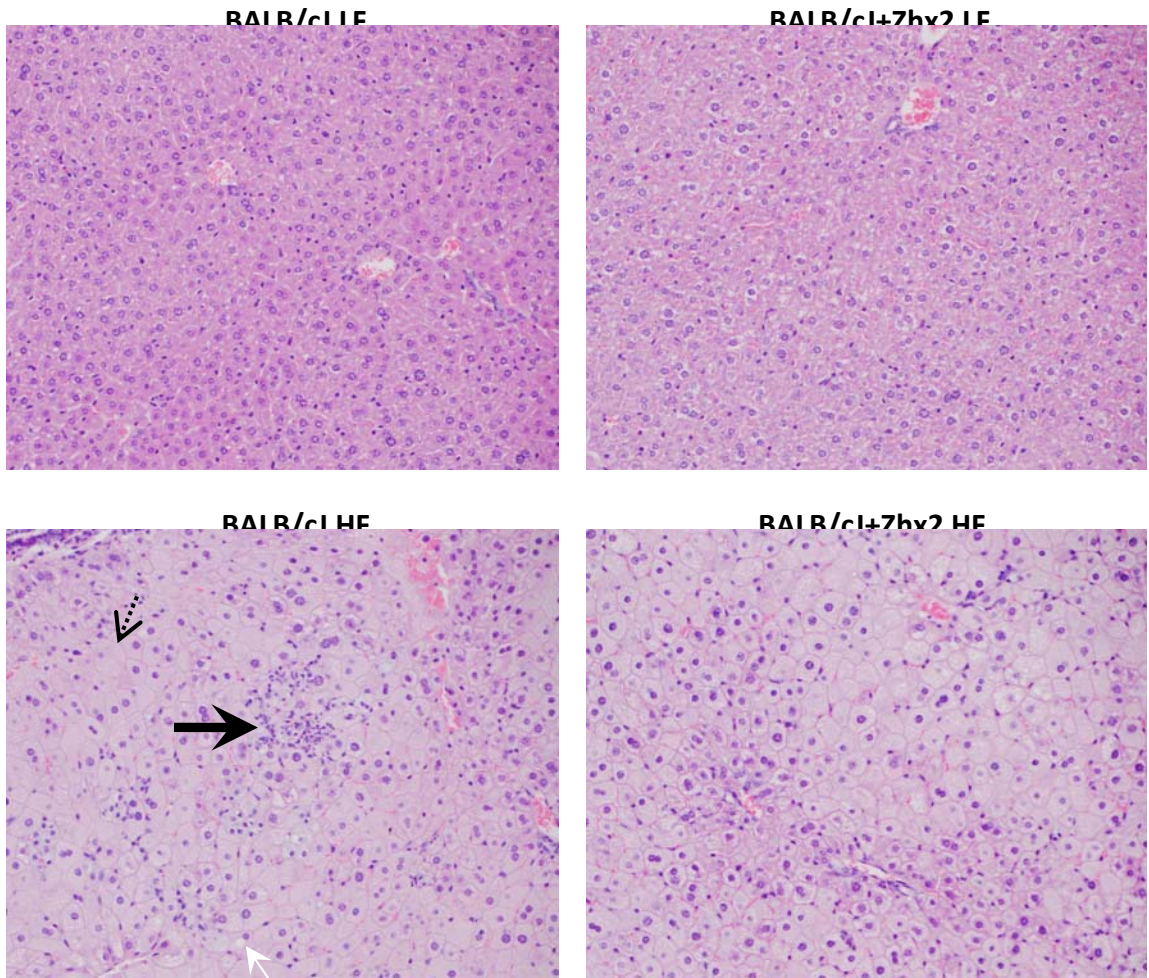


Figure 25. TNF α is induced during HF feeding and is not strain specific. **A.** RNA collected from BALB/cJ or BALB/cJ+Zhx2 livers on chow (LF) or high fat chow (HF) and **B.** BALB/cJ and BALB/c on chow or HF chow were analyzed through real-time RT-PCR for expression of Tumor Necrosis Factor alpha (TNF α). Ribosomal gene L30 was used as a normalizing control for all samples. Average from n=5 for each group was calculated and plotted as mean+standard deviation. In all cohorts on chow TNF α levels are very low and no difference is observed between strains. HF chow induces TNF α expression significantly (p<0.05) however, there again is no strain specific difference suggesting inflammation may not be the cause for liver damage. [56]

A.



B.

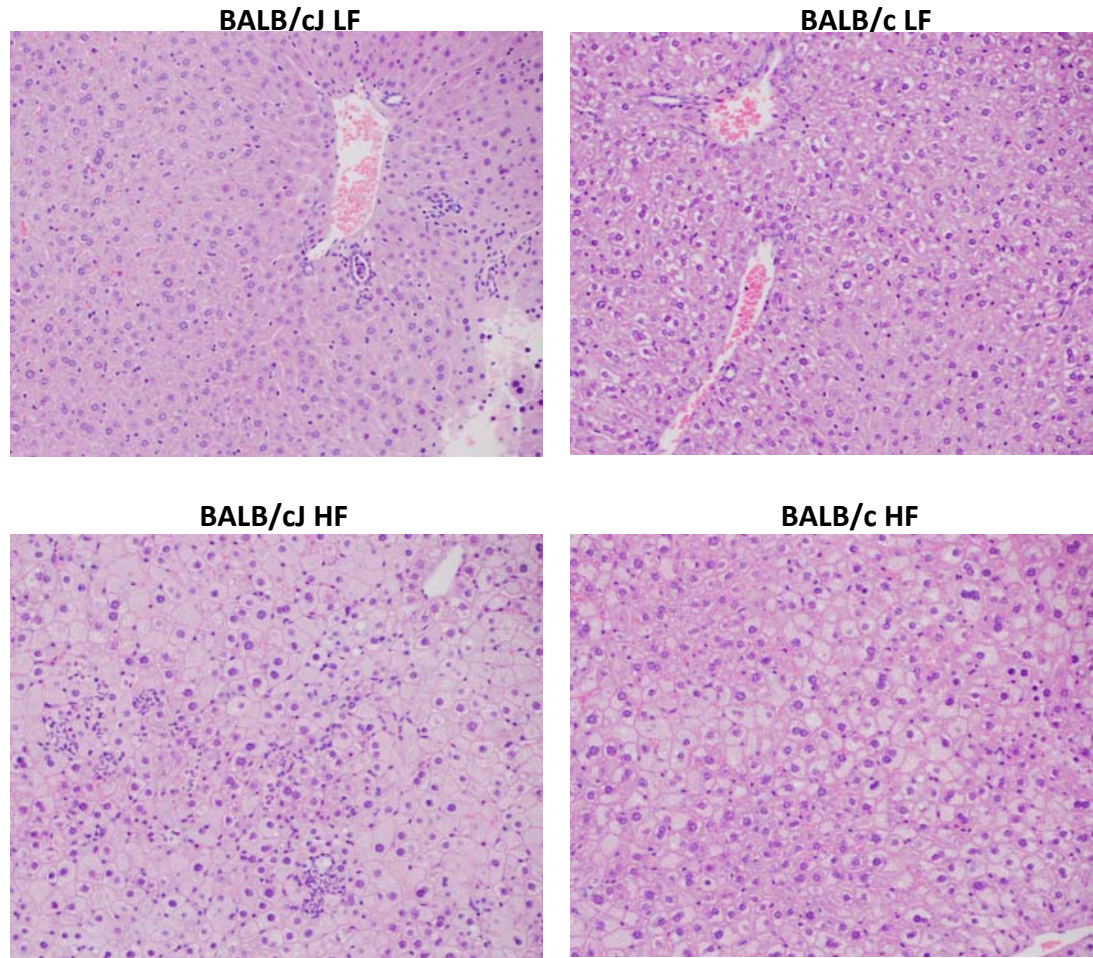
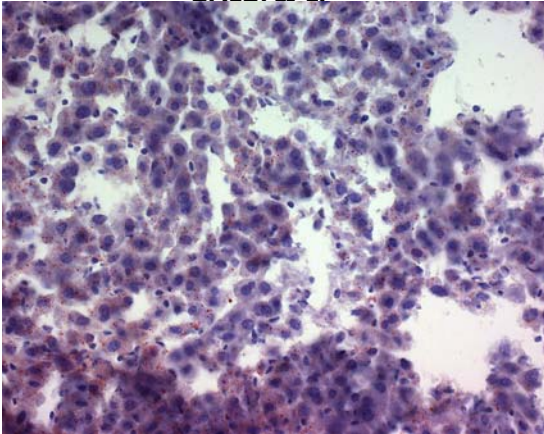


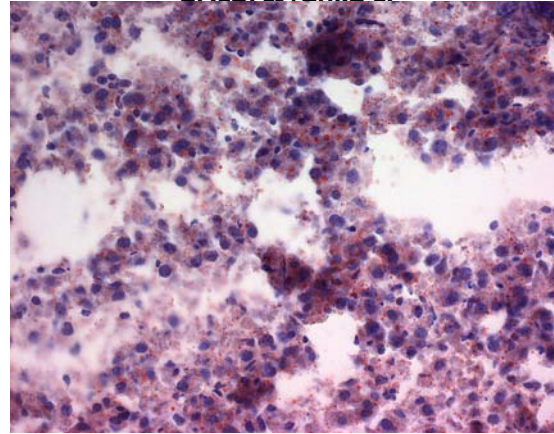
Figure 26. Liver Histology. Livers from each mouse in the cohorts **A.** BALB/cJ and BALB/cJ+Zhx2 on chow and HF chow and **B.** BALB/cJ and BALB/c on chow and HF were formalin fixed and paraffin-embedded. Sections from each were stained with H&E to examine overall histology. All mice on chow are normal as assessed by a blinded analysis by a pathologist. All mice on the high fat diet exhibit steatosis (white arrow) as well as hepatocyte ballooning (dashed arrow). Neutrophil abscesses are also present (black arrow) in the livers suggesting increased inflammation. BALB/cJ mice in particular were found to have <1% macrovesicular. These parameters in BALB/c mice were much less severe suggesting Zhx2 protects the liver. Magnification 20X.

A.

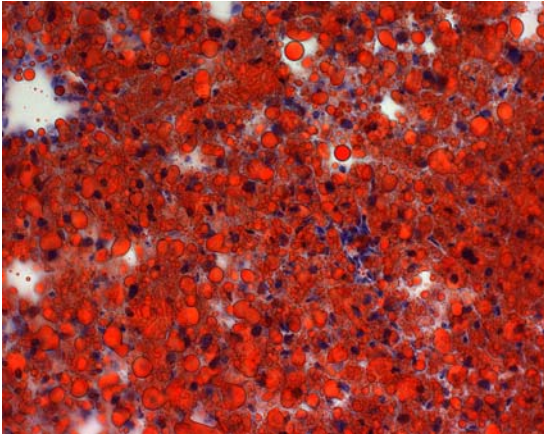
BALB/cJ LF



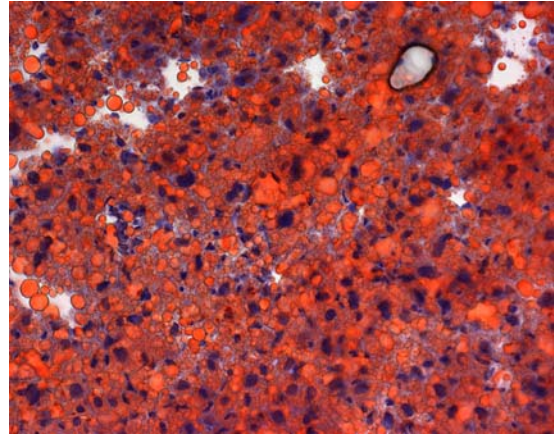
BALB/cJ+Zhx2 LF



BALB/cJ HF



BALB/cJ+Zhx2 HF



B.

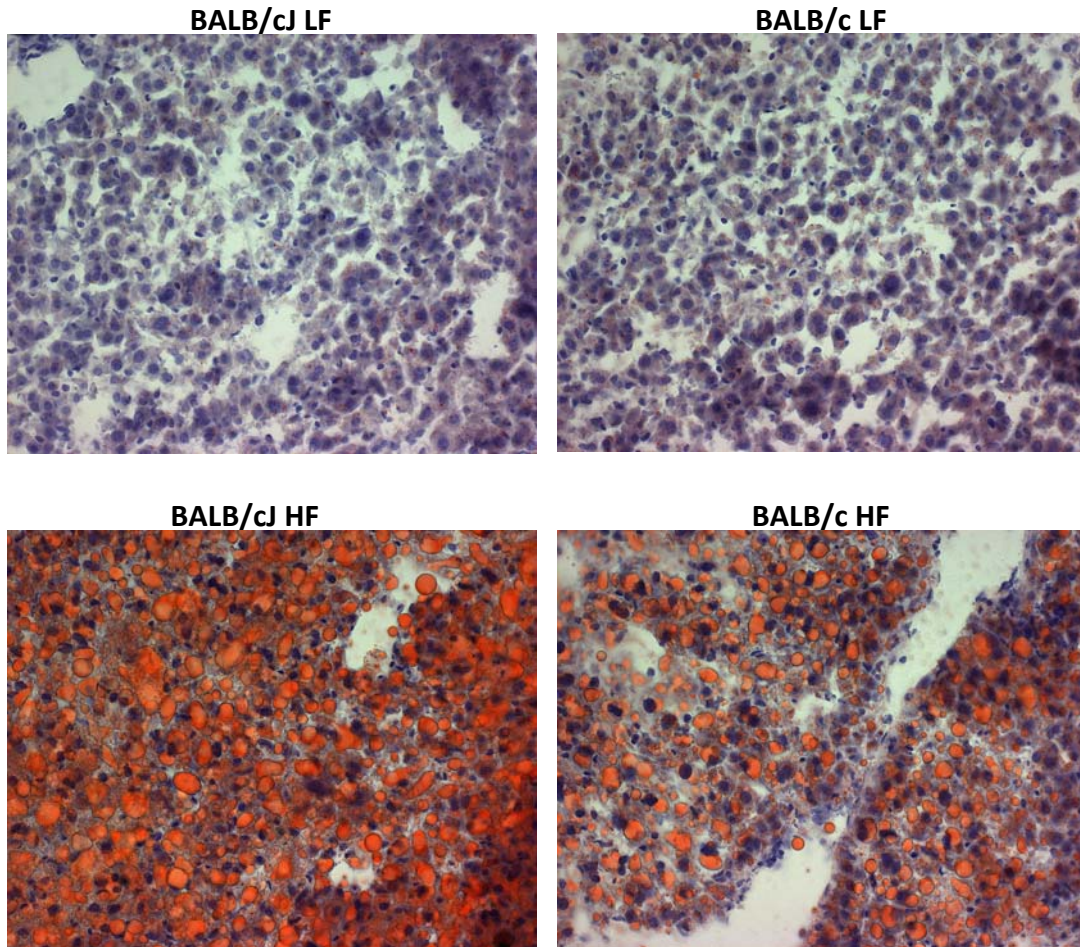


Figure 27. Oil Red O Staining is less severe in mouse livers expressing Zhx2. Frozen livers from the cohorts A. BALB/cJ and BALB/cJ+Zhx2 on chow or HF and B. BALB/cJ and BALB/c on chow or HF. Livers were sectioned at 10 um thickness and stained with Oil Red O in propylene glycol which stains lipids red. Livers were also counterstained with haematoxylin to stain nuclei blue. Images show that all mice on chow have small and few lipid droplets. BALB/cJ livers on HF have very dark red and widespread Oil Red O staining. Livers expressing Zhx2 (BALB/cJ+Zhx2 and BALB/c) have less robust staining suggesting these livers have less lipid accumulation. (Magnification, 20X)

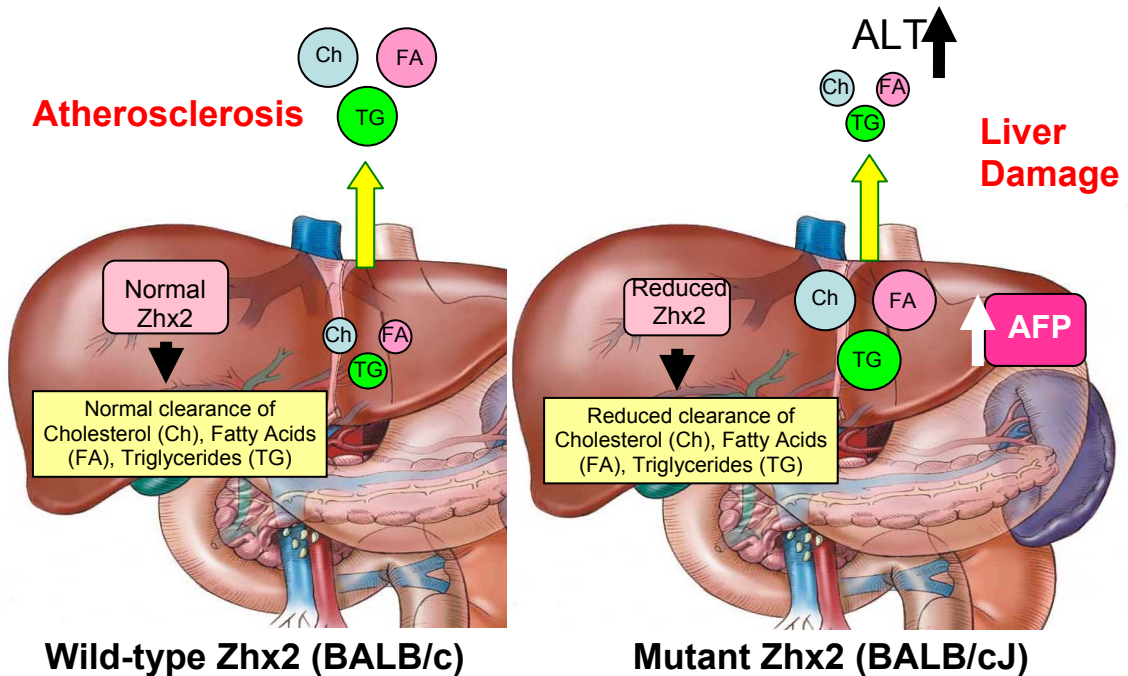


Figure 28. Model of Zhx2 and high fat diet-induced liver damage. In mice on a high fat chow, lipids including fatty acids, cholesterol and triglycerides are transported to the liver from the intestine. In BALB/c mice with normal Zhx2 levels (left panel) these lipids are exported out of the liver leading to excess lipids in the serum causing atherosclerotic plaque formation. In BALB/cJ mice (right panel), reduced Zhx2 levels lead to dysregulation of target genes resulting in reduced clearance of lipids from the liver. While decreased serum lipid levels reduce atherosclerosis incidence, excess hepatic lipids damage the liver and elevate AFP expression and serum ALT levels.

CHAPTER 6

Conclusions and Future Directions

The liver is an essential organ needed for maintenance of metabolic homeostasis and xenobiotic clearance. Therefore, the liver has become a organ for studying gene regulation in development and disease. In our case, we focus on the liver-specific gene AFP to understand developmental gene regulation as well as liver cancer gene expression changes. Early work with AFP by the Tilghman lab identified three upstream enhancers as well as a regulator of AFP postnatal repression [87, 197]. All three enhancers are important for the developmental hepatic expression for both AFP and its family member Albumin [198]. Characterization of E3 in particular by Peyton and Ramesh found pericentral activity in adult livers similar to endogenous pericentral zonal genes providing the first evidence of cis-acting elements governing zonal regulation [89]. *Zhx2*, the postnatal repressor of AFP, was identified in linkage mapping and recently implicated in other diseases including atherosclerosis. While my work has provided further insights into E3 regulation and atherosclerosis associated *Zhx2*, future studies will build upon these observations to better understand these mechanisms of regulation.

E3, RGS^e and β -catenin: Liver zonation is poorly understood, however an accepted mechanism is that extracellular signals are linked to transcriptional regulation. E3 and RGS^e are the first known cis-acting elements that exhibit zonality and therefore provide a model system for understanding the regulation. AFP is not a zonal gene and slight differences between E3 and RGS^e activity suggest they might not be regulated in exactly the same manner. However, one major similarity is the regulation by β -catenin. Previous studies showed an association of β -catenin activity with overall liver zonal gene expression [36, 128]. Utilization of hepatocyte specific β -catenin knockout (J. Butler, manuscript in preparation) showed both enhancer transgenes lost all activity in β -catenin^{-/-} livers.

My study presented here identified TCF4 sites at the 3' end in both enhancers through which β -catenin activates the enhancers. The degree of evolutionary

conservation of this site in both enhancers suggests an important function. This is especially evident in E3 since the sequence surrounding the TCF4 site immediately loses the high degree of conservation. Mutating the TCF4 site in the enhancers prevents increased activity from β -catenin; however, basal activities of these mutant enhancers is higher compared to wildtype enhancer constructs. In the mouse liver I hypothesize mutating this site in E3 and RGS^e will increase their activity into non-pericentral hepatocytes. Without active Wnt/ β -catenin signaling, bound TCF4 acts as a repressor through recruitment of co-repressors [61]. If TCF4 is unable to bind to its site, the enhancer should become de-repressed.

One aspect that continues to confound researchers is the mechanism that drives the formation of zonation in the liver. While the gradient hypothesis is the most popular, it remains difficult to study. Ultimately, oxygen and nutrient concentrations are highest at the portal triad and lowest at the central vein creating a gradient of potential signaling molecules [44]. Unfortunately, the exact molecules that signal intracellularly to alter gene expression remain unknown. β -catenin, while known as a regulator of zonation, has recently been found to be important in maintaining stem cells in their niches that generally have low oxygen concentrations. It was found that hypoxia inducible factor 1 alpha (HIF-1 α) activated β -catenin needed for maintenance [67]. I hypothesize that lower O₂ concentrations at the central vein increases β -catenin activity in the pericentral hepatocytes. To test this hypothesis Hep3B cells, with low endogenous β -catenin activity, could be transfected with TOP-Flash and treated with cobalt chloride (CoCl₂) to mimic a hypoxic environment. If hypoxia activates β -catenin, I would expect an increase in TOP-Flash luciferase levels. I would also expect similar results if wildtype E3- or RGS^e-pGL3 plasmids were used as the reporter construct. Mutated TCF4 sites within E3 and RGS^e enhancer constructs would blunt the activation by β -catenin activity.

AFP and β -catenin have very similar expression profiles throughout development of the liver [199]. Developing fetal liver express high levels of AFP and β -catenin until they are repressed around birth. AFP and β -catenin are also re-activated during liver regeneration for appropriate expansion and repopulation of hepatocytes [71]. Mutations in the negative complex or in β -catenin causing over-activation are often found

in cancers [200]. HCC reactivates AFP and a number of HCC samples harbor these activating β -catenin mutations [72]. To date, no association has been found between AFP and β -catenin in HCC [201]. However, in my study, I found that β -catenin regulates AFP during development since knockout β -catenin d1 livers have decreased AFP mRNA levels. During development, AFP is methylated until it is activated in hepatoblasts [202]. No studies have examined epigenetic changes at the AFP locus after birth. I hypothesize that the AFP gene undergoes epigenetic alterations after birth that contributes to low expression in adult liver. DNA methylation could be analyzed through bisulfite sequencing at both endogenous E3 and the promoter at time points in the liver beginning after birth continuing to adulthood. DNA methylation is typically preceded by other chromatin changes including nucleosome positioning. This type of regulation occurs very early in development when the stem cell markers OCT4 and Nanog are silenced. When the upstream enhancer of OCT4 and proximal Nanog enhancer become occupied with nucleosomes the element becomes less accessible to activating proteins [203]. ChIP analysis of histones 3 and 4 (H3 and H4) on the regulatory cis-elements of AFP could be done pre- and postnatally to determine if nucleosome positioning changes especially at the endogenous enhancers. It is possible that other pathways during HCC development activate AFP more robustly at earlier time points compared to β -catenin and this regulation occurs within the other regulatory elements. Ras and myc are oncogenes that are often activated in HCC [204]. Tumor suppressor p53 is often found repressed either through mutations or hypermethylation which can also occur in HCC [205]. AFP upstream region at -3.6kb is found to bind to myc and the AFP promoter has a Foxa/p53 binding site [206, 207]. Both could therefore be potential sites for AFP activation in HCC independent of β -catenin status.

E3, RGS^e and nuclear receptors. Both the AFP E3 and RGS^e enhancers are approximately 300-350bp in length which can potentially contain many protein binding sites. Besides the TCF4 site at the 3'-end, E3 contains three characterized binding sites at the 5'-end. Only the nuclear receptor site is involved in maintaining pericentral activity of E3 which binds ROR α and Rev-erb proteins [133]. In my study I show ROR α is an important positive regulator of E3 because ROR α null mice have dramatically reduced

E3 transgene activity. There is not a complete loss of E3 activity like in β -catenin knockout livers suggesting that there is possibly another factor that is able to activate the enhancer but not to the level of ROR α . Based on previous data from the lab as well as studies performed in chapter 4, β -catenin and ROR α are needed for full activity of E3. β -catenin has recently been shown to interact with nuclear receptors [158] so it is conceivable that there may be an interaction between β -catenin and ROR α occurring in E3. In EMSA, ROR α binds to the E3 NR site probe. Including the active β -catenin antibody resulted in a supershifted band suggesting an interaction between ROR α and β -catenin. These interactions unfortunately were not corroborated utilizing co-immunoprecipitations (Butler, J. unpublished). One interesting observation made by J. Butler was that ROR α has a slight zonal pattern of expression where the highest levels of proteins, detected by immunofluorescence, are in pericentral hepatocytes. Based on some recent data there also may be an indirect interaction between ROR α and β -catenin via HIF-1 α . As mentioned above, HIF-1 α has been shown to activate β -catenin, but it can also activate ROR α [208]. Moreover, ROR α is able to activate HIF-1 α creating a feed-forward mechanism of continuous activation [209]. I hypothesize that ROR α is able to activate HIF-1 α which activates β -catenin in pericentral hepatocytes. To test this hypothesis in vitro, Hep3B cells could be co-transfected with ROR α expression plasmid and TOP-Flash control plasmid. If β -catenin is activated luciferase from TOP-flash will increase. To date no ROR α binding site is identified for RGS^e so the results should be similar to TOP-flash. If this is occurring in the mouse liver then immunohistochemistry for active β -catenin in ROR^{-/-} mice should also be reduced.

When Peyton linked E3 to E2 in the reporter transgene the regulation occurring at E3 was dominant, and again, only active in pericentral hepatocytes [90]. Mutation of the nuclear receptor in the E3-E2- β gl-D^d changed the pattern of activity to that of E2 demonstrating active repression of E3 is occurring in the non-pericentral hepatocytes [90]. The proteins Rev-erb α and Rev-erb β are known repressors and can bind to the NR site along with the activator ROR α . My data is consistent with SS transgenics in that the NR mutation in E3-E2-pGL3 is not responsive to either Rev-erb α or Rev-erb β but surprisingly maintains responsiveness to ROR α . This could be due to added activity

provided by E2 which is not as well characterized or studied enhancer. To rule out ROR α binding in E2, binding site analysis with TESS could identify any putative orphan nuclear receptor sites. If any exist, EMSA can show whether the ROR α protein is capable of binding to any putative sites. Transient transfection with E2 alone in pGL3, if activated by ROR α , would show an increase in activity when ROR α is co-transfected. I have also determined a cloning strategy to place SS mutant E3 alone in pGL3. While it may have low baseline activity, if my hypothesis is correct, activity will increase in presence of ROR α . Our working hypothesis is that in pericentral hepatocytes ROR α is bound to the nuclear receptor site while Rev-erb α or Rev-erb β is bound in non-pericentral hepatocytes. To test this hypothesis we are currently generating mice that contain the MHC Class 1 L^d gene driven by the PEPCK promoter; so transgene expression is only detected in periportal hepatocytes [210]. Transgene positive mice will then be bred to E3- β gl-D^d mice to establish mice with reporter genes expressed in periportal (L^d) and pericentral (D^d) hepatocytes. One could cell sort these two different subsets of hepatocytes and run ChIP analysis on E3 transgene for the presence of ROR α or the Rev-erb proteins. Another approach, using mRNA-seq, could identify differential expression of genes between periportal and pericentral hepatocytes possibly elucidating novel factors involved with zonal regulation.

RGS^e is not as well characterized compared to E3, but binding site analysis has not identified a ROR α binding site. Endogenous GS expression is slightly reduced in ROR^{-/-} livers suggesting other factors may be involved in its regulation. Based on these observations, if RGS^e mice were bred to ROR null mice, I would not predict any transgene changes in activity with or without ROR α . The nuclear receptor HNF4 α seems to have a greater impact on GS expression compared to data from ROR α . HNF4 α knockout livers increase GS expression into non-pericentral hepatocytes detected by immunohistochemistry suggesting HNF α represses GS in non-pericentral hepatocytes [50]. Unfortunately, the binding sites identified and characterized in GS were not located within the enhancer [211]. Through similar binding site analysis with TESS I have identified a putative HNF4 α binding site within RGS^e. In vitro tests could be performed first, co-transfecting increasing HNF4 α with wildtype RGS^e-pGL3 and HNF4 α mutant

RGS^c to determine if this site was functional and responsive to HNF4 α . I predict, if mutated, RGS^c activity *in vivo* would increase into non-pericentral hepatocytes much like that of the NR mutation in E3.

Our model for zonal regulation also includes these different factors, β -catenin and nuclear receptors exerting their function through an enhancer element. GS is not the only zonal genes in which enhancers have been identified. More recently, four putative enhancer elements were identified upstream of the OAT transcription start site. Cloning each individual enhancer showed activity in the eye, gut, and kidney (J. Butler). Unfortunately, the final putative enhancer was not found to have any liver activity (data not shown). We are currently exploring other putative enhancer elements found within RhBG and I predict one will recapitulate zonal activity. While our focus has been mainly on pericentral genes, not much is known about the zonal regulation of periportal genes. PEPCK, as mentioned before, is regulated by many nutritional and hormonal signals [39], but the factor to silence it in pericentral hepatocytes remains unknown. Linking the promoter to a heterologous gene drives the expression to a zonal pattern much like that of the endogenous gene. Therefore, I predict periportal genes are zonally regulated primarily through the promoter. The PEPCK-L^d transgenic mice could then serve as a model system to understanding periportal gene regulation.

Zhx2 and Cholesterol Homeostasis. Overall lipid homeostasis is important since imbalance can lead to disease such as obesity, diabetes, atherosclerosis and NAFLD. While the QTL study shows presence of Zhx2 causes susceptibility to an atherogenic diet due to increased serum cholesterol and triglyceride levels, my data suggests it also protects against liver damage. Liver damage introduces additional consequences for if it becomes chronic, NAFLD can progress to cirrhosis which is known to promote the development of HCC [186]. My data also validates gene expression changes, similar to those observed in the Lusis microarray, identifying novel Zhx2 targets. Lpl, Cyp8B1 and Elovl3 fall within this category, but have slightly different expression patterns based on Zhx2 status and type of diet. While the mechanism for how Zhx2 regulates its target genes remains unknown studying additional targets provides new models for understanding the mechanism. The postnatal repression of AFP by Zhx2 occurs in the

250 proximal promoter therefore, the prediction is these new target genes would also be regulated through their promoters. PCR amplified promoters from each of these genes could be placed in the pGL3 enhancer construct and co-transfected in hepatoma cell lines along with a Zhx2 expression plasmid. I would expect constructs with Lpl and Cyp8B1 promoters would have reduced luciferase levels with the addition of Zhx2. Transfection studies with AFP and Zhx2 have proven difficult since the 10 to 20-fold difference seen between BALB/cJ and other strains is not recapitulated with this system. The maximum reduction Zhx2 exerts at the 250 bp AFP promoter is 2-fold. Examining the Elov13 promoter would provide a better model system since addition of Zhx2 would have an opposite effect, causing an increase in luciferase levels. If all of the promoters respond, gradual deletions of promoter regions could localize elements responsive to Zhx2 that can then be compared amongst all the target genes to determine a consensus sequence. Throughout the years no binding of Zhx2 to the AFP promoter has been detected suggesting a post-transcriptional regulation by Zhx2. Unspliced AFP is found in the cytoplasm in the presence of Zhx2 (Turcios, L and Peterson, M. unpub data). To see if this occurs in the new targets several primers can be designed to monitor inclusion of introns in the mRNA.

Damage occurs in the liver multiple ways due to its encounter with many molecules and substances on a daily basis. Though the mechanisms of hepatocyte stress and cell death may differ, HCC development is a common end result of chronic damage. For NAFLD, in particular, chronic inflammation is not the only causative source of hepatic injury. In my study, I have shown serum ALT and AFP are induced in BALB/cJ during a high fat feeding. I predict based on these parameters that there is increased cell death during the high fat feeding, the most occurring in BALB/cJ livers, which can be quantified using Tunel staining of paraffin embedded sections. Lipids are known to cause damage through stress and metabolism. Lipid oxidation of fatty acids can produce reactive-oxygen species (ROS) that lead to cell death [212]. Since BALB/cJ mice seem to accumulate more lipids based on Oil Red O, I would hypothesize ROS levels are dramatically increased compared to BALB/c and BALB/cJ+Zhx2 during high fat feeding. Liver damage activates stellate cells which deposit collagen as a regeneration mechanism. Years ago QTL mapping of BALB/cJ and A/J mice located a site on mouse chromosome

15 associated with fibrosis, a similar region to the location of *Zhx2*. BALB/cJ mice exhibit more severe fibrosis which could be occurring in this diet study [213]. Collagen deposition can be easily detected and quantified using staining techniques on paraffin-embedded sections. I would predict that BALB/cJ hepatic collagen levels would be higher than BALB/c livers on the high fat chow. This too may also be due to *Zhx2* expression within stellate cells. Therefore, I would not predict a difference in collagen levels between BALB/cJ and BALB/cJ+*Zhx2* since the transgene is only expressed in hepatocytes.

The liver is not the only tissue found important for the progression of metabolic diseases. Macrophages are a major culprit for they develop into foam cells when phagocytosing lipids, which adds to atherosclerotic plaques [214]. Dysregulation of fat storage or increased inflammation occurring in the adipose tissue has major implications in the metabolic diseases [215]. Due to its ubiquitous expression profile [96] and implications in other diseases [101, 106, 216], it is possible the *Zhx2* may be playing regulatory roles in these other tissues that have not yet been identified. We have recently obtained *Zhx2* floxed mice from the KnockOut Mouse Project (KOMP) in which their targeting vector inserted loxP sites flanking exon 3, the coding region of *Zhx2*. These mice can then breed to mice containing cre-recombinase driven by various promoters to delete *Zhx2* in a tissue specific manner. Based on our laboratory's interests we would first knock-out *Zhx2* in hepatocytes. If these mice are placed on an atherogenic diet I would predict a phenotype similar to that of BALB/cJ; lower serum lipid levels compared to wildtype animals, low incidence of atherosclerotic plaques, increased serum ALT and AFP mRNA levels. Tissue specific *Zhx2* deletion could elucidate *Zhx2* regulatory roles during high fat feeding in other metabolic tissues mentioned above. *Zhx2* knockout may in fact provide a better model since BALB/cJ mice are a knock-down of *Zhx2* in protein and mRNA levels compared to either BALB/c or BALB/cJ+*Zhx2*.

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Curriculum Vitae

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EDUCATION

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PUBLICATIONS

Kojima F, Kapoor M, Yang L, **Fleishaker EL**, Ward MR, Monrad SU, Kottangada PC, Pace CQ, Clark JA, Woodward JG, Crofford LJ. Defective generation of a humoral immune response is associated with a reduced incidence and severity of collagen-induced arthritis in microsomal Prostaglandin E Synthase-1 null mice. J Immunol. 2008 June 15; 180(12):8361-8

Opel, KL, **Fleishaker, EL**, Nicklas, JA, Buel, E., McCord, BR. Evaluation and quantification of nuclear DNA from human telogen hairs. J Forensic Sci. 2008 Jul; 53(4):853-7. Erratum in: J Forensic Sci. 2008 Nov; 53(6):1501

POSTERS

April ,2011, Experimental Biology Meeting, Washington,D.C. Erica Fleishaker, James Butler, Martha Peterson, Brett Spear. Beta-catenin and nuclear receptors regulate zonal AFP enhancer expression. Poster Presentation

March 22,2011, Markey Cancer Center Research Day, Lexington, KY. Erica Fleishaker, James Butler, Martha Peterson, Brett Spear. Beta-catenin and nuclear receptors regulate zonal AFP enhancer expression. Poster Presentation

October, 2010, Microbiology, Immunology, Molecular Genetics Department Retreat, Lexington, KY. Erica Fleishaker, James Butler, Martha Peterson, Brett Spear. Beta-catenin and nuclear receptors regulate zonal AFP enhancer expression. Poster Presentation

April 27, 2010, Experimental Biology Meeting, Anaheim, CA. Poster presentation entitled “Zhx2 protects the liver from high fat diet-induced liver damage”. Erica L. Fleishaker, Martha Peterson, Brett T. Spear

April 14, 2010, Markey Cancer Center Research Day, Lexington, KY. Poster presentation entitled “Zhx2 protects the liver from high fat diet-induced liver damage”. Erica L. Fleishaker, Martha Peterson, Brett T. Spear.

PRESENTATIONS

February 4, 2011 – “AFP enhancer 3: Insights into liver zonality” Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky

March 5, 2010 – “New insights into Zhx2 and liver function” Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky

April 24, 2009 – “Zhx2: A Novel Regulator of Hepatic Lipid Metabolism” Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky

September 21, 2007 – “Alternative Methods to Ameliorate Pathology of Duchenne’s Muscular Dystrophy” Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky

AWARDS

March 22, 2011, Markey Cancer Center Research Day, Lexington, KY. 1st place basic science graduate student poster.