ROLE OF SRC PHOSPHORYLATION OF FXR IN BILE ACID REGULATION

BY

DANIEL RYERSON

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Molecular and Integrative Physiology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2017

Urbana, Illinois

Doctoral Committee:

Professor Jongsook Kim Kemper, Chair
Professor David Shapiro
Associate Professor Lori Raetzman
Assistant Professor Sayeepriyadarshini Anakk

Abstract

Bile acids are physiological detergents which aid in the absorption of dietary lipids and lipid soluble vitamins but also function as fed state signaling molecules. Elevated bile acid levels in the liver can lead to cholestatic injury, primary biliary cirrhosis, fibrosis, and liver cancer; therefore, these levels must be tightly regulated. The farnesoid X receptor (FXR) is the primary bile acid nuclear receptor and acts as the master regulator of bile acid homeostasis, preventing liver damage caused by bile acid accumulation. FXR does this by regulating the expression of many target genes in the gut and liver including the intestinal hormone fibroblast growth factor 19 (*FGF19*) and orphan nuclear receptor small heterodimer partner (*SHP*). In response to elevated hepatic bile acid levels FXR, acting directly as well as through FGF19 and SHP, inhibits the synthesis of bile acids, downregulates bile acid importers, upregulates bile acid exporters along with genes involved in bile acid conjugation and detoxification. These important roles of FXR are highlighted by the phenotypic effects observed in FXR knockout ($FXR^{-/-}$) mice. $FXR^{-/-}$ mice display elevated bile acid pool size as well as elevated serum bile acid levels. Additionally, $FXR^{-/-}$ mice show signs of liver damage and develop spontaneous tumors as they age. Understanding how FXR receives signals and translates them into transcriptional responses to mediate these diverse cellular effects will be important for the development of therapeutic agents to treat cholestatic liver disorders.

One mechanism through which FXR activity is regulated is signal-induced post-translational modifications. FXR has been shown to undergo multiple types of post-translational modifications including phosphorylation, methylation, acetylation and sumoylation in response to physiological and pathological signals. These modifications affect FXR in many ways including modulating subcellular localization, stability, DNA binding, interaction with

transcriptional coregulators and affecting the expression of FXR target genes in a gene selective manner. Mutation of a single amino acid, disrupting one of these post-translational modifications, has been shown to dramatically alter FXR function. Interestingly, some of these post-translational modifications have been shown to be misregulated in models of disease, which highlights the importance of understanding the molecular mechanisms through which FXR is post-translationally modified.

In this study a new post-translational modification of FXR was identified which profoundly impacts FXR transcriptional activity. Unbiased mass spectrometry based proteomic analysis showed that tyrosine-67 of FXR is rapidly phosphorylated in liver hepatocytes in response to treatment with either natural bile acids or FGF19. Biochemical analysis paired with bioinformatic tools identified Src as the kinase responsible for this post-translational modification. Feeding mice a diet supplemented with the primary bile acid cholic acid (CA) led to interaction between FXR and Src as well as phosphorylation of FXR. Further studies showed that Src interacts with the DNA binding domain of FXR specifically. *In vitro* kinase assays utilizing purified Src protein coupled with studies utilizing siRNA knockdown of Src demonstrated that Src is both necessary and sufficient for FXR phosphorylation. Adenoviral reconstitution of wild type and tyrosine-67 phosphorylation deficient mutant (Y67F) FXR in isolated primary mouse hepatocytes (PMH) showed that disruption of this phosphorylation site led to a decrease in FXR/RXR interaction and decreased expression of a subset of FXR target genes involved in bile acid regulation, particularly bile salt export pump (*BSEP*) and *SHP*. Disruption of this site *in vivo* also led to elevated bile acid levels, elevated liver enzyme levels, and increased macrophage infiltration; all signs of liver damage. Additionally, when challenged in models simulating cholestasis, these signs of liver damage are dramatically elevated in mice

expressing Y67F-FXR. These *in vivo* studies demonstrate that disruption of the FXR tyrosine-67 site drastically impairs FXR's ability to regulate its target genes, maintain bile acid homeostasis, and protect the liver from bile acid induced toxicity.

In conclusion, this study identified a previously unknown phosphorylation site of FXR which is mediated by Src. We further showed that this phosphorylation is critical for FXR function, maintenance of bile acid homeostasis, and protecting the liver against bile toxicity; with loss of this phosphorylation site leading to the development of liver damage *in vivo*. The profound effects FXR tyrosine-67 phosphorylation has on FXR transcriptional activity and metabolic outcomes suggest that this site and the kinase leading to its phosphorylation may prove to be innovative targets for the treatment of hepatobiliary and cholestatic diseases.

Table of Contents

Chapter One

Background and Significance

1.1 FXR is a member of the nuclear receptor family

Nuclear receptors (NRs) are a family of ligand activated transcription factors which regulate expression of networks of target genes involved in diverse physiological functions (Nagy and Schwabe 2004, Mangelsdorf et al., 1995). NRs can be divided into three subgroups based on their physiological ligands (Sonoda et al., 2008). The first class of NRs contains the classic endocrine receptors which are characterized by their very high affinity to ligands. This class includes the receptors for steroid hormones, thyroid hormone, and vitamins A and D and plays key roles in regulation of the endocrine system. The second and third classes of NRs were identified through their sequence homology to the endocrine NRs but had no identified ligand. Receptors whose physiological ligands are not known are referred to as orphan NRs. Subsequently, many of the ligands for these orphan NRs have been identified and these are called adopted NRs. The adopted NRs make up the second class of NRs and are characterized by their low affinity for their respective ligands. This class includes receptors for dietary lipids and xenobiotics and plays important roles in regulating glucose and lipid homeostasis (Parks et al., 1999; Willy et al., 1995; Kliewer 2003; Lehmann et al., 1997; Kilewer et al 1997; Repa and Mangelsdorf 1999; Chiang 2002). The third class of NRs contains the remaining orphan NRs whose ligands have not yet been identified.

FXR is an adopted NR that is highly expressed in the liver, intestine, kidney and adrenal glands with lower levels found in heart and adipose tissues (Forman et al., 1995, Lu et al., 2001, Zhang et al., 2002, Seol et al., 1995). In humans, four functional isoforms of FXRα (FXRα1,

FXRα2, FXRα3, FXRα4) are encoded by a single gene. FXRα3 and FXRα4 are also referred to as FXRβ1 and FXRβ2. The four different forms of FXR arise from the use of two separate promoter sequences and alternative splicing of exons 5 and 6 (Zhang et al., 2002; Huber et al., 2002). Most FXR target genes are regulated in an isoform independent manner. The physiological importance of the different FXR isoforms has not been established.

Upon activation, NRs bind as monomers or dimers to specific DNA sequences called response elements and regulate gene transcription (Chiang 2002; Chawla et al., 2001). FXR usually functions as a heterodimer with 9-*cis*-retinoid X receptor (RXR) when binding to its response element (Claudel et al., 2002). The FXR response element (FXRE) generally consists of two copies of a six nucleotide sequence (AGGTCA) arranged in one of three orientations: as an inverted repeat separated by a single nucleotide (IR1), as a direct repeat separated by four nucleotides (DR4), or as an everted repeat separated by eight nucleotides (ER8); however FXR has also shown the ability to bind as a monomer to FXRE half sites (Claudel et al., 2002; Edwards et al., 2002; Kalaany et al., 2006; Zhang and Edwards 2008).

NR activation is often stimulated by the binding of agonists to the ligand binding domain. In the absence of ligand, NRs are associated with corepressor proteins and are inactive. Upon binding of a ligand, NRs undergo a conformational change which leads to activation through several methods including release of corepressors, dimerization, nuclear translocation, and recruitment of coactivators (Repa and Mangelsdorf 2000; Sonoda et al., 2008).

1.2 FXR in bile acid homeostasis

FXR was originally named because of its affinity for farnesol metabolites, however recent work has shown that at physiological levels bile acids act as the major ligand for FXR

(Makishima et al., 1999; Zhang and Edwards 2008; Goodwin et al., 2000). Several endogenous bile acids have been identified as agonists of FXR to varying levels. The order of potency for FXR activation by the endogenous bile acids cholic acid (CA), chenodeoxycholic acid (CDCA) deoxycholic acid (DCA) and lithocholic acid (LCA) is CDCA > LCA = DCA > CA. In addition to the endogenous FXR ligands several synthetic FXR agonists including GW4064, fexaramine, 6-a-ethyl-chenodeoxycholic acid (ECDCA) and obeticholic acid (OCA) have been developed both as investigative tools to examine FXR functions as well as potential pharmaceutical treatments (Goodwin et al., 2000; Ali et al., 2015; Fang et al., 2015).

Bile acid synthesis is regulated through feedback inhibition of the enzyme cholesterol 7 alpha-hydroxylase (*CYP7A1*), which catalyzes the rate limiting step in the conversion of cholesterol into bile acids. This inhibition is mediated by FXR. The mechanism of feedback regulation starts when bile acids reabsorbed in the ileum are transported through the enterohepatic circulation to the liver, the major site of bile acid synthesis. The elevated bile levels in the liver activate FXR though ligand binding. The activated FXR then heterodimerize with RXR and binds to FXREs on the promoter of its target gene small heterodimer partner (*SHP*), inducing expression. SHP goes on to interact with the nuclear receptors liver receptor homolog-1 (LRH-1) and hepatocyte nuclear factor 4 α (HNF4α) which are required for *CYP7A1* expression. SHP binding to LRH-1 and HNF4α inhibits their activity thereby inhibiting *CYP7A1* expression and bile acid synthesis (Russell 2003). In addition to directly binding to the *SHP* promoter and inducing transcription of *SHP* in the liver, FXR also induces expression of the hormone fibroblast growth factor 19 (*FGF19*) in intestinal cells which binds to the FGFR4/β-Klotho receptor on hepatocytes, activating kinase pathways to stabilize SHP and further inhibiting *CYP7A1* expression (Inagaki et al., 2005; Miao et al., 2009).

Accumulation of bile acids in the liver can have toxic effects, which requires the liver to have a number of pathways to protect itself from bile acid accumulation. In addition to downregulating the rate limiting enzyme in bile acid synthesis, FXR also regulates a number of genes involved in bile acid transport and detoxification. FXR inhibits expression of sodium taurocholate cotransporting polypeptide (*NTCP*) which is involved in the transport of bile acids into the liver from intestinal cells (Kok et al., 2003; Sinal et al., 2000). FXR also increases expression of transporters involved in the export of bile acids from the liver into the bile duct, such as bile salt export pump (*BSEP*), multidrug resistance-associated protein 2 (*MRP2*), and multidrug resistance P-glycoprotein 3 (*MDR3*) (Plass et al., 2002; Ananthanarayanan et al, 2001; Kast et al., 2002; Trauner and Boyer 2003). Furthermore, FXR increases the expression of genes responsible for the conjugation and detoxification of bile acids including sulfotransferase family 2A member 1 (*SULT2A1*), cytochrome P450 3A4 (*CYP3A4*), and UDP glucuronosyltransferase family 2 member B4 (*UGT2B4*) (Song et al., 2001; Barbier et al., 2003; Gnerre et al., 2004). Taken all together, in response to elevated bile acid levels FXR inhibits bile

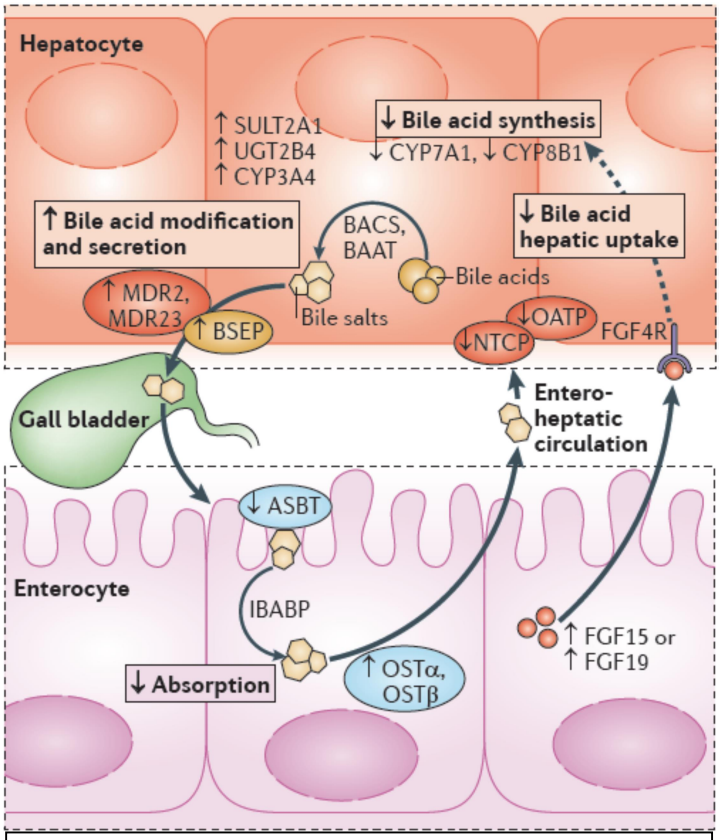


Figure 1.1: FXR regulation of bile acid homeostasis.

Adapted from Calkin and Tontonoz (2012) Transcriptional integration of metabolism by the nuclear sterol activated receptors LXR and FXR Nat Rev Mol Cell Biol 13(4):213-24.

acid synthesis and limits bile acid import into the liver while simultaneously increasing the conjugation, detoxification, and export of bile acids, thereby protecting the liver from toxic effects of increased bile acid levels as summarized in figure 1.1.

1.3 FXR in lipid and glucose regulation

In addition to its important role in the regulation of bile acid homeostasis, FXR has been shown to regulate genes involved in numerous other metabolic pathways. Deletion of FXR in transgenic mice results in a number of deleterious effects including increases in plasma triglycerides, free fatty acids, LDL and HDL proteins, insulin insensitivity, glucose intolerance, reduced hepatic glycogen, and impaired liver regeneration (Sinal et al., 2000; Cariou et al., 2006; Cariou et al.,2005; Inagaki et al., 2006; Huang et al., 2006). Consistent with these results, treatment of diabetic mice with FXR agonists has been shown to significantly improve insulin sensitivity and plasma glucose levels (Zhang et al., 2006). Additionally administration of bile acids to human patients has been shown to have the desirable effect of decreasing plasma triglyceride and LDL levels (Bateson et al., 1978; Fiorucci et al., 2007). Unfortunately FXR activation also produces the undesirable outcome of reducing HDL levels (Sinal et al., 2000). Recent work has begun to elucidate the molecular mechanisms through which FXR activation regulates lipid and glucose metabolism. FXR activation induces the expression of phospholipid transfer protein (PLTP) which is involved in HDL remodeling (Urizar et al., 2000). Activation of FXR also reduces the expression of apoA-I, the major apolipoprotein of HDL (Claudel et al., 2002). Additionally FXR induces expression of lipoprotein lipase activator genes apoC-II and apoA-V while inhibiting expression of lipoprotein lipase inhibitor apoC-III (Kast et al., 2001; Prieur et al., 2003; Claudel et al., 2003). FXR activation also increases fat oxidation by enhancing the expression of *PPARα* (Pineda Torra et al., 2003). FXR activity modulates glucose

metabolism through the regulation of the gluconeogenic genes phosphoenolpyruvate carboxykinase (*PEPCK*), glucose 6-phosphatase (*G6Pase*), and fructose-1,6-bisphosphatase (*FBP1*) (Yamagata et al., 2004; De Fabiani et al., 2003). FXR's roles in lipid and glucose metabolism have led some to suggest that FXR may be a novel target for the treatment of metabolic diseases (Duran-Sandoval et al., 2005).

1.4 Novel functions of FXR

Recent work has shown that FXR also plays key roles in many other physiological processes including autophagy, liver regeneration, inflammation, and regulation of cell proliferation and tumor suppression. Autophagy is a cellular recycling system which is activated in low nutrient conditions. When cells are deprived of nutrients cellular components are broken down to serve as energy sources. In fasting conditions, the transcriptional activators cAMP response element-binding protein (CREB) and peroxisome proliferator-activated receptor-α (*PPARα*) upregulate the hepatic autophagy gene network. CREB functions by recruiting the coactivator CREB regulated transcription coactivator 2 (CRTC2) to autophagy genes. While, PPARα works by heterodimerizing with RXR to increase transcription of autophagy genes through a distinct but complimentary mechanism. FXR has been shown to play a key role in regulating autophagy. FXR acts as a fed state sensor, activating in response to the elevation of bile acid levels after ingestion of a meal. Activation of FXR disrupts the interaction of CREB and CRTC2 and competes with PPARα for RXR binding, thereby inhibiting the expression of key autophagy genes, including those for autophagy-related protein 7 (*Atg7*), Unc51 like autophagy activating kinase 1 (*Ulk1*), transcription factor EB (*TFEB*), and microtubule-associated protein 1A/1B-light chain 3 (*LC-3*) (Seok et al., 2014; Lee et al., 2014).

FXR deficiency strongly inhibits liver growth in response to damage either through partial hepatectomy or chemical induction of apoptosis through CCl_4 treatment (Meng et al., 2010). Conversely, FXR overexpression promotes liver regeneration and stimulates liver repair after CCl_4 treatment. FXR's stimulation of liver regeneration is due to its ability to activate forkhead box M1 (*FOXM1b*) which is a key cell cycle regulator.

FXR activity has also been shown to help regulate inflammation. Activation of FXR with synthetic ligands has been shown to attenuate inflammation in mice fed methionine and choline-deficient diets. FXR has also been shown to interact with and regulate the expression of NFκB, a key regulator of inflammation (Gadaleta et al., 2011). Additionally, FXR regulates the expression of orosomucoid-1 (*AGP*) acute-phase protein which is secreted into the plasma in response to liver inflammation.

In addition to their metabolic phenotype, $FXR^{-/-}$ mice also spontaneously develop hepatocellular carcinoma (HCC) suggesting FXR acts as a tumor suppressor in normal tissue. Conversely, treatment of human HCC cells with FXR and RXR ligands inhibits growth and causes apoptosis. The exact mechanisms for FXR tumor suppressing effects are not well defined although possible contributors include SHP mediated increased of apoptosis, repression of bile acid synthesis, and signal transducer and activator of transcription 3 (*STAT3*) inactivation through increased expression of suppressor of cytokine signaling 3 (*SOCS3*). These observations describe the molecular mechanisms through which FXR modulates various physiological, homeostatic and metabolic pathways. However in addition to understanding the molecular mechanisms of FXR's physiological effects, it is important to understand how the activity of FXR is modulated.

1.5 Bile acid synthesis

Bile acids are amphipathic molecules which play important roles in digestion and nutrient signaling. Bile acids can be categorized into either primary or secondary bile acids based on where they are synthesized. Primary bile acids are synthesized from cholesterol in the hepatocytes of the liver. In humans, the primary bile acids are CA and CDCA while in mice an additional step converts CDCA into muricholic acid (MCA). The primary bile acids are converted into the secondary bile acids by bacterial cells in the intestine. CA and CDCA are converted to DCA and LCA, respectively (Ridlon and Hylemon 2006; Ridlon et al., 2013).

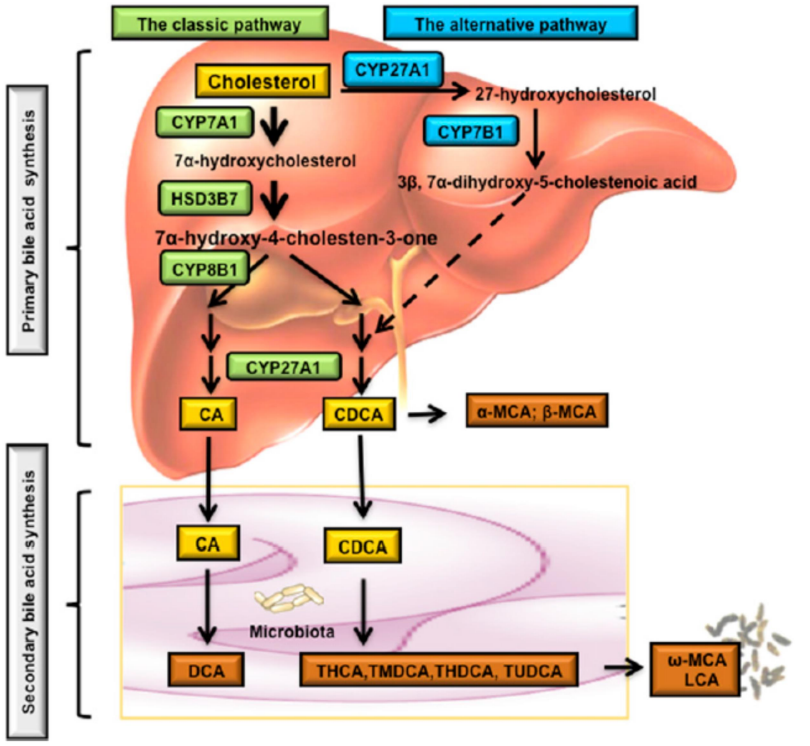


Figure 1.2: Bile acid synthesis pathways.

Adapted from Li T, Chiang JY. (2014) Bile acid signaling in metabolic disease and drug therapy Pharmacol Rev 66(4): 948-83.

There are two main pathways through which the bile acids are synthesized, which are summarized in figure 1.2. These pathways are the classical pathway, sometimes called the neutral pathway, and the alternative pathway, also known as the acidic pathway. In humans, the classical pathway accounts for more than 90% of total bile acid production and is considered the major bile acid biosynthetic pathway. The classical pathway begins with the conversion of cholesterol to 7α-

hydroxy-cholesterol by CYP7A1 (Myant and Mitropoulos, 1977). This is the rate limiting step in this biosynthetic pathway and CYP7A1 expression is heavily regulated through multiple mechanisms which will be discussed later. In humans, the classical pathway creates both CA and CDCA and the ratio of these is determined by the activity of another important biosynthetic enzyme, sterol 12α-hydroxylase (CYP8B1). Higher levels of CYP8B1 activity push the pathway towards creation of CA while lower levels of CYP8B1 lead to more CDCA production. A series of enzymatic reactions follow to produce the primary bile acids. In humans, the alternative pathway is thought to account for less than 10% of total bile acid production under normal physiologic conditions. The alternative pathway creates mainly CDCA and begins with the conversion of cholesterol to 27-hydroxy-cholesterol by sterol 27-hydroxylase (CYP27A1) in the inner membrane of the mitochondria. While the conversion of cholesterol to 27-hydroxy-cholesterol is the first step of this pathway, transport of cholesterol into the mitochondria is the rate limiting step for the alternative pathway (Pandak et al., 2002). A series of enzymatic reactions follow to produce CDCA in humans or MCA in mice. The alternate pathway is believed to be more active in individuals with cirrhotic liver disease (Gupta et al., 2001; Axelson and Sjovall 1990).

1.6 Bile acid physiology

After synthesis in the liver, bile acids are conjugated to glycine or taurine by bile acid coenzyme A:amino acid N-acyltransferase (BAAT) to decrease their hydrophobicity (Shonsey et al., 2005). Bile acids can also be conjugated to sulfate or glucuronidated by sulfotransferase 2A1 (SULT2A1) or the UDP glucuronosyltransferase family 2 proteins (UGT2B4 and UGT2B7), respectively, which decreases the toxicity and increases fecal excretion of bile acids. After conjugation, bile acids are secreted into the bile ducts through transporters such as BSEP and

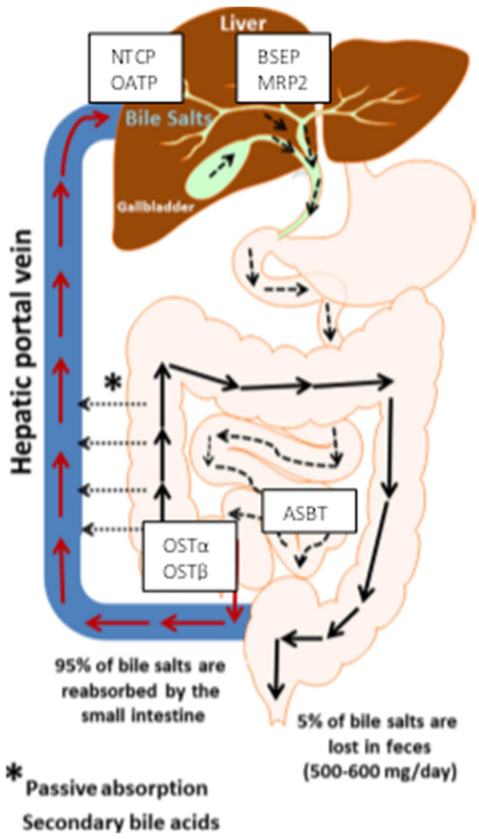


Figure 1.3: Bile acid circulation.

Adapted from Chiang. (2009) Bile acids: regulation of synthesis J Lipid Res 50(10): 1955-66

MRP2 and travel to the gallbladder where they are stored and concentrated (Boyer and Nathanson 1999). The hormone cholecystokinin, which is released when food enters the intestine, stimulates contraction of the gallbladder pushing the bile acids into the small intestine where they play a crucial role in solubilization and absorption of lipids and lipid soluble vitamins (Vlahcevic et al., 1996). Bile acids travel through the small intestine and are reabsorbed in the terminal ileum through the apical sodium dependent transporter (ASBT). The reabsorbed bile acids are then transported out of the intestinal cells and into the portal circulation via the heteromeric organic solute transporter α/β (OSTα/β) (Dawson et al., 2010). Bile acids that escape this reabsorption are deconjugated by bacterial bile salt hydrolases found in the gut microbiota. In the large intestine, bacterial 7α-hydroxylase converts CA and CDCA into the secondary bile acids DCA and LCA respectively. These free bile acids can travel through plasma membranes without the need for transporters and are then passively reabsorbed by colonocytes of the large intestine or excreted in the feces. Absorbed bile acids return to the liver through the portal circulation and are transported into the hepatocytes by the NTCP and organic anion transporting protein (OATP) transporters. Here the bile acids are again secreted into the bile ducts and return to the gallbladder to be used when needed. Bile acids will

repeat this process, summarized in figure 1.3, traveling through the enterohepatic circulation several times each day.

1.7 Bile acid signaling

In addition to their role in nutrient absorption, recent work has shown that bile acids also act as signaling molecules. Bile acids have been shown to activate several nuclear receptors including FXR, Pregnane X Receptor (PXR), and Vitamin D Receptor (VDR). As described above, FXR acts as the primary bile acid biosensor and major regulator of bile acid homeostasis. Activation of the other nuclear receptors, PXR and VDR, appears to function to protect cells from hydrophobic bile acids by inducing expression of genes such as cytochrome P450, family 3, subfamily A (*CYP3A*), which metabolize the bile acids into more hydrophilic metabolites (Xie et al., 2001; Makishima et al., 2002).

Bile acids also activate membrane bile acid receptors including several G protein coupled receptors (GPCRs) such as TGR5 and sphingosine 1-phosphate receptor 2 (S1PR2) (Staudinger et al., 2001; Makishima 2002; Watanabe et al., 2006; Thomas et al., 2009). TGR5 was the first GPCR to be identified as activated by bile acids. TGR5 is widely expressed in human tissues including the liver, gall bladder, kidneys, spleen, brown adipose tissue, and brain (Keitel and Haussinger 2012; Kawamata et al., 2003; Marutama et al., 2002; Maruyama et al., 2006; Vassileva et al., 2006). In the liver, TGR5 is not abundantly expressed in hepatocytes but is localized in Kupffer cells, cholangiocytes, sinusoidal endothelial cells (SEC), and gall bladder smooth muscle cells (Keitel et al., 2007; Keitael et al., 2008; Keitel et al., 2009; Keitel et al., 2010; Keitel and Haussinger 2011; Lavoie et al., 2010). Activation of TGR5 has been shown to have many diverse functions. TGR5 activation has been shown to inhibit the immune response

and cytokine release through inhibition of NFκB in macrophages and Kupffer cells (Keitel et al., 2008; Pols et al., 2011; Wang et al., 2011). Activation of TGR5 in SEC and biliary epithelial cells protects against bile acid induced apoptosis (Keitel and Haussinger 2011). TGR5 activation in the smooth muscle of the gall bladder leads to muscle relaxation and gallbladder filling through activation of protein kinase A (PKA) (Lavoie et al., 2010; Li et al., 2011). TGR5 also plays important roles in regulating energy metabolism through activating iodothyronine deiodinase in brown adipocytes leading to increases in thyroid hormone (Watanabe et al., 2006) and increasing secretion of glucagon-like peptide from enteroendocrine cells (Thomas et al., 2009). Activation of another GPCR, S1PR2, by conjugated bile acids activates the insulin signaling AKT and extracellular signal–regulated kinase (ERK) 1/2 pathways in hepatocytes (Studer et al., 2012). S1PR2 also plays an important role in hepatic lipid regulation. Additionally S1PR2 is upregulated in cholangiocarcinoma (CCA), a cancer of the biliary tract that is associated with chronic cholestasis and elevated levels of conjugated primary bile acids. Excess stimulation of S1PR2 promotes cell proliferation, migration, and invasion in CCA cell lines all of which are inhibited through treatment with S1PR2 antagonists (Liu et al., 2014).

Bile acids have also been shown to activate a number of other cellular signaling pathways including cyclic AMP synthesis, calcium mobilization, protein kinase C (PKC), *c-jun* N-terminal kinase (JNK), and epidermal growth factor (EGF) (Stravitz et al., 1996; Gupta et al., 2001; Rao et al 2002; Kawamata et al., 2003; Dent et al 2005; Nguyen and Bouscarel 2008). Through these pathways, bile acids have been linked to a wide variety of important biological functions ranging from lipid, lipoprotein, fatty acid, and bile acid synthesis to glucose metabolism and even regulation of intestinal bacterial growth.

1.8 FGF19 signaling

In addition to directly activating signaling pathways, bile acids have been shown to regulate the expression of the endocrine hormone fibroblast growth factor 19 (*FGF19*). *FGF19*, or its ortholog in mice fibroblast growth factor 15 (*FGF15*), is expressed in the small intestine, kidney, gallbladder, brain, skin, cartilage, retina, placenta and umbilical cord (Nishimura et al., 1999; Xie et al., 1999). When bile acid levels become elevated in the intestine, in response to a meal, they induce expression of *FGF19* in the intestine which is then released into the portal circulation and binds to its receptors on the hepatocytes of the liver. FGF19 binding requires a two component receptor consisting of fibroblast growth factor receptor 4 (FGFR4) and β-Klotho which are coexpressed in hepatocytes. Upon FGF19 binding, FGFR4/β-Klotho activate multiple kinase pathways including the ERK and JNK kinase pathways which ultimately lead to decreased expression of *CYP7A1* and inhibition of bile acid synthesis (Holt et al., 2003; Miao et al., 2009). FGF19$^{-/-}$ and FGFR4$^{-/-}$ transgenic mice show elevated bile pool sizes and increased expression of *CYP7A1* (Yu et al., 2000). In addition to its role in regulating bile acid synthesis, FGF19 signaling plays an important role in gallbladder filling.

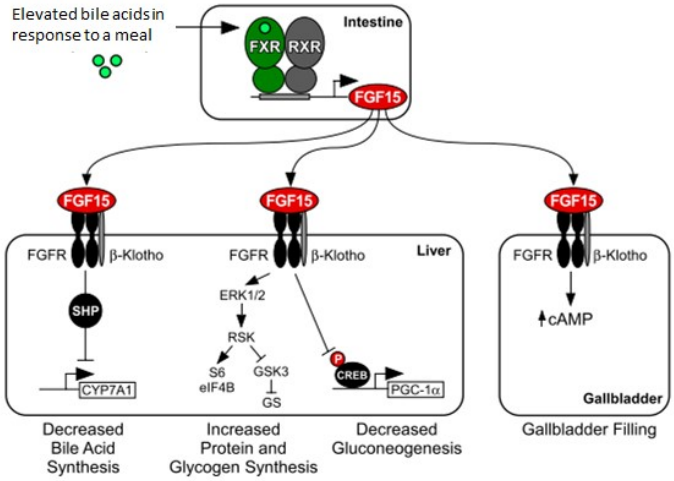


Figure 1-4. FGF19 signaling.

Adapted from Potthoff MJ, Kliewer SA, and Mangelsdorf DJ. (2012) Endocrine fibroblast growth factors 15/19 and 21: from feast to famine. Genes Dev. 15;26(4):312-24

FGF19 also plays a key role in metabolism. Mice overexpressing FGF19 show many beneficial metabolic effects including decreased body weight, decreased fat mass, lower serum glucose

and insulin levels, and improved glucose tolerance and insulin sensitivity (Tomlinson et al., 2002). These metabolic effects have been attributed to activation of the ERK pathway. Activation of ERK then increases protein synthesis through activation of the ribosomal protein s6 (S6) and eukaryotic translation initiation factor 4B (eIF4B). ERK stimulation also increases glycogen synthesis through inhibition of glycogen synthase kinase (GSK). FGF19 signaling also decreases gluconeogenesis through inhibition of CREB phosphorylation which inhibits the expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1α*). The roles of FGF19 signaling in metabolism are summarized in figure 1.4. Due to these important roles in regulating metabolic homeostasis many studies have suggested FGF19 may be an interesting drug target for the treatment of enterohepatic metabolic diseases (Nies et al, 2016; Owen et al., 2015; Reue et al 2014; Zhang et al., 2015).

1.9 Emerging role of gut microbiota in bile acid regulation

Increasing evidence indicates that in addition to altering the composition of the bile acid pool through the generation of secondary bile acids in the intestines, the gut microbiota can also regulate bile acid synthesis in the liver (Sayin et al., 2013). Utilizing mice bred in a germ-free environment, which lack normal gut microbiota, it was shown that microbiota depletion altered bile acid synthesis by decreasing intestinal expression of *FGF15* in a FXR dependent manner. Additionally, changes in the gut microbiota though the use of antibiotics or probiotics have similarly been shown to cause changes in bile acid synthesis (Sayin et al., 2013; Degirolamo et al., 2014; Li et al., 2013). Conversely, bile acid pool size and composition have been shown to play a role in regulating the make-up of the gut microbiome. Bile acids have both direct antimicrobial effects and indirect effects through FXR-induced expression of antimicrobial peptides (Inagaki et al., 2006; Vavassori et al., 2009). Consistent with these findings, rats fed a

diet rich in CA were shown to undergo significant alterations to the composition of the gut microbiome (Islam et al., 2011). Additionally, studies have shown that transplantation of gut microbiota from obese mice to germ-free mice can lead to increased fat accumulation, while exposure to gut microbiota from lean mice prevents this (Ridaura et al., 2013). Transplantation with gut bacteria from lean or obese mice also caused significant changes in bile pool composition as well as expression of FXR and its targets. Taken together these findings have led some to suggest utilizing bile acids as a way to target the gut microbiome to treat obesity (Le Chatelier et al., 2013).

1.10 Regulation of FXR through post-translational modifications

Post-translational modifications are modifications made to proteins, usually through the addition of a functional group to the side chain of an amino acid, which can have a profound effect on the fate and function of a protein. Recently a number of post-translational modifications of FXR have been shown to produce dramatic changes in FXR activity and several have been associated with significant phenotypic changes *in vivo* (mapped out in Figure 1.5). Several phosphorylation sites have been identified in FXR including serine-135, serine-154, and threonine-442 (Gineste et al., 2008; Frankenberg et al., 2008). Phosphorylation at serine-135 and serine-154 by PKC increases FXR interaction with the coactivator PGC-1α resulting in greater transactivation activity of FXR (Gineste et al., 2008). Phosphorylation of threonine-442 by PKCζ and subsequent interaction with familial intrahepatic cholestasis 1 (FIC1) increases both FXR transactivation activity and FXR localization to the nucleus, where it functions to modulate gene expression (Frankenberg et al., 2008). Phosphorylation of FXR at serine-250 by AMP-activated protein kinase (AMPK) decreases its ability to recruit transcriptional coactivators, such as steroid receptor coactivator-2 and nuclear receptor coactivator 6, decreasing expression of

FXR target genes (Lien et al., 2014). Metformin, a well-known drug used for the treatment diabetes, is an activator of AMPK and has been shown to exacerbate liver injury in mouse models of cholestasis and patients with advanced liver cirrhosis (Miralles-Linares et al., 2012; Lien et al., 2014). FXR has also been shown to be methylated at lysine-206 by SET domain containing lysine methyltransferase SETD7 (SET7/9) (Balasubramaniyan et al., 2012). Methylation of FXR at this site increases its transactivation of two well-known target genes, *SHP* and *BSEP*. Additionally two acetylation sites of FXR have been identified at lysine-157 and lysine-217. These sites are dynamically regulated by the acetylase P300 and the NAD-dependent deacetylase sirtuin-1 (SIRT1) (Kemper et al., 2009). Acetylation at these sites has several effects including increasing stability of FXR, inhibiting heterodimerization with RXR, decreasing DNA binding and causing an overall decrease in the transactivation activity of FXR. In obese mice FXR acetylation levels are highly elevated, likely due to low activity and levels of SIRT1, exacerbating dyslipidemia, hyperglycemia, and insulin insensitivity in these mice. (Kemper et al., 2009). FXR has also been shown to be sumoylated at lysine-277 by protein inhibitor of activated STAT protein gamma (PIASγ). Sumoylation of FXR leads to its interaction with NFκB and repression of pro-inflammatory genes (Kim et al.,2015). An interesting interplay between acetylation and sumoylation of FXR occurs. When FXR is hyperacetylated it is no longer able to interact with PIASγ leading to an inhibition of sumoylation. Hyperacetylation of FXR is observed in obese mouse models. This suggests that the inhibition of FXR sumoylation in obese conditions may play a role in the observed inflammatory signaling seen in obesity. Finally, FXR can be O-GlcNAcylated at serine-62 in response to high levels of glucose by O-GlcNAc transferase. O-GlcNAcylation leads to increased FXR protein stability, transcriptional

activity, and chromatin binding through SMRT inactivation. This leads to an increase in FXR target gene expression.

The misregulation of FXR post-translational modifications in disease states highlights the importance of understanding the molecular mechanisms through which FXR activity is regulated. This level of understanding will give us the tools to identify novel pharmacological targets and possibly develop treatments for metabolic diseases. Some post-translational modifications of FXR modulate its regulation of specific subsets of target genes. This poses a uniquely attractive possibility of being able to activate beneficial FXR target genes, responsible for increased glucose tolerance, insulin sensitivity, and decreased plasma triglycerides and LDL, while not inducing undesirable FXR metabolic effects such as lowering of HDL.

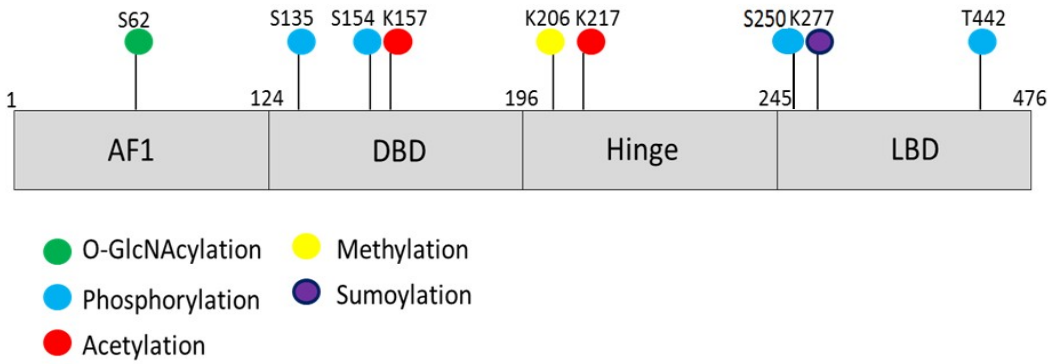


Figure 1-5. Map of post-translational modifications of FXR.
Adapted from Kemper JK. (2011) Regulation of FXR transcriptional activity in health and disease: Emerging roles of FXR cofactors and post-translational modifications. Biochim Biophys Acta 1812(8):842-50.

1.11 References

Ali AH, Carey EJ, Lindor KD. (2015) Recent advances in the development of farnesoid X receptor agonists. Ann Transl Med. 3(1): 5.

Ananthanarayanan M, Balasubramanian N, Makishima M, Mangelsdorf DJ, Suchy FJ. (2001) Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. J Biol Chem. 276(31):28857-65.

Axelson M, Sjovall J. (1990) Potential bile acid precursors in plasma–possible indicators of biosynthetic pathways to cholic and chenodeoxycholic acids in man. J Steroid Biochem. 36:631–40.

Balasubramaniyan N, Ananthanarayanan M, Suchy FJ. (2012) Direct methylation of FXR by Set7/9, a lysine methyltransferase, regulates the expression of FXR target genes. Am J Physiol Gastrointest Liver Physiol. 302(9):G937-47.

Barbier O, Torra IP, Sirvent A, Claudel T, Blanquart C, Duran-Sandoval D, Kuipers F, Kosykh V, Fruchart JC, Staels B. (2003) FXR induces the UGT2B4 enzyme in hepatocytes: a potential mechanism of negative feedback control of FXR activity. Gastroenterology. 124(7):1926-40.

Bateson MC, Maclean D, Evans JR, Bouchier IA. (1978) Chenodeoxycholic acid therapy for hypertriglyceridaemia in men. Br J Clin Pharmacol. 5(3):249-54.

Boyer JL, Nathanson MH. (1999) Bile formation. In Schiff's Diseases of the Liver. Schiff ER, Sorrell MF, and Maddrey WC, editors. Lippincott-Raven, New York, NY. 119–146.

Calkin AC, Tontonoz P. (2012) Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR. Nat Rev Mol Cell Biol. 13(4):213-24.

Cariou B, van Harmelen K, Duran-Sandoval D, van Dijk TH, Grefhorst A, Abdelkarim M, Caron S, Torpier G, Fruchart JC, Gonzalez FJ, Kuipers F, Staels B. (2006) The farnesoid X receptor modulates adiposity and peripheral insulin sensitivity in mice. J Biol Chem. 281(16):11039-49.

Cariou B, van Harmelen K, Duran-Sandoval D, van Dijk TH, Grefhorst A, Bouchaert E, Fruchart JC, Gonzalez FJ, Kuipers F, Staels B. (2005) Transient impairment of the adaptive response to fasting in FXR-deficient mice. FEBS Lett. 579(19):4076-80.

Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ. (2001) Nuclear receptors and lipid physiology: opening the X-files. Science. 294(5548):1866-70.

Chiang JY. (2009) Bile acids: regulation of synthesis. J Lipid Res. 50(10):1955-66.

Chiang JY. (2002) Bile acid regulation of gene expression: roles of nuclear hormone receptors. Endocr Rev. 23(4):443-63.

Claudel T, Inoue Y, Barbier O, Duran-Sandoval D, Kosykh V, Fruchart J, Fruchart JC, Gonzalez FJ, Staels B. (2003) Farnesoid X receptor agonists suppress hepatic apolipoprotein CIII expression. Gastroenterology. 125(2):544-55.

Claudel T, Sturm E, Duez H, Torra IP, Sirvent A, Kosykh V, Fruchart JC, Dallongeville J, Hum DW, Kuipers F, Staels B. (2002) Bile acid-activated nuclear receptor FXR suppresses apolipoprotein A-I transcription via a negative FXR response element. J Clin Invest. 109(7):961-71.

Dawson PA, Hubbert ML, Rao A. (2010) Getting the mOST from OST: role of organic solute transporter, OSTalpha–OSTbeta, in bile acid and steroid metabolism. Biochim Biophys Acta. 1801:994–1004.

De Fabiani E, Mitro N, Gilardi F, Caruso D, Galli G, Crestani M. (2003) Coordinated control of cholesterol catabolism to bile acids and of gluconeogenesis via a novel mechanism of transcription regulation linked to the fasted-to-fed cycle. J Biol Chem. 278(40):39124-32.

Degirolamo C, Rainaldi S, Bovenga F, Murzilli S, Moschetta A. (2014) Microbiota modification with probiotics induces hepatic bile acid synthesis via downregulation of the Fxr-Fgf15 axis in mice. Cell Rep. 7(1):12-8.

Dent P, Fang Y, Gupta S, Studer E, Mitchell C, Spiegel S, Hylemon PB. (2005) Conjugated bile acids promote ERK1/2 and AKT activation via a pertussis toxin-sensitive mechanism in murine and human hepatocytes. Hepatology. 42(6):1291-9.

Duran-Sandoval D, Cariou B, Fruchart JC, Staels B. (2005) Potential regulatory role of the farnesoid X receptor in the metabolic syndrome. Biochimie. 87(1):93-8.

Edwards PA, Kast HR, Anisfeld AM. (2002) BAREing it all: the adoption of LXR and FXR and their roles in lipid homeostasis. J. Lipid Res. 43(1):2-12.

Fang S, Suh JM, Reilly SM, Yu E, Osborn O, Lackey D, Yoshihara E, Perino A, Jacinto S, Lukasheya Y, Atkins AR, Khvat A, Schnabl B, Yu RT, Brenner DA, Coulter S, Liddle C, Schoonjans K, Olefsky JM, Saltiel AR, Downes M, Evans RM. (2015) Intestinal FXR agonism promotes adipose tissue browning and reduces obesity and insulin resistance. Nat Med. 21(2):159-65.

Fiorucci S, Rizzo G, Donini A, Distrutti E, Santucci L. (2007) Targeting farnesoid X receptor for liver and metabolic disorders. Trends Mol Med. 13(7):298-309.

Forman BM, Goode E, Chen J, Oro AE, Bradley DJ, Perlmann T, Noonan DJ, Burka LT, McMorris T, Lamph WW, Evans RM, Weinberger C. (1995) Identification of a nuclear receptor that is activated by farnesol metabolites. Cell. 81(5):687-93.

Gadaleta RM, Oldenburg B, Willemsen EC, Spit M, Murzilli S, Salvatore L, Klomp LW, Siersema PD, van Erpecum KJ, van Mil SW. (2011)
Activation of bile salt nuclear receptor FXR is repressed by pro-inflammatory cytokines activating NF-κB signaling in the intestine. Biochim Biophys Acta. 1812(8):851-8.

Gérard P. (2016) Gut microbiota and obesity. Cell Mol Life Sci. 73(1):147-62.

Gnerre C, Blättler S, Kaufmann MR, Looser R, Meyer UA. (2004) Regulation of CYP3A4 by the bile acid receptor FXR: evidence for functional binding sites in the CYP3A4 gene. Pharmacogenetics. 14(10):635-45.

Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi C, Wilson JG, Lewis MC, Roth ME, Maloney PR, Wilson TM, Kliewer SA. (2000) A regulatory cascade of the nuclear receptors FXR, SHP-1 and LRH-1 represses bile acid biosynthesis. Mol Cell. 6(3):517-26.

Gupta S, Stravitz RT, Dent P, Hylemon PB. (2001) Down-regulation of cholesterol 7alpha-hydroxylase (CYP7A1) gene expression by bile acids in primary rat hepatocytes is mediated by the c-Jun N-terminal kinase pathway. J Biol Chem. 276(19):15816-22.

He C, Shan Y, Song W. (2015) Targeting gut microbiota as a possible therapy for diabetes. Nutr Res. 35(5):361-7.

Holt JA, Luo G, Billin AN, Bisi J, McNeill YY, Kozarsky KF, Donahee M, Wang DY, Mansfield TA, Kliewer SA, Goodwin B, Jones SA. (2003) Definition of a novel growth factor-dependent signal cascade for the suppression of bile acid biosynthesis. Genes Dev. 17(13):1581-91.

Huang W, Ma K, Zhang J, Qatanani M, Cuvillier J, Liu J, Dong B, Huand X, Moore DD. (2006) Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration. Science. 312(5771):233-6.

Huber RM, Murphy K, Miao B, Link JR, Cunningham MR, Rupar MJ, Gunyuzlu PL, Haws TF, Kassam A, Powell F, Hollis GF, Young PR, Mukherjee Rm Burn TC. (2002) Generation of multiple farnesoid-X-receptor isoforms through the use of alternative promoters. Gene. 290(1-2):35-43.

Human Microbiome Project and Consortium (2012) Structure, function and diversity of the healthy human microbiome. Nature 486: 207–214

Inagaki T, Choi M, Moschetta A, Peng L, Cummins CL, McDonald JG, Luo G, Jones SA, Goodwin B, Richardson JA, Gerard RD, Repa JJ, Mangelsdorf DJ, Kliewer SA. (2005) Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. Cell Metab. 2(4):217-25.

Inagaki T, Moschetta A, Lee YK, Peng L, Zhao G, Downes M, Yu RT, Shelton JM, Richardson JA, Repa JJ, Mangelsdorf DJ, Kliewer SA. (2006) Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor. Proc Natl Acad Sci USA. 103(10):3920-5.

Islam KB, Fukiya S, Hagio M, Fujii N, Ishizuka S, Ooka T, Ogura Y, Hayashi T, Yokota A. (2011) Bile acid is a host factor that regulates the composition of the cecal microbiota in rats. Gastroenterology. 141(5):1773-81.

Kalaany NY, Mangelsdorf DJ. (2006) LXRS and FXR: the yin and yang of cholesterol and fat metabolism. Annu Rev Physiol. 68:159-91.

Kast HR, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, Tontonoz P, Kliewer S, Willson TM, Edwards PA. (2002) Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. J Biol Chem. 277(4):2908-15.

Kast HR, Nguyen CM, Sinal CJ, Jones SA, Laffitte BA, Reue K, Gonzalez FJ, Willson TM, Edwards PA. (2001) Farnesoid X-activated receptor induces apolipoprotein C-II transcription: a molecular mechanism linking plasma triglyceride levels to bile acids. Mol Endocrinol. 15(10):1720-8.

Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y, Hinuma S, Fujisawa Y, Fujino M. (2003) A G protein-coupled receptor responsive to bile acids. J Biol Chem. 278(11):9435-40.

Keitel V, Cupisti K, Ullmer C, Knoefel WT, Kubitz R, Häussinger D. (2009) The membrane-bound bile acid receptor TGR5 is localized in the epithelium of human gallbladders. Hepatology. 50:861-70.

Keitel V, Donner M, Winandy S, Kubitz R, Häussinger D. (2008) Expression and function of the bile acid receptor TGR5 in Kupffer cells. Biochem Biophys Res Commun. 372:78-84.

Keitel V, Häussinger D. (2012) Perspective: TGR5 (Gpbar-1) in liver physiology and disease. Clinics and Research in Hepatology and Gastroenterology. 36(5):412-9.

Keitel V, Häussinger D. (2011) TGR5 in the biliary tree. Dig Dis. 29:45-7.

Keitel V, Reinehr R, Gatsios P, Rupprecht C, Gorg B, Selbach O, Häussinger D, Kubitz R. (2007) The G-protein coupled bile salt receptor TGR5 is expressed in liver sinusoidal endothelial cells. Hepatology. 45:695-704.

Keitel V, Ullmer C, Häussinger D. (2010) The membrane-bound bile acid receptor TGR5 (Gpbar-1) is localized in the primary cilium of cholangiocytes. Biol Chem. 391:785-9.

Kemper JK. (2011) Regulation of FXR transcriptional activity in health and disease: Emerging roles of FXR cofactors and post-translational modifications. Biochim Biophys Acta 1812(8):842-50.

Kim DH, Xiao Z, Kwon S, Sun X, Ryerson D, Tkac D, Ma P, Wu SY, Chiang CM, Zhou E, Xu HE, Palvimo JJ, Chen LF, Kemper B, Kemper JK. (2015) A dysregulated acetyl/SUMO switch of FXR promotes hepatic inflammation in obesity. EMBO J. 34(2):184-99.

Kliewer SA. (2003) The nuclear pregnane X receptor regulates xenobiotic detoxification. J Nutr. 133(7 Suppl):2444S-2447S.

Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, Lehmann JM. (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc Natl Acad Sci USA. 94(9):4318-23.

Kok T, Hulzebos CV, Wolters H, Havinga R, Agellon LB, Stellaard F, Shan B, Schwarz M, Kuipers F. (2003) Enterohepatic circulation of bile salts in farnesoid x receptor deficient mice: efficient intestinal bile salt absorption in the absence of ileal bile acid-binding protein. J Biol Chem. 278(43):41930-7.

Lavoie B, Balemba OB, Godfrey C, Watson CA, Vassileva G, Corvera CU, Nelson MT, Mawe GM. (2010) Hydrophobic bile salts inhibit gallbladder smooth muscle function via stimulation of GPBAR1 receptors and activation of KATP channels. J Physiol. 588:3295-305.

Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, Almeida M, Arumugam M, Batto JM, Kennedy S, Leonard P, Li J, Burgdorf K, Grarup N, Jørgensen T, Brandslund I, Nielsen HB, Juncker AS, Bertalan M, Levenez F, Pons N, Rasmussen S, Sunagawa S, Tap J, Tims S, Zoetendal EG, Brunak S, Clément K, Doré J, Kleerebezem M, Kristiansen K, Renault P, Sicheritz-Ponten T, de Vos WM, Zucker JD, Raes J, Hansen T; MetaHIT consortium, Bork P, Wang J, Ehrlich SD, Pedersen O. (2013) Richness of human gut microbiome correlates with metabolic markers. Nature. 500(7464):541-6.

Lee JM, Wagner M, Xiao R, Kim KH, Feng D, Lazar MA, Moore DD. (2014) Nutrient-sensing nuclear receptors coordinate autophagy. Nature. 4;516(7529):112-5.

Lehmann JM, Kliewer SA, Moore LB, Smith-Oliver TA, Oliver BB, Su JL, Sundseth SS, Winegar DA, Blanchard DE, Spencer TA, Willson TM. (1997) Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. J Biol Chem. 272(6):3137-40.

Li F, Jiang C, Krausz KW, Li Y, Albert I, Hao H, Fabre KM, Mitchell JB, Patterson AD, Gonzalez FJ. (2013) Microbiome remodeling leads to inhibition of farnesoid X receptor signaling and decreased obesity. Nat Commun. 4:2384.

Li T, Chiang JY. (2014) Bile acid signaling in metabolic disease and drug therapy Pharmacol Rev 66(4): 948-83.

Li T, Holmstrom SR, Kir S, Umetani M, Schmidt DR, Kliewer SA, Mangelsdorf DJ. (2011) The G-protein coupled bile acid receptor, TGR5, stimulates gallbladder filling. Mol Endocrinol. 25:1066-71.

Lien F, Berthier A, Bouchaert E, Gheeraert C, Alexandre J, Porez G, Prawitt J, Dehondt H, Ploton M, Colin S, Lucas A, Patrice A, Pattou F, Diemer H, Van Dorsselaer A, Rachez C, Kamilic J, Groen AK, Staels B, Lefebvre P. (2014) Metformin interferes with bile acid homeostasis through AMPK-FXR crosstalk. J Clin Invest.124(3):1037-51.

Liu R, Zhao R, Zhou X, Ling X, Campbell DJ, Zhang X, Zhang L, Shi R, Wang G Pandak WM, Sirica AE, Hylemon PB, Zhou H. (2014) Conjugated bile acids promote cholangiocarcinoma cell invasive growth via activation of sphingosine 1-phosphate receptor 2. Hepatology. 60(3):908-18.

Lu TT, Repa JJ, Mangelsdorf DJ. (2001) Orphan nuclear receptors as eLiXiRs and FiXeRs of sterol metabolism. J. Biol. Chem. 276:37735–37738.

Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR, Mangelsdorf DJ. (2002) Vitamin D receptor as an intestinal bile acid sensor. Science. 296(5571):1313-6.

Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV Lustig KD, Mangelsdorf DJ, Shan B. (1999) Identification of a nuclear receptor for bile acids. Science. 284(5418):1362-5.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. (1995) The nuclear receptor superfamily: the second decade. Cell. 83: 835–839.

Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, Nakamura T, Itadani H, Tanaka K. (2002) Identification of membrane-type receptor for bile acids (M-BAR). Biochem Biophys Res Commun. 298:714-9.

Maruyama T, Tanaka K, Suzuki J, Miyoshi H, Harada N, Nakamura T, Miyamoto Y, Kanatani A, Tamai Y. (2006) Targeted disruption of G-protein coupled bile acid receptor 1 (Gpbar1/M-Bar) in mice. J Endocrinol. 191:197-205.

Meng Z, Wang Y, Wang L, Jin W, Liu N, Pan H, Liu L, Wagman L, Forman BM, Huang W. (2010) FXR regulates liver repair after CCl4-induced toxic injury. Mol Endocrinol. 5:886-97.

Miao J, Xiao Z, Kanamaluru D, Min G, Yau PM, Veenstra TD, Ellis E, Strom S, Suino-Powell K, Xu HE, Kemper JK. (2009) Bile acid signaling pathways increase stability of Small Heterodimer Partner (SHP) by inhibiting ubiquitin-proteasomal degradation. Genes Dev. 23(8):986-96.

Miralles-Linares F, Puerta-Fernandez S, Bernal-Lopez MR, Tinahones FJ, Andrade RJ, Gomez-Huelgas R. (2012) Metformin-induced hepatotoxicity. Diabetes Care. 35(3):e21.

Mulak A, Bonaz B. (2015) Brain-gut-microbiota axis in Parkinson's disease. World J Gastroenterol. 21(37):10609-20.

Myant NB and Mitropoulos KA (1977) Cholesterol 7 alpha-hydroxylase. J Lipid Res 18:135–153.

Nagy L, Schwabe JW. (2004) Mechanism of the nuclear receptor molecular switch. Trends Biochem. Sci. 29:317–324.

Nguyen A, Bouscarel B. (2008) Bile acids and signal transduction: role in glucose homeostasis. Cell. Signal. 20**:**2180-2197.

Nies VJ, Sancar G, Liu W, van Zutphen T, Struik D, Yu RT, Atkins AR, Evans RM, Jonker JW, Downes MR. (2016) Fibroblast Growth Factor Signaling in Metabolic Regulation. Front Endocrinol (Lausanne). 19;6:193.

Nishimura T, Utsunomiya Y, Hoshikawa M, Ohuchi H, Itoh N. (1999) Structure and expression of a novel human FGF, FGF-19, expressed in the fetal brain. Biochim Biophys Acta. 1444(1):148-51.

Owen BM, Mangelsdorf DJ, Kliewer SA. (2015) Tissue-specific actions of the metabolic hormones FGF15/19 and FGF21. Trends Endocrinol Metab 26(1):22-9.

Pandak WM, Ren S, Marques D, Hall E, Redford K, Mallonee D, Bohdan P, Heuman D, Gil G, Hylemon P. (2002) Transport of cholesterol into mitochondria is rate-limiting for bile acid synthesis via the alternative pathway in primary rat hepatocytes. J Biol Chem. 277:48158–64.

Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Wilson TM Zavacki AM, Moore DD, Lehmann JM. (1999) Bile acids: natural ligands for an orphan nuclear receptor. Science. 284(5418):1365-8.

Pineda Torra I, Claudel T, Duval C, Kosykh V, Fruchart JC, Staels B. (2003) Bile acids induce the expression of the human peroxisome proliferator-activated receptor alpha gene via activation of the farnesoid X receptor. Mol Endocrinol. 17(2):259-72.

Plass JR, Mol O, Heegsma J, Geuken M, Faber KN, Jansen PL, Müller M. (2002) Farnesoid X receptor and bile salts are involved in transcriptional regulation of the gene encoding the human bile salt export pump. Hepatology. 35(3):589-96.

Pols TW, Nomura M, Harach T, Lo SG, Oosterveer MH, Thomas C, Rizzo G, Gioiello A, Adorini L, Pellicciari R, Auwerx J, Shoonjans K. (2011) TGR5 activation inhibits atherosclerosis by reducing macrophage inflammation and lipid loading. Cell Metab. 14:747-57.

Prieur X, Coste H, Rodriguez JC. (2003) The human apolipoprotein AV gene is regulated by peroxisome proliferator-activated receptor-alpha and contains a novel farnesoid X-activated receptor response element. J Biol Chem. 278(28):25468-80.

Qiao L, Studer E, Leach K, McKinstry R, Gupta S, Decker R, Kukreja R, Valerie K, Nagarkatti P, El Deiry W, Molkentin J, Schmidt-Ullrich R, Fisher PB, Grant S, Hylemon PB, Dent P. (2001) Deoxycholic acid (DCA) causes ligand-independent activation of epidermal growth factor receptor (EGFR) and FAS receptor in primary hepatocytes: inhibition of EGFR/mitogen-activated protein kinase-signaling module enhances DCA-induced apoptosis. Mol Biol Cell. 12:2629–45.

Rao YP, Studer EJ, Stravitz RT, Gupta S, Qiao L, Dent P, Hylemon PB. (2002) Activation of the Raf-1/MEK/ERK cascade by bile acids occurs via the epidermal growth factor receptor in primary rat hepatocytes. Hepatology. 35(2):307-14.

Repa JJ, Mangelsdorf DJ. (1999) Nuclear receptor regulation of cholesterol and bile acid metabolism. Curr Opin Biotechnol. 10(6):557-63.

Reue K, Lee JM, Vergnes L. (2014) Regulation of bile acid homeostasis by the intestinal Diet1-FGF15/19 axis. Curr Opin Lipidol. 25(2):140-7.

Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, Griffin NW, Lombard V, Henrissat B, Bain JR, Muehlbauer MJ, Ilkayeva O, Semenkovich CF, Funai K, Hayashi DK, Lyle BJ, Martini MC, Ursell LK, Clemente JC, Van Treuren W, Walters WA, Knight R, Newgard CB, Heath AC, Gordon JI. (2013) Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science. 341(6150):1241214

Ridlon JM, Hylemon PB. (2006) A potential role for resistant starch fermentation in modulating colonic bacterial metabolism and colon cancer risk. Cancer Biol Ther. 5:273-4.

Ridlon JM, Ikegawa S, Alves JM, Zhou B, Kobayashi A, Iida T, Mitamurka K, Tanabe G, Serrano M, De Guzman A, Cooper P, Buck GA, Hylemon PB. (2013) Clostridium scindens: a human gut microbe with a high potential to convert glucocorticoids into androgens. J Lipid Res. 54:2437–49.

Roskoski R. (2004) Src protein-tyrosine kinase structure and regulation. Biochem Biophys Res Commun. 324(4): 1155-1164.

Roskoski R. (2015) Src protein-tyrosine kinase structure, mechanism, and small molecule inhibitors. Pharmacol Res. 94C:9-25

Russell DW. (2003) The enzymes, regulation, and genetics of bile acid synthesis. Annu Rev Biochem. 72:137-74.

Sayin SI, Wahlström A, Felin J, Jäntti S, Marschall HU, Bamberg K, Angelin B, Hyötyläinen T, Orešič M, Bäckhed F. (2013) Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. Cell Metab. 17(2):225-35.

Seok S, Fu T, Choi SE, Li Y, Zhu R, Kumar S, Sun X, Yoon G, Kang Y, Zhong W, Ma J, Kemper B, Kemper JK. (2014) Transcriptional regulation of autophagy by an FXR-CREB axis. Nature. 516(7529):108-11.

Seol W, Choi HS, Moore DD. (1995) Isolation of proteins that interact specifically with the retinoid X receptor: two novel orphan receptors. Mol. Endocrinol. 9(1):72–85.

Shonsey EM, Sfakianos M, Johnson M, He D, Falany CN, Falany J, Merkler DJ, Barnes S. (2005) Bile acid coenzyme A: amino acid N-acyltransferase in the amino acid conjugation of bile acids. Methods Enzymol. 400:374-94.

Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. (2000) Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. Cell. 102(6):731-44.

Sofi A, Silverman AL, Khuder S, Garborg K, Westerink JM, Nawras A. (2013) Relationship of symptom duration and fecal bacteriotherapy in Clostridium difficile infection-pooled data analysis and a systematic review. Scand J Gastroenterol. 48(3):266-73.

Song CS, Echchgadda I, Baek BS, Ahn SC, Oh T, Roy AK, Chatterjee B. (2001) Dehydroepiandrosterone sulfotransferase gene induction by bile acid activated farnesoid X receptor. J Biol Chem. 276(45):42549-56.

Sonoda J, Pei L, Evans RM. (2008) Nuclear receptors: decoding metabolic disease. FEBS Letters. 582(1):2-9.

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH, Kliewer SA. (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. Proc Natl Acad Sci USA. 98(6):3369-74.

Stravitz RT, Rao YP, Vlahcevic ZR, Gurley EC, Jarvis WD, Hylemon PB. (1996) Hepatocellular protein kinase C activation by bile acids: implications for regulation of cholesterol 7 alpha-hydroxylase. Am J Physiol. 271(2 Pt 1):G293-303.

Studer E, Zhou X, Zhao R, Wang Y, Takabe K, Nagahashi M, Pandak WM, Dent P, Spiegel S, Shi R, Xu W, Liu X, Bohdan P, Zhang L, Zhou H, Hylemon PB. (2012) Conjugated bile acids activate the sphingosine-1-phosphate receptor 2 in primary rodent hepatocytes. Hepatology. 55:267–76.

Thomas C, Gioiello A, Noriega L, Strehle A, Oury J, Rizzo G, Macchiarulo A, Yamamoto H, Mataki C, Pruzanski M, PellicciariR, Auwerx J, Schoonjans K. (2009) TGR5-mediated bile acid sensing controls glucose homeostasis. Cell Metabolism. 10:167–77.

Tomlinson E, Fu L, John L, Hultgren B, Huang X, Renz M, Stephan JP, Tsai SP, Powell-Braxton L, French D, Stewart TA. (2002) Transgenic mice expressing human fibroblast growth factor-19 display increased metabolic rate and decreased adiposity. Endocrinology. 143(5):1741-7.

Trauner M, Boyer JL. (2003) Bile salt transporters: molecular characterization, function, and regulation. Physiol Rev. 83(2):633-71.

Urizar NL, Dowhan DH, Moore DD. (2000) The farnesoid X-activated receptor mediates bile acid activation of phospholipid transfer protein gene expression. J Biol Chem. 275(50):39313-7.

Vassileva G, Golovko A, Markowitz L, Abbondanzo SJ, Zeng M, Yang S, Hoos L, Tetzloff G, Levitan D, Murgolo NJ, Keane K, Davis HR Jr, Hedrick J, Gustafson EL. (2006) Targeted deletion of Gpbar1 protects mice from cholesterol gallstone formation. Biochem J. 398:423-30.

Vavassori P, Mencarelli A, Renga B, Distrutti E, Fiorucci S. (2009) The bile acid receptor FXR is a modulator of intestinal innate immunity. J Immunol. 183(10):6251-61.

Vlahcevic ZR, Heuman DM, Hylemon PB. (1996) Physiology and pathophysiology of enterohepatic circulation of bile acids. In Hepatology: A textbook of Liver Disease. Zakim D and Boyer TD, editors. W. B. Sanders Co., Philadelphia, PA. 376-417.

Wang YD, Chen WD, Yu D, Forman BM, Huang W. (2011) The Gprotein coupled bile acid receptor, Gpbar1 (TGR5), negatively regulates hepatic inflammatory response through antagonizing nuclear factor kappa light-chain enhancer of activated B cells (NF-kappaB) in mice. Hepatology. 54:1421-32.

Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, Messaddeg N, Harney JW, Ezaki O, Kodama T, Schoonjans K, Bianco AC, Auwerx J. (2006) Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. Nature. 439:484–9.

Willy PJ, Umesono K, Ong ES, Evans RM, Heyman RA, Mangelsdorf DJ. (1995) LXR, a nuclear receptor that defines a distinct retinoid response pathway. Genes Dev. 9(9):1033-45.

Xie MH, Holcomb I, Deuel B, Dowd P, Huang A, Vagts A, Foster J, Liang J, Brush J, Gu Q, Hillan K, Goddard A, Gurney AL. (1999) FGF-19, a novel fibroblast growth factor with unique specificity for FGFR4. Cytokine. 11(10):729-35.

Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, Waxman DJ, Evans RM. (2001) An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. Proc Natl Acad Sci. 98(6):3375-80.

Yamagata K, Daitoku H, Shimamoto Y, Matsuzaki H, Hirota K, Ishida J, Fukamizu A. (2004) Bile acids regulate gluconeogenic gene expression via small heterodimer partner-mediated repression of hepatocyte nuclear factor 4 and Foxo1. J Biol Chem. 279(22):23158-65.

Yu C, Wang F, Kan M, Jin C, Jones RB, Weinstein M, Deng CX, McKeehan WL. (2000) Elevated cholesterol metabolism and bile acid synthesis in mice lacking membrane tyrosine kinase receptor FGFR4. J Biol Chem. 275(20):15482-9.

Zhang F, Yu L, Lin X, Cheng P, He L, Li X, Lu X, Tan Y, Yang H, Cai L, Zhang C. (2015) Minireview: Roles of Fibroblast Growth Factors 19 and 21 in Metabolic Regulation and Chronic Diseases. Mol Endocrinol. (10):1400-13.

Zhang Y, Edwards PA. (2008) FXR signaling in metabolic disease. FEBS Lett. 582(1):10-8.

Zhang Y, Kast-Woelbern HR, Edwards PA. (2002) Natural structural variants of the nuclear receptor farnesoid X receptor affect transcriptional activation. J. Biol. Chem. 278(1):104-10.

Zhang Y, Lee FY, Barrera G, Lee H, Vales C, Gonzalez FJ, Willson TM, Edwards PA. (2006) Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice. Proc Natl Acad Sci USA. 103(4):1006-11.

Zhou H, Hylemon PB. (2006) Bile acids are nutrient signaling hormones. Steroids. 86:62-8.

Chapter Two

Phosphorylation of FXR by Src and its role in bile acid regulation

2.1 Abstract

FXR is the primary bile acid nuclear receptor that acts as the master regulator of bile acid homeostasis. Despite extensive studies on the physiological pathways FXR regulates, how FXR activity is modulated through post-translational modification is not fully understood. Here we report a new post-translational modification of FXR. Unbiased mass spectrometry based proteomic analysis identified tyrosine-67 as a novel phosphorylation site in FXR which is phosphorylated in response to treatment with either the endogenous bile acid CDCA or the post-prandial signaling molecule FGF19. Bioinformatic in silico analysis showed that this site is highly conserved across vertebrates and contains a phosphorylation motif for Src, a tyrosine kinase that is known to be regulated by FXR. Biochemical analysis showed that treatment with CDCA or FGF19 led to Src activation and that *in vivo* feeding of a diet rich in the primary bile acid CA led to an increased interaction between FXR and Src, as well as phosphorylation of FXR. It was also observed that *in vitro* Src interacts with the DNA binding domain of FXR and is both sufficient and necessary for FXR phosphorylation. We showed that disruption of FXR phosphorylation impaired the ability of FXR to heterodimerize with RXR and to regulate the expression of its target genes *SHP* and *BSEP*.

In conclusion, this study identifies a new post-translational modification of FXR at tyrosine-67 which profoundly regulates its ability to heterodimerize with RXR and transcriptionally regulate its target genes. Further we show that Src is responsible for this

phosphorylation and describe the pathway through which endogenous bile acid signaling leads to dynamic phosphorylation of FXR. Understanding how this post-translational modification is regulated and its effects on FXR activity may provide useful tools and therapeutic targets for understanding and treating cholestatic diseases.

2.2 Introduction

FXR is a bile acid activated NR that is highly expressed in liver, intestine, kidney and adrenal glands and is a key regulator of many important physiological processes. The primary role of FXR is the maintenance of bile acid homeostasis (Makishima et al., 1999; Zhang and Edwards 2008; Goodwin et al., 2000). In response to elevated bile acid levels FXR downregulates genes important for bile acid synthesis while upregulating genes important for bile acid conjugation, detoxification, and transport out of the liver (Russell 2003, Ananthanarayanan et al, 2001; Trauner and Boyer 2003; Song et al., 2001; Barbier et al., 2003; Gnerre et al., 2004). FXR regulates these genes either through direct effects or through upregulation of SHP, an atypical nuclear receptor lacking a DBD. SHP functions by binding to other NRs disrupting their activity and thereby inhibiting expression of target genes. FXR's role in maintaining bile acid homeostasis is to protect the liver from the toxic effects of high concentrations of bile acids. The importance of FXR and SHP in maintaining bile acid homeostasis can be seen clearly in FXR/SHP double knockout mice which show dramatically elevated hepatic and serum bile acid levels as well as bile induced liver damage (Anakk et al., 2011).

In addition to responding to elevated bile acid levels, FXR activity has been shown to be regulated by several post-translational modifications. FXR can be acetylated by the histone acetyl transferase P300 at lysine-157 and lysine-217. Acetylation of FXR at these sites has been

shown to increase FXR's stability while decreasing its ability to dimerize with RXR and bind DNA causing an overall decrease in FXR activity. Under normal conditions the acetylation levels of FXR are tightly regulated by P300 and SIRT1. In some pathological settings, such as obesity, FXR can be hyperacetylated which results in inhibition of its activity and deleterious metabolic outcomes (Kemper et al., 2009). FXR can also be phosphorylated at serine-135, serine-154, and threonine-442. PKC has been shown to phosphorylate FXR at serine-135 and serine-154 which increases its interaction with its coactivator PGC-1α and increase its transactivation activity (Gineste et al., 2008). Phosphorylation of FXR at threonine-442 is mediated by PKCζ which leads to interaction with FIC1 and an increase in FXR's translocation to the nucleus and FXR transactivation activity (Frankenberg et al., 2008). Understanding how FXR's activity is regulated by post-translational modifications and the signaling pathways that lead to these modifications can provide insight into the development of and possible treatments for many bile acid and metabolic diseases.

Recent studies have linked FXR activity and the function of the tyrosine kinase Src. Src is a non-receptor tyrosine kinase which plays important roles in cell differentiation, proliferation, and survival (Thomas and Brugge 1997). Src is ubiquitously expressed in vertebrates with higher levels found in brain, osteoclasts, and platelets (Brown and Cooper 1996, Frame 2002, Levin et al., 2004). Src is generally bound to endosomes, perinuclear membranes, secretory vesicles and the cytoplasmic side of the plasma membrane where it interacts with a variety of growth factor and integrin receptors (Brown and Cooper 1996, Thomas and Brugge 1997); however Src has also been detected in the nucleus (Zhao et al., 1992). The Src protein from N to C terminus harbors: a N terminal myristoyl group, a unique domain, an SH3 domain, an SH2 domain, an SH2-kinase linker, a protein tyrosine kinase domain, and a C terminal regulatory

segment (Brown and Cooper 1996, Thomas and Brugge 1997). Myristoylation of Src facilitates its attachment to membranes and is required for its function (Brown and Cooper 1996). The SH3 domain is involved in protein-protein interactions and, along with the SH2 domain, plays an important role in the regulation of Src activity.

Regulation of Src activity is tightly regulated through intramolecular interactions between the SH2 and SH3 domains and two regulatory phosphorylation sites, tyrosine-416 in the activation loop which activates Src and tyrosine-527 which inhibits Src activity. Under resting conditions over 90% of Src is phosphorylated at tyrosine-527 and is therefore inactive (Zheng et al., 2000). The first step to activation is displacement of phosphotyrosine-527 from the SH2 domain, which can occur in response to interaction with other proteins or ligands, and is called unlatching. In the unlatched conformation of Src, the intramolecular forces holding Src in the repressed state are no longer stabilized (Harrison 2003). This open structure of Src allows for dephosphorylation of tyrosine-527 through interaction with phosphatases like protein tyrosine phosphatase α (PTP α) and autophosphorylation of tyrosine-416 by another Src molecule (Brown and Cooper 1996). Phosphorylation of tyrosine-416 stabilizes Src in its active state.

Recent work has shown that treatment of intestinal cancer cells with the synthetic FXR ligand GW4064 prevented phosphorylation of Src at tyrosine-416 and therefore prevented Src from phosphorylating its downstream targets. This FXR mediated inhibition of Src also lead to a decrease in cell proliferation and tumor growth. Conversely, treatment with the FXR antagonist guggulsterone led to an increase in Src activity through phosphorylation of tyrosine-416 resulting in an increase in cell proliferation (Peng et al., 2012). In this study we present evidence that FXR and Src interaction also occurs in the liver. However, while in the intestine FXR inhibits

Src activity, here we show that in the liver Src can phosphorylate FXR in response to bile acid signaling leading to FXR activation.

2.3 Materials and Methods

Reagents

Antibodies for FXR (sc-13063, sc-1204) were purchased from Santa Cruz Biotech, Pan phospho-tyrosine antibodies (8954S, 9411S) and Src antibodies (2108S) were purchased from Cell Signaling. M2 antibody and M2 conjugated to agarose beads (A2220) were obtained from Sigma.

Cell culture

PMH and monkey kidney cells (COS-1) were maintained in M199 and DMEM media, respectively. Media was supplemented with 100 U/ml penicillin G, streptomycin, and 10% fetal bovine serum.

Primary mouse hepatocyte isolation

C56BL/6J or B6.129X1(FVB)-Nr1h4^{tm1Gonz}/J (FXR$^{-/-}$) mice were placed in a sealed container with absorbent material containing isoflurane. When mice have stopped breathing, but the heart is still beating, an incision was made in the abdomen exposing the liver. The infusion needle was inserted into the hepatic portal vein and the inferior vena cava was cut. Twenty-five ml of 37^{o}C Perfusion buffer 1; 142 mM NaCl, 6.5 mM KCl, 10 mM Hepes, and 2.5 mM EGTA (pH 7.4); was perfused into the liver at a rate of 10 ml/min administered by a pump. This was followed by perfusion of 25 ml 37^{o}C Perfusion buffer 2; 66.7 mM NaCl, 6.7 mM KCl, 100mM Hepes, 4.8 mM CaCl2 (pH 7.6), 0.016 g/ml BSA and 0.8 mg/ml collagenase. Immediately

following the second perfusion, the liver was removed and placed in 10 ml Medium 199 (Sigma). The liver was then perforated using an 18 gauge needle and cells released were collected. The liver was washed in 20 ml additional Medium 199 and the collected cells were filtered through a cell strainer (Falcon). Cells were centrifuged for 5 min at 500 rpm and the supernatant was removed. Cells were then resuspended in Medium 199 and layered on top of a mixture of 7 ml percoll (Sigma) and 8ml Medium 199. Cells were then centrifuged for 5 min at 1250 rpm and the supernatant was removed. Cells were resuspended in Medium 199 supplemented with 100 U/ml penicillin G, streptomycin and 10% fetal bovine serum and plated for experimentation.

***In vivo* experiments**

C57BL/6J mice were purchased from Jackson laboratory. All *in vivo* experiments were done using mice from 12-16 weeks of age. Food was removed from the cages for 10 hours (7:00AM-5:00PM) after which mice were refed with either normal chow or chow supplemented with 0.5% cholic acid (Harlan Teklad) for 3 hours. After refeeding mice were sacrificed and FXR immunoprecipitated from liver nuclear extracts was measured by western blot. All animal use was approved by the Institutional Animal Care and Use and Biosafety Committees at the University of Illinois at Urbana-Champaign and was in accordance with National Institutes of Health guidelines.

In cell phosphorylation assays

PMH were grown in 6 well plates for phosphorylation assays. Cells were infected with an adenoviral vector expressing flag tagged FXR. Following infection cells were incubated overnight in serum free media containing 100 U/ml penicillin G-streptomycin then treated with

fed state signaling molecules at physiologically relevant doses; CDCA (50μM), GW4064 (200nM), FGF19 (50ng/μl), or insulin (100nM) for 15 minutes. Three minutes prior to the end of treatment cells were treated with 100 μM of the phosphatase inhibitor pervanadate. Cells were then harvested and lysed in 500 μl RIPA buffer containing protease and phosphatase inhibitors. After sonication and incubation on ice for 10 minutes cells were centrifuged and lysates were collected. One μg of either control IgG or M2 antibody was added and cells were incubated for 30 minutes at 4^{o}C. Twenty-five μl of 25% protein G-sepharose slurry was added and incubated at 4^{o}C for 3.5 hours. Immunoprecipitates were washed three times with lysis buffer. The levels of FXR tyrosine phosphorylation were measured by western blotting using anti-phosphotyrosine antibodies.

GST pulldown assays

Domain fragments of FXR (AF1, DBD, Hinge, and LBD) were cloned into the PGEX-4T-1 vector and transfected into *E. coli* BL21 cells to express fusion Glutathione S-transferase (GST) FXR domain fragments. GST-tagged domains were purified with glutathione sepharose beads. One hundred ng of purified Src (Millipore, Inc) was allowed to interact with 1μg GST fusion domains for 3 hours at 4^{o}C. Complexes were then washed three times with Co-IP buffer; Hepes 20 mM, KCl 100 mM, glycerol 10%, EDTA 0.1 mM, NP40 0.05%. Levels of Src in complex with FXR domain fragments were then measured via western blot.

***In vitro* phosphorylation assays**

COS-1 cells were infected with adenoviral vectors overexpressing flag-tagged FXR. FXR protein was purified through immunoprecipitation as described above. Purified FXR was

incubated with Src protein (Millipore, Inc) at 30^{o}C for 30 minutes. Afterwards FXR phosphorylation levels were measured via western blot.

RNA isolation

PMH from C57BL/6J mice were plated in 6 well plates. Cells were treated with vehicle, CDCA (50μM), or GW4064 (200nM) for 6 hours. RNA was isolated in Trizol reagent (Qiagen) according to the manufactures instructions. Relative gene expression levels were measured by qRTPCR analysis with SYBR Green (Roche).

2.4 Results

FXR is tyrosine phosphorylated in response to bile acid feeding.

FXR is a key regulator of many metabolic processes throughout the body (Russell 2003; Fang et al., 2015; Kemper 2011) and its activity has been shown to be dramatically modulated by post-translational modifications (Gineste et al., 2008; Frankenberg et al., 2008 Kemper et al., 2009; Kemper 2011). To better understand how FXR is post-translationally modified in response to bile acid signaling under physiological conditions, C57BL/6J mice were fed either normal chow or chow supplemented with 0.5% cholic acid for 3 hours. FXR protein from the livers of these mice was then immunoprecipitated and phosphorylation levels were measured via western blotting. Interestingly, it was found that FXR protein from mice fed a cholic acid rich diet showed high levels of tyrosine phosphorylation whereas mice fed normal chow showed minimal if any tyrosine phosphorylation (Figure 2.1). No significant changes were seen in serine or threonine phosphorylation levels in response to cholic acid feeding. These results suggest that not only is there a previously unidentified tyrosine phosphorylation site on FXR but that it is also regulated in response to bile acid signaling.

Treatment with bile acid or FGF19 increases FXR tyrosine phosphorylation.

To further investigate what signaling leads to FXR tyrosine phosphorylation, PMHs isolated from $FXR^{-/-}$ mice were infected with adenoviral vectors expressing flag tagged FXR. These cells were then treated with bile acid or fed state signaling molecules. FXR protein was immunoprecipitated and tyrosine phosphorylation levels were determined via western blot. FXR from cells treated with the endogenous bile acid CDCA or the post-prandial hormone FGF19 both showed dramatically increased tyrosine phosphorylation levels whereas cells treated with vehicle, the synthetic FXR ligand GW4064, or insulin did not exhibit FXR phosphorylation (Figure 2.2). These data suggest that FXR is being phosphorylated specifically in response to bile acids and fed state bile acid signaling molecules.

Tyrosine phosphorylation of FXR in response to bile acid treatment is transiently increased.

Post-translational modifications are generally dynamically regulated allowing the target protein to modulate its activity in response to a stimulus and return to its basal state afterwards. Imbalance of this regulation can lead to pathological situations (Kemper et al., 2009). To understand the regulation of FXR tyrosine phosphorylation, PMHs expressing flag tagged FXR were treated with CDCA or FGF19 at various time points. FXR was then immunoprecipitated and phosphorylation levels were measured via western blot. FXR tyrosine phosphorylation levels were dramatically increased as early as 5 minutes after treatment and peaked at 10 minutes. The phosphorylation levels dropped significantly after the 15 minute time point and were near basal levels by 30 minutes post treatment (Figure 2.3). These data show that FXR tyrosine phosphorylation is occurring very rapidly and is being tightly regulated.

Src interacts with the DNA binding domain of FXR.

To identify possible kinases that may phosphorylate FXR, the bioinformatic tools at Human Protein Reference Database (HPRD) were used. HPRD recognized tyrosine phosphorylation site motifs of five kinases and one phosphatase (Table 2.1). Of particular note, there were several Src kinase and Src family kinase motifs found on FXR. Recent studies have indicated that FXR can modulate the activity of Src in intestinal cells (Peng et al., 2012). To explore if FXR and Src could interact, separate domains of FXR tagged to GST were expressed *in vitro* (Figure 2.4A). A GST pulldown assay was performed in which each domain of FXR was incubated with purified Src. We found the Src interacted specifically with the DNA binding domain (DBD) of FXR and no other domains (Figure 2.4B). These data show that there is a strong interaction that takes place between FXR and Src in one specific domain of FXR.

FXR interacts with the tyrosine kinase Src *in vivo* in response to cholic acid feeding.

To examine if Src could be playing a role in FXR tyrosine phosphorylation in the liver, co-immunoprecipitation assays were carried out. FXR from nuclear extracts of mice fed either normal chow or cholic acid supplemented chow was immunoprecipitated under non-denaturing conditions. The levels of Src were then measured via western blot. Src was found to interact with FXR in samples from mice fed a cholic acid supplemented diet but not in mice fed normal chow (Figure 2.5). These data show that Src interacts with FXR *in vivo* in response to cholic acid feeding, the same conditions under which FXR is phosphorylated, suggesting that Src may play a role in the phosphorylation of FXR.

Src is activated by bile acid and FGF19 treatment.

Several recent studies have examined the effect of bile acid signaling on Src activation in the intestine, however, the role of hepatic Src in bile acid signaling is poorly understood. To explore the how CDCA and FGF19 treatment might affect Src activation in the liver, PMH from C57BL/6J mice were isolated and treated with CDCA or FGF19. At various time points, Src activation was measured via western blot. Both CDCA and FGF19 treatment lead to increases in Src tyrosine-416 phosphorylation at 5 minutes post treatment, with phosphorylation returning to basal levels at an hour post treatment (Figure 2.6). These data are consistent with the timing of FXR phosphorylation showing that Src is active when FXR is being phosphorylated.

Src is sufficient to phosphorylate FXR *in vitro*.

To determine whether activated Src could phosphorylate FXR, COS-1 cells were infected with adenoviral vectors overexpressing flag-tagged FXR. FXR protein was purified through immunoprecipitation and incubated with a constitutively active form of Src protein (Millipore, Inc). Western blot analysis showed that FXR protein incubated with Src protein and ATP was tyrosine phosphorylated while controls without ATP or Src were not (Figure 2.7). These data indicate that activated Src has the ability to phosphorylate FXR *in vitro*.

Loss of Src impairs FXR tyrosine phosphorylation.

To determine whether Src was required for FXR tyrosine phosphorylation in cells, PMH were isolated and transfected with either control siRNA or siRNA targeting Src. These cells were then treated with CDCA (Figure 2.8A) or FGF19 (Figure 2.8B). FXR protein and phosphorylation levels were determined via western blot. The siSrc RNA was able to achieve a significant decrease in Src expression with between a 70% and 90% reduction in protein levels

observed. This decreased expression was accompanied by a dramatic loss of FXR phosphorylation. These data suggest that Src is required for FXR tyrosine phosphorylation in response to CDCA and FGF19 treatments.

Identification of FXR tyrosine phosphorylation site.

Identification of the site of FXR tyrosine phosphorylation site is key to understanding how this phosphorylation is regulated and the effects that phosphorylation has on FXR. In order to identify the phosphorylation site, PMH from $FXR^{-/-}$ mice were isolated and infected with vectors expressing flag tagged FXR. The cells were then treated with CDCA or FGF19 and the FXR protein was immunoprecipitated. The purified FXR protein was then analyzed by tandem mass spectrometry to identify potential phosphorylation sites. Through this analysis three potential tyrosine phosphorylation sites were identified; tyrosine-46, tyrosine-49, and tyrosine-67 (Figure 2.9A). The bioinformatics program NetPhos 3.1 was also used to screen for potential phosphorylation sites. NetPhos 3.1 analyzed every tyrosine residue of FXR and gave each a prediction score based on the likelihood of the site being phosphorylated. While tyrosine-46 and 49 both received scores indicating possible phosphorylation, tyrosine-67 was by far the most highly predicted site (Table 2.2). According to HPRD software these 3 tyrosine sites also contain Src and Src family kinase motifs (Table 2.1) further supporting the theory that they may be potential phosphorylation sites of FXR. To examine which of these sites plays a role in FXR signal dependent phosphorylation, plasmid constructs were created mutating each of these sites. This constructs were then transfected into PMH which were treated with FGF19. Western blot analysis showed that mutation of the tyrosine-67 phosphorylation site dramatically reduced FXR tyrosine phosphorylation while mutation of either tyrosine-46 or 49 lead to no observable change in phosphorylation (Figure 2.9B). Additionally, sequence alignment shows that FXR tyrosine-67

is highly conserved between species (Table 2.3) suggesting that this site may be important for FXR function. Taken together these data indicate that tyrosine-67 is the major phosphorylation site of FXR.

Mutation of FXR tyrosine-67 leads to loss of transactivation activity.

Previous work has shown that phosphorylation of FXR can play a key role in its ability to regulate its target gene expression (Gineste et al., 2008). To examine if tyrosine-67 is important for FXR transactivation activity, a luciferase reporter assay was done. COS-1 cells were transfected with a reporter plasmid containing the luciferase gene driven by either a promoter containing the FXRE (Figure 2.10A) or by the endogenous SHP promoter (Figure 2.10B) along with an expression plasmid containing the FXR heterodimer partner RXR and varying concentrations of expression plasmids containing either wild type FXR or the phosphorylation deficient mutant Y67F-FXR. The cells were then treated with CDCA or FGF19, the signals shown to activate FXR tyrosine-67 phosphorylation. Transfection of the wild type FXR plasmid was shown to increase the expression of luciferase in a dose dependent manner whereas cells transfected with the Y67F-FXR plasmid showed significantly lower levels of activation. These data suggest that tyrosine-67 phosphorylation plays an important role in the ability of FXR to transactivate both its FXRE and the endogenous promoter of its well-known target SHP.

Loss of tyrosine-67 phosphorylation impairs the interaction of FXR with its heterodimer partner RXR.

Heterodimerization with RXR is a key step in FXR activation (Claudel et al., 2002) and has been shown to be regulated by the post-translational state of FXR (Kemper et al., 2009). To determine if tyrosine-67 phosphorylation plays a role in FXR/RXR interaction, co-

immunoprecipitation experiments were done. PMH cells from $FXR^{-/-}$ mice were infected with adenoviral vector expressing flag tagged wild type or Y67F-FXR and treated with CDCA. RXR protein was immunoprecipitated and samples were immunoblotted to measure interaction with the flag tagged FXR. Significantly less interaction was observed between RXR and Y67F-FXR than with wild type FXR (Figure 2.11A). To ensure that this decreased interaction is caused by loss of phosphorylation and not a conformational change caused by the tyrosine to phenylalanine mutation, siRNA knockdown experiments were used to determine if decreased Src levels altered FXR/RXR interactions. PMH cells from $FXR^{-/-}$ mice were infected with adenoviral vector expressing flag tagged wild type. These cells were then transfected with either control siRNAs or siSrc. These cells were then treated as above. PMHs transfected with siSrc showed significantly less interaction between FXR and RXR than those transfected with control siRNAs (Figure 2.11B). These data show that loss of FXR tyrosine-67 phosphorylation causes a dramatic decrease in FXR/RXR interaction, likely contributing to the loss of transactivation activity seen in previous experiments.

Mutation of tyrosine-67 impairs the ability of FXR to regulate genes involved in bile acid homeostasis.

To determine if the previously seen decreases in heterodimerization and transactivation activity lead to changes in FXR target gene expression, PMH from $FXR^{-/-}$ mice were isolated and subsequently infected with adenoviral vectors expressing either wild type FXR or the phosphorylation deficient Y67F-FXR. These cells were treated with CDCA or FGF19 for 6 hours at which point RNA was isolated from the cells. Expression of various FXR target genes was measured by qPCR. Cells expressing Y67F-FXR showed decreases in expression of FXR target genes *SHP* and *BSEP* but not *MRP2* or *MDR1* (Figure 2.12). These data suggest that loss

of FXR tyrosine-67 phosphorylation affects FXR target gene expression in a gene selective manner.

2.5 Discussion

In this work we identify a new phosphorylation site of FXR at tyrosine-67, demonstrate that bile acid and FGF19 signaling activate the kinase Src to produce this modification, and explore the mechanism through which phosphorylation of FXR regulates its activity by altering its ability to heterodimerize with RXR and transcriptionally regulate target genes.

It is well known that bile acids can activate numerous signaling pathways including nuclear receptor and kinase signaling both through direct activation of target receptors and though upregulation of the peptide hormone FGF19 by FXR. (Staudinger et al 2001, Makishima 2002; Stravitz et al., 1996; Gupta et al., 2001; Rao et al 2002; Kawamata et al., 2003; Dent et al 2005). However, what is not known is whether these signaling pathways in turn feedback to modulate FXR in the liver through post-translational modification. Here we show that feeding mice a diet rich in CA leads to a significant increase in tyrosine phosphorylation of FXR in the liver. To differentiate between the numerous pathways activated by CA feeding, PMH were isolated from C57BL/6J mice and treated directly with fed state signaling molecules. We observed that treatment with either the natural FXR ligand CDCA or FGF19 both lead to dramatic increases in FXR tyrosine phosphorylation. CDCA and FGF19 are known to activate distinct membrane receptors important for bile acid signaling, suggesting crosstalk between these receptors or convergence downstream of these membrane receptors may be important for FXR tyrosine phosphorylation. Of note, a recent study (Li et al., 2014) identified protein-tyrosine phosphatase 2C (SHP-2) as a coordinator of bile acid and FGF19 signaling. SHP-2 is a phosphatase that acts as a key downstream target of FGF19 signaling. Loss of SHP-2 impaired

both FGF19 downstream signaling as well as the ability of FXR to activate SHP expression in response to ligand binding. Li et al., suggests a currently unidentified factor might mediate this interplay between SHP-2 and FXR. As dephosphorization of Src at tyrosine-527 is a key step in its activation, it is possible that Src is this unidentified factor. These factors make SHP-2 an interesting target for future studies looking at FXR tyrosine phosphorylation.

A consequence of FXR phosphorylation identified in this study is an increase in FXR/RXR interaction. This is consistent with other studies looking at post-translational modifications of FXR and other NRs which have similarly shown that these modifications can often lead to changes in the recruitment of binding partners and transcriptional cofactor complexes (DH Kim et al., 2015; Seok et al., 2013; Miao et al., 2009). It is well known that FXR transactivation of many of its target genes requires dimerization with RXR. This provides a mechanism through which phosphorylation of FXR at tyrosine-67 could modulate the expression of its target genes. Interestingly, recent studies have shown that FXR can also regulate target genes through transrepression, often acting without RXR binding. FXR transrepression activity, while originally thought to be very rare, has been shown to be important for a large fraction of FXR target genes, notably FXR repression of autophagy and inflammatory genes (Lee et al., 2012; Seok et al., 2014; Kim et al., 2015). Future studies examining whether FXR tyrosine-67 phosphorylation has an effect on the expression of these RXR independent target genes will be interesting.

Previous work has suggested that crosstalk between FXR-mediated bile acid signaling and Src kinase signaling occurs in intestinal cells (Peng et a., 2012) although the exact mechanism for this crosstalk remains unclear. Our data indicate that, in the liver, FXR can interact with Src directly through its DBD and that this interaction is increased in the presence of

bile acids. These findings on the interactions between hepatic FXR and Src may provide insights for future studies into the possible mechanisms through which intestinal FXR regulates Src activity. In addition to interacting with Src, our tandem mass spectrometry analysis identified multiple potential FXR phosphorylation sites, several containing Src phosphorylation motifs. While in the liver our results show that tyrosine-67 was the major site of phosphorylation, in different cellular contexts other sites may play a larger role.

Recently there have been many studies exploring FGF19 as a potential target for treating metabolic diseases, including, hepatobiliary diseases and diabetes (Owen et al., 2015; Degirolamo et al., 2016; Kliewer and Mangelsdorf 2015). While much is known about the beneficial effects and pathways activated by FGF19 signaling, nothing is known about how FGF19 signaling is terminated. Understanding this mechanism will be important if FGF19 is to be used as a therapeutic drug, as chronic activation of FGF19 is associated with liver and intestinal tumorigenesis (Nicholes et al., 2002; Desnoyers et al., 2008). Previous studies have shown that FXR helps to prime the liver for FGF19 signaling by increasing the expression of β-Klotho, a key coreceptor necessary for FGF19 binding (Fu et al., 2016). Future studies with this project will explore the possibility that after FGF19 signaling has been received by the liver, FXR is phosphorylated which then acts in a gene selective manner to decrease expression of FGF19 signaling genes, forming a negative feedback loop to maintain homeostasis. Understanding exactly how tyrosine-67 phosphorylation gene selectively regulates FXR target genes may provide potential therapeutic targets for this pathway and as well as other bile acid related diseases.

2.6 Tables and Figures

Figure 2.1

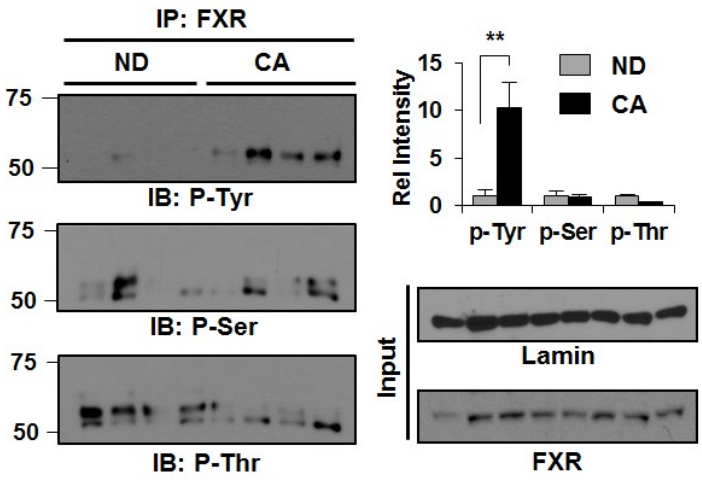


Figure 2.1: FXR is tyrosine phosphorylated in response to bile acid feeding. C57BL/6J mice were fasted for 10 hours then refed with normal chow diet (ND) or a diet containing 0.5% cholic acid (CA) for 3 hours. Nuclear extracts from the livers of these mice were prepared. FXR protein was immunoprecipitated and then immunoblotted using antibodies specific for proteins phosphorylated at serine, threonine, or tyrosine residues. This work was completed by Dong Hyun Kim with help from Daniel Ryerson.

Figure 2.2

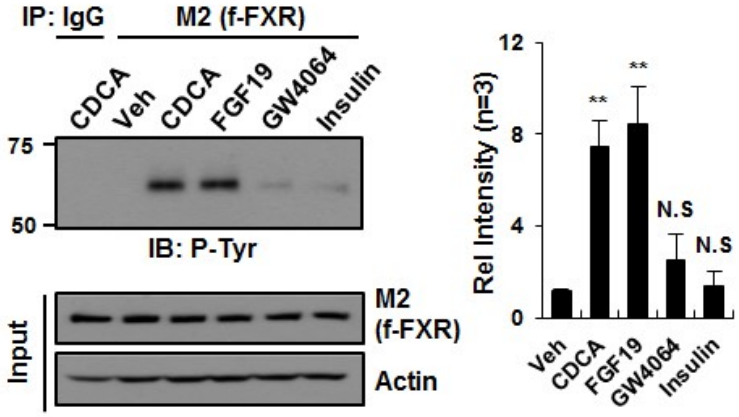
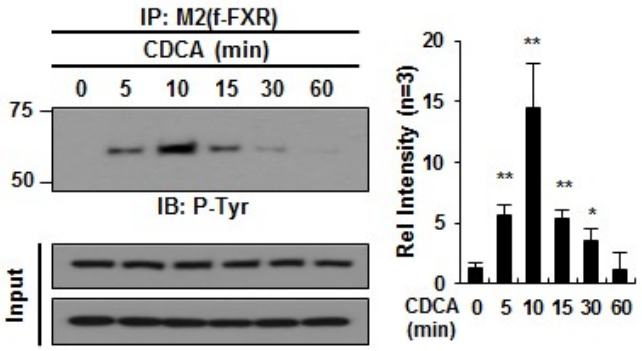


Figure 2.2: Treatment with bile acids or FGF19 increases FXR tyrosine phosphorylation. PMH cells isolated from $FXR^{-/-}$ mice were infected with an adenoviral vector expressing flag tagged FXR protein (f-FXR). Cells were then treated with several signaling molecules important for bile acid and fed state signaling: CDCA (50 μM), FGF19 (50 ng/μl), GW4064 (200 nM), and Insulin (100 nM). Cells were treated for 12 minutes followed by 3 minutes of treatment with the phosphatase inhibitor pervanadate. Cells were lysed and FXR protein was immunoprecipitated using M2 antibody conjugated to agarose beads. Tyrosine phosphorylation levels of FXR were determined through western blot analysis. This work was completed by Daniel Ryerson with help from Dong Hyun Kim.

Figure 2.3

A)



B)

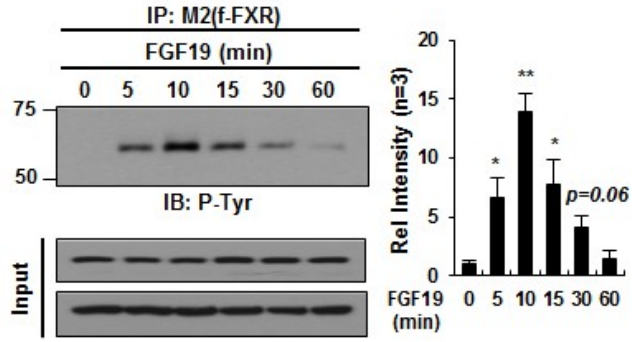
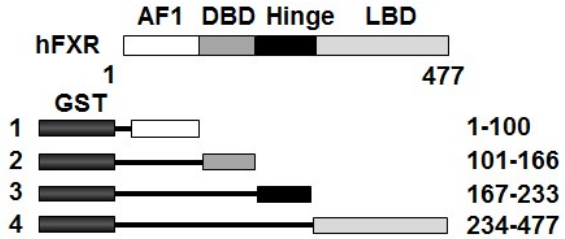


Figure 2.3: Tyrosine phosphorylation of FXR in response to treatment is transiently increased. PMH cells isolated from $FXR^{-/-}$ mice were infected with adenoviral vector expressing flag tagged FXR protein (f-FXR). Infected cells were then treated with A) CDCA (50 μM) or B) FGF19 (50 ng/μl) for the indicated times. Prior to lysing, cells were treated with the phosphatase inhibitor pervanadate for 3 minutes. Cells were lysed and FXR protein was immunoprecipitated using M2 antibody conjugated to agarose beads. Tyrosine phosphorylation levels of FXR were determined through western blot analysis. This work was completed by Daniel Ryerson with help from Dong Hyun Kim.

Figure 2.4

A)



B)

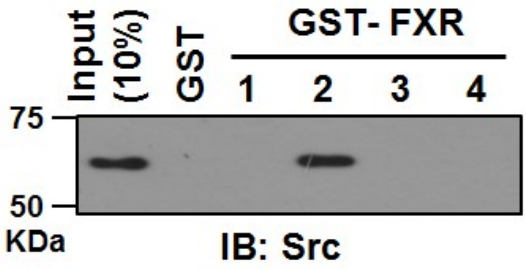


Figure 2.4: Src interacts with the DNA binding domain of FXR. A) GST tagged domains of human FXR were expressed in BL21 cells. AF1, DBD, Hinge, and LBD domains were purified with glutathione beads. B) Equal amounts of each purified FXR domain were incubated with purified Src. Protein complexes were then precipitated in a GST pulldown assay. Interaction of FXR and Src was measured through western blot using a Src antibody. This work was completed by Daniel Ryerson.

Figure 2.5

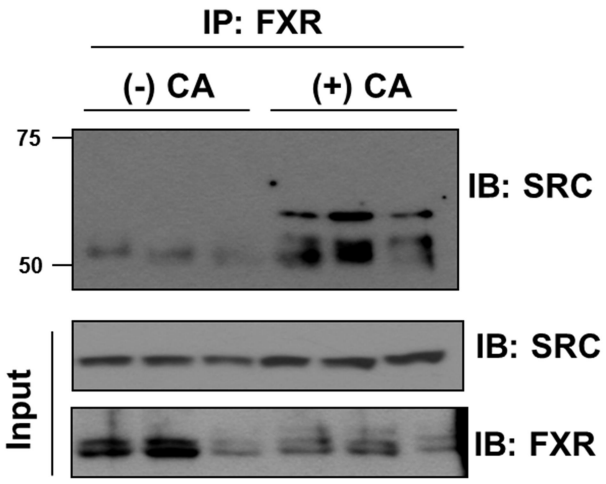
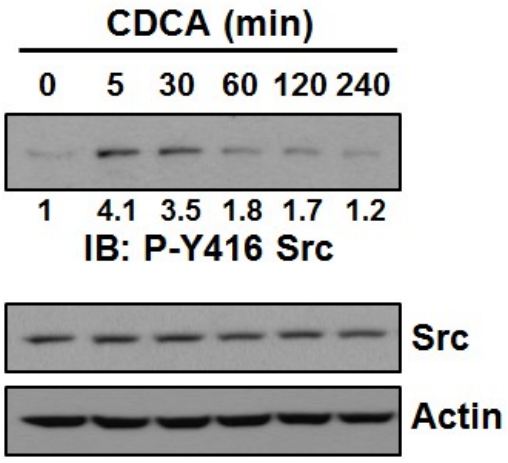


Figure 2.5: FXR interacts with the tyrosine kinase Src *in vivo* in response to cholic acid feeding. C57BL/6J mice were fasted for 10 hours then refed with normal chow diet (-CA) or a diet containing 0.5% cholic acid (+CA) for 3 hours. Nuclear extracts from the livers of these mice were prepared. FXR protein was immunoprecipitated under non-denaturing conditions and samples were immunoblotted using Src antibody. This work was completed by Dong Hyun Kim.

Figure 2.6

A)



B)

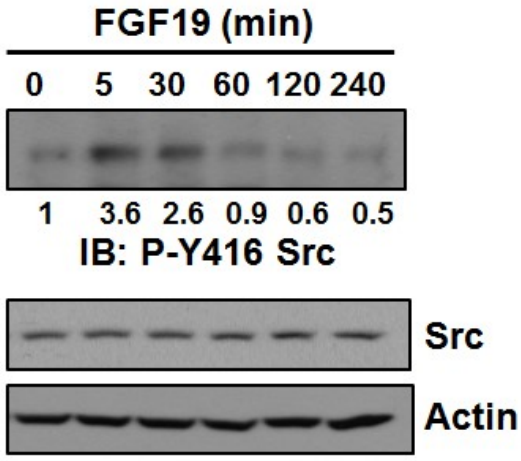


Figure 2.6: Src is activated by bile acid and FGF19 treatment. PMH isolated from C57BL/6J were treated with A) CDCA (50 µM) or B) FGF19 (50 ng/µl) for the indicated times. The cells were lysed and Src protein was immunoprecipitated. Src activation was measured by tyrosine-416 phosphorylation levels determined through western blot analysis. This work was completed by Dong Hyun Kim with help from Daniel Ryerson.

Figure 2.7

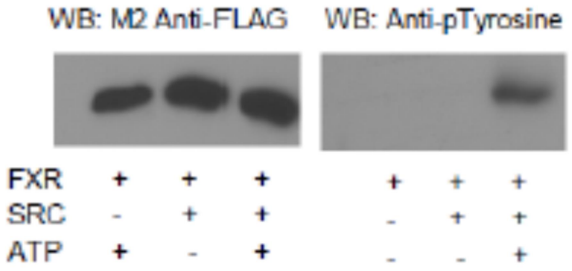
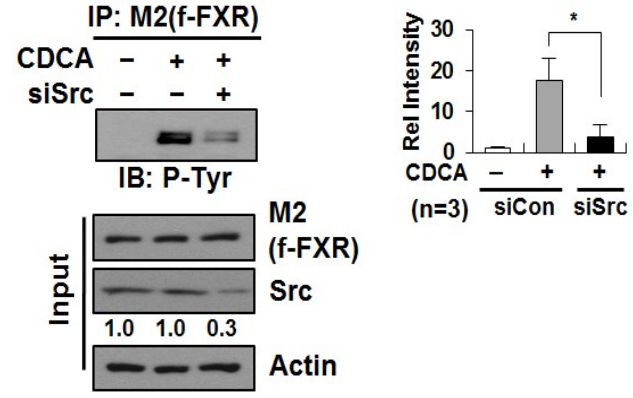


Figure 2.7: Src is sufficient to phosphorylate FXR *in vitro*. Flag tagged FXR protein was immunoprecipitated from COS-1 cells following adenoviral infection. Purified FXR protein was then incubated with 10 ng purified Src for 30 minutes at 30^{o}C and phosphorylation levels were measured via western blot. This work was completed by Daniel Ryerson.

Figure 2.8

A)



B)

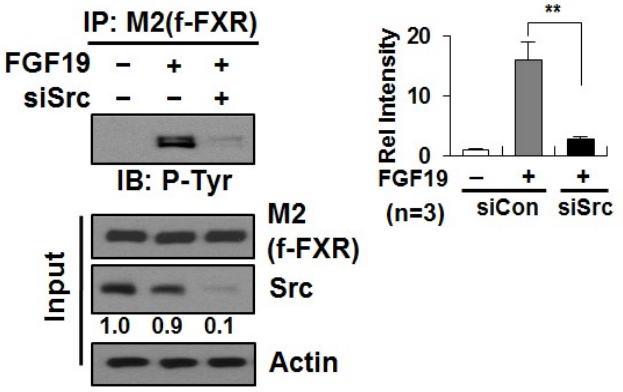
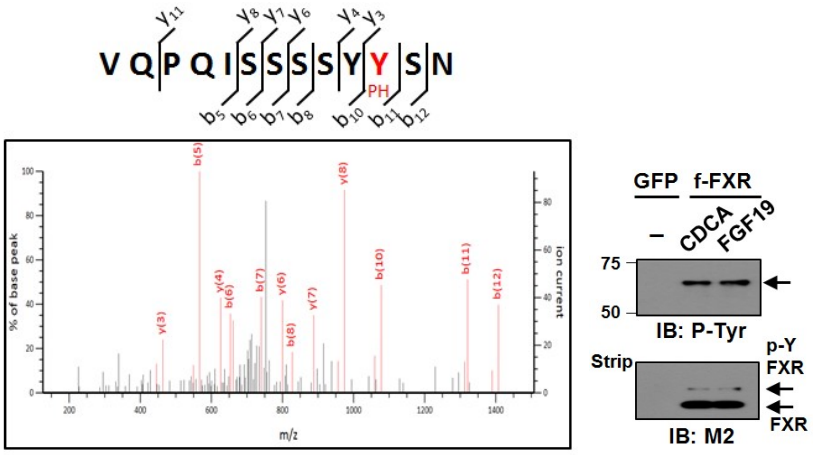


Figure 2.8: Loss of Src impairs FXR tyrosine phosphorylation. PMH cells isolated from $FXR^{-/-}$ mice were infected with adenoviral vector expressing flag tagged FXR protein (f-FXR). Infected cells were then transfected with either control siRNAs or siSrc. These cells were then treated with A) CDCA (50 µM) or B) FGF19 (50 ng/µl). FXR was then immunoprecipitated and tyrosine phosphorylation levels were measured via western blot. This work was completed by Dong Hyun Kim with help from Daniel Ryerson.

Figure 2.9

A)



B)

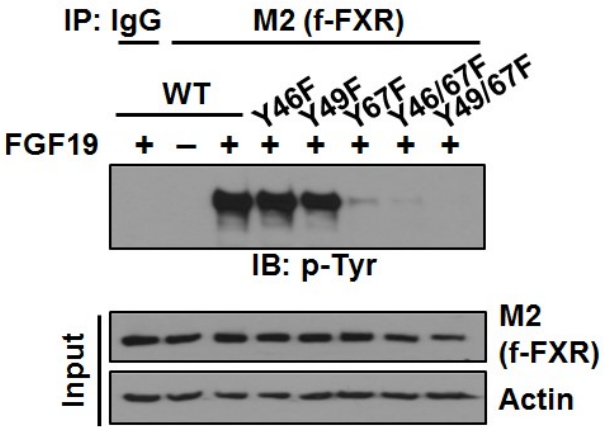
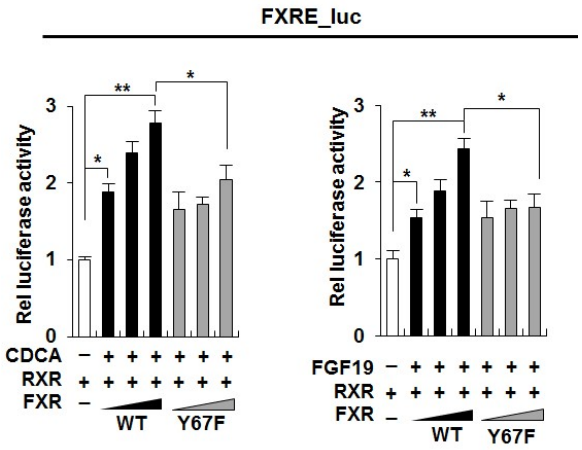


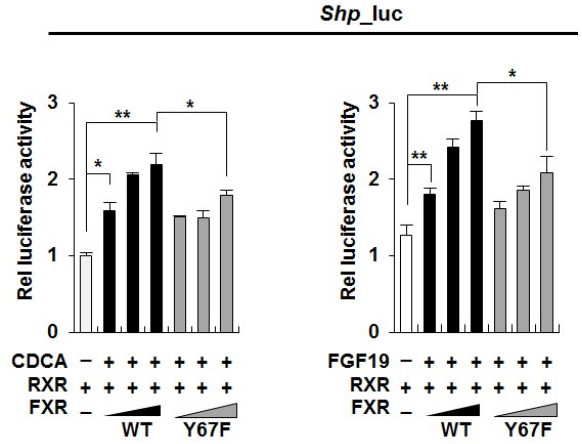
Figure 2.9: Identification of FXR tyrosine phosphorylation site. A) PMH cells isolated from FXR$^{-/-}$ mice were infected with an adenoviral vector expressing flag tagged FXR protein (f-FXR). Infected cells were then treated with CDCA (50µM) or FGF19 (50ng/µl). FXR was then immunoprecipitated and phosphorylation sites were identified through tandem mass spectrometry. B) Plasmids expressing phosphorylation deficient FXR mutants for the sites identified through tandem mass spectrometry were created. PMH from FXR$^{-/-}$ mice were then transfected with these phosphorylation mutant plasmids and treated with FGF19. FXR protein was immunoprecipitated and tyrosine phosphorylation levels were measured via western blot. This work was completed by Dong Hyun Kim with help from Daniel Ryerson.

Figure 2.10

A)



B)



C)

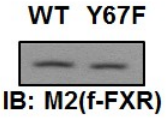


Figure 2.10: Mutation of FXR Y67 leads to loss of transactivation activity. COS-1 cells were cotransfected with increasing amounts of plasmid containing either wild type FXR or Y67F-FXR mutant, RXR, and a luciferase reporter plasmid driven by A) the FXR response element or B) the endogenous SHP promoter. The cells were then treated with either CDCA (50 μM) or FGF19 (50 ng/μl) for 6 hours. After treatment luciferase levels were measured with Luciferase Assay System (Promega, Inc) according to the manufacturer's directions. C) FXR levels were measured via western blot to show equal levels of expression between wild type and Y67F-FXR. This work was completed by Dong Hyun Kim with help from Daniel Ryerson.

Figure 2.11

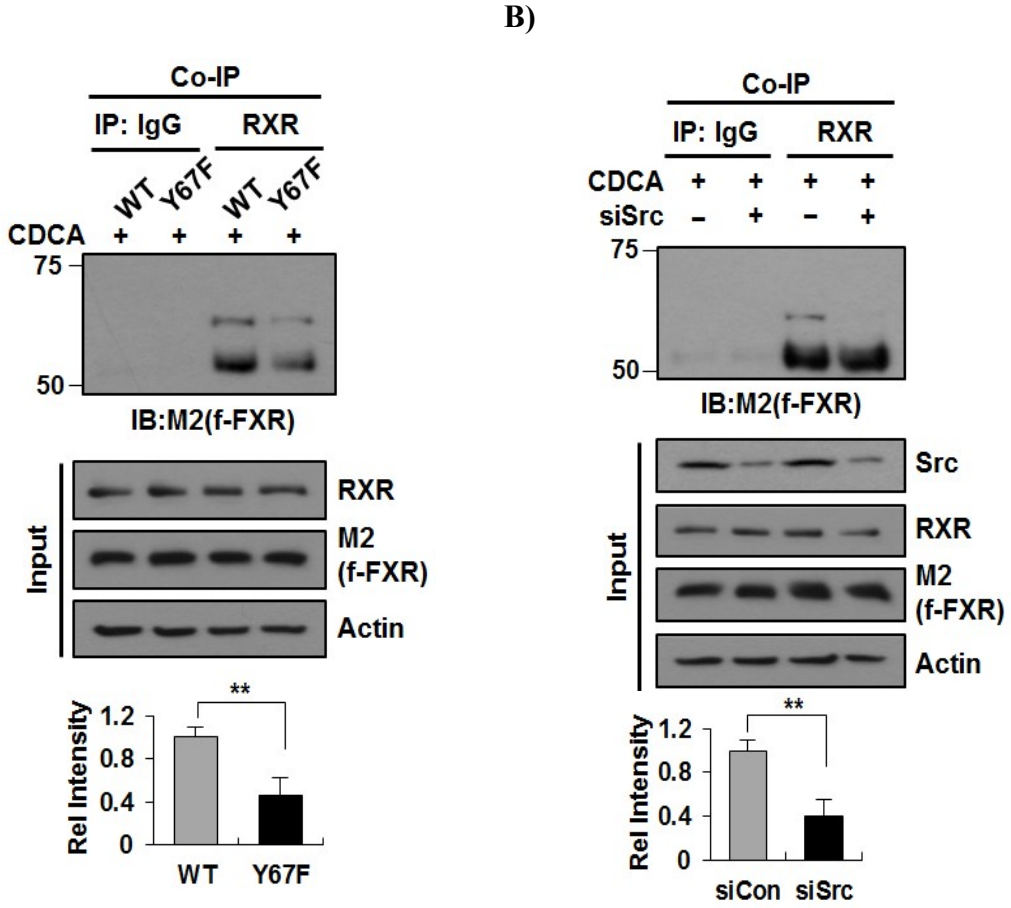


Figure 2.11: Loss of tyrosine-67 phosphorylation impairs the interaction of FXR with its heterodimer partner RXR. PMH cells were isolated from $FXR^{-/-}$ mice. A) These cells were infected with adenoviral vector expressing flag tagged wild type or Y67F-FXR. Infected cells were then treated with CDCA (50 μM). After treatment, cells were lysed and RXR protein was immunoprecipitated under non-denaturing conditions. Samples were immunoblotted using M2 antibody. B) Cells were infected with adenoviral vector expressing flag tagged wild type FXR. Infected cells were then transfected with either control siRNAs or siSrc. These cells were then treated with CDCA (50 μM). After treatment cells were lysed and RXR protein was immunoprecipitated under non-denaturing conditions. Samples were immunoblotted using M2 antibody. This work was completed by Dong Hyun Kim with help from Daniel Ryerson.

Figure 2.12

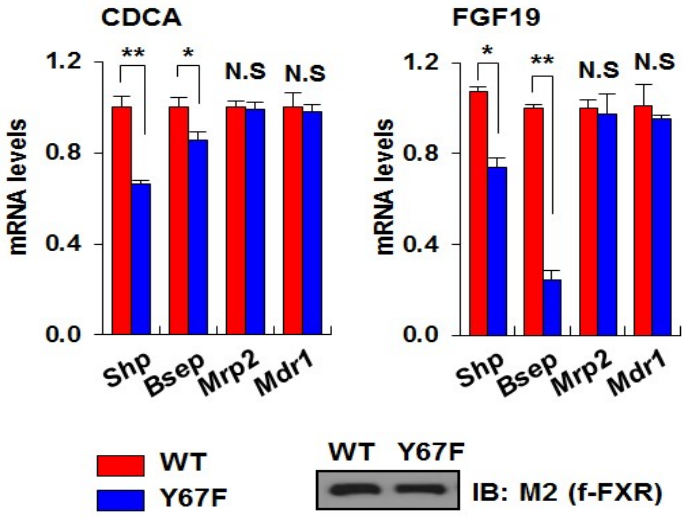


Figure 2.12: Mutation of tyrosine-67 impairs the ability of FXR to regulate genes involved in bile acid homeostasis. PMH from $FXR^{-/-}$ mice were isolated and subsequently infected with adenoviral vectors expressing either wild type FXR or the phosphorylation deficient Y67F-FXR. Cells were then treated CDCA (50 µm) or FGF19 (50 ng/ul) for 6 hours. RNA from the treated cells was isolated and cDNA was made. Expression of various FXR target genes was measured by qPCR analysis. Expression levels of FXR were measured via western blot. This work was done by Dong Hyun Kim with help from Daniel Ryerson.

Table 2.1

Position in query protein	Sequence in query protein	Corresponding motif described in the literature (phosphorylated residues in red)	Features of motif described in the literature
44 – 46	EPY	[E/D]XpY	SHP1 phosphatase substrate motif
46 - 47	YS	pY[A/G/S/T/E/D]	Src kinase substrate motif
49 - 50	YS	pY[A/G/S/T/E/D]	Src kinase substrate motif
49 - 52	YSNV	pYXX[L/I/V]	JAK2 kinase substrate motif
49 - 54	YSNVQF	pYXXXX[F/Y]	ALK kinase substrate motif
65 - 70	SYYSNL	[I/V/L/S]XpYXX[L/I]	Src family kinase substrate motif
66 - 67	YY	[E/D/Y]pY	TC-PTP phosphatase substrate motif
67 - 68	YS	pY[A/G/S/T/E/D]	Src kinase substrate motif
67 - 70	YSNL	pYXX[L/I/V]	JAK2 kinase substrate motif
67 - 72	YSNLGF	pYXXXX[F/Y]	ALK kinase substrate motif
78 - 81	EEWY	[E/D]XXpY	ALK kinase substrate motif
79 - 81	EWY	[E/D]XpY	SHP1 phosphatase substrate motif
81 - 82	YS	pY[A/G/S/T/E/D]	Src kinase substrate motif
81 - 86	YSPGIY	pYXXXX[F/Y]	ALK kinase substrate motif
86 - 87	YE	pY[A/G/S/T/E/D]	Src kinase substrate motif
94 - 97	ETLY	[E/D]XXpY	ALK kinase substrate motif
139 - 142	YNAL	pYXX[L/I/V]	JAK2 kinase substrate motif
172 - 174	DMY	[E/D]XpY	SHP1 phosphatase substrate motif
195 - 198	ECMY	[E/D]XXpY	ALK kinase substrate motif
198 - 199	YT	pY[A/G/S/T/E/D]	Src kinase substrate motif
198 - 201	YTGL	pYXX[L/I/V]	JAK2 kinase substrate motif
262 - 264	DSY	[E/D]XpY	SHP1 phosphatase substrate motif

Table 2.1 (cont.)

363 - 365	DEY	[E/D]XpY	SHP1 phosphatase substrate motif
363 - 366	DEYI	X[E/D]pYX	EGFR kinase substrate motif
363 - 366	DEYI	X[E/D]pY[I/L/V]	EGFR kinase substrate motif
364 - 365	EY	[E/D/Y]pY	TC-PTP phosphatase substrate motif
365 - 370	YITPMF	pYXXXX[F/Y]	ALK kinase substrate motif
371 - 376	SFYKSI	[I/V/L/S]XpYXX[L/I]	Src family kinase substrate motif
373 - 376	YKSI	pYXX[L/I/V]	JAK2 kinase substrate motif
384 - 386	EEY	[E/D]XpY	SHP1 phosphatase substrate motif
384 - 387	EEYA	X[E/D]pYX	EGFR kinase substrate motif
385 - 386	EY	[E/D/Y]pY	TC-PTP phosphatase substrate motif
386 - 387	YA	pY[A/G/S/T/E/D]	Src kinase substrate motif
386 - 389	YALL	pYXX[L/I/V]	JAK2 kinase substrate motif
398 - 401	DRQY	[E/D]XXpY	ALK kinase substrate motif

Table 2.1: Analysis of phosphorylation site motifs found on FXR. The amino acid sequence of FXR was input into the human protein reference database (Prasad et al., 2009) and analyzed for known tyrosine kinase motifs. Analysis was completed by Daniel Ryerson.

Table 2.2

Tyrosine Site Phosphorylation Prediction

Position	Sequence	Score
46	EVEPYSQYS	0.694
49	PYSQYSNVQ	0.643
66	SSSSYYSNL	0.893
67	SSSYYSNLG	0.968
73	NLGFYPQQP	0.028
81	PEEWYSPGI	0.438
86	SPGIYELRR	0.320
97	AETLYQGET	0.727
137	RASGYHYNA	0.830
139	SGYHYNALT	0.114
161	KNAVYKCKN	0.634
174	VMDMYMRRK	0.606
198	AECMYTGLL	0.196
264	IMDSYNKQR	0.284
365	ISDEYITPM	0.836
373	MFSFYKSIG	0.397
386	TQEEYALLT	0.673
401	PDRQYIKDR	0.222

Table 2.2: Analysis of predicted phosphorylation sites on FXR. The amino acid sequence of FXR was input into the NetPhos 3.1 server (Blom et al., 1999) and analyzed for potential tyrosine phosphorylation sites. The most highly predicted site of tyrosine phosphorylation is bolded. Analysis was completed by Daniel Ryerson.

Table 2.3

FXR Sequences

Human	54	FPQVQPQISSSSY**Y**SNLGFYPQQPEEW	80
Mouse	64	FPQVQPQISSSSY**Y**SNLGFYPQQPEDW	90
Rat	50	FPQVQPQISSSSY**Y**SNLGFYPQQPEDW	76
Monkey	64	FPQVQPQISSSSY**Y**SNLGFYPQQPEEW	90
Dog	63	FPQVQPQISSSSY**Y**SNLGFYPQHPEEW	89
Cow	64	FPQVQPQISSSSY**Y**SNVGFYPQQPEEW	90
Chicken	55	FPQVQPQISSPPY**Y**SNLGFYPPQHEEW	81
Zebrafish	59	YTSGEPSMSSPSY**Y**SSQHCYSQYGAEE	85

Table 2.3: Alignment of FXR tyrosine phosphorylation site across species. The amino acid sequence of FXR proteins from different species, obtained from NCBI database, was aligned using ClustalW2 alignment tool to determine sequence similarity to human FXR tyrosine-67. Analysis was completed by Daniel Ryerson.

2.7 References

Anakk S, Watanabe M, Ochsner SA, McKenna NJ, Finegold MJ, Moore DD. (2011) Combined deletion of Fxr and Shp in mice induces Cyp17a1 and results in juvenile onset cholestasis. J Clin Invest. 121(1):86-95.

Ananthanarayanan M, Balasubramanian N, Makishima M, Mangelsdorf DJ, Suchy FJ. (2001) Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. J Biol Chem. 276(31):28857-65.

Barbier O, Torra IP, Sirvent A, Claudel T, Blanquart C, Duran-Sandoval D, Kuipers F, Kosykh V, Fruchart JC, Staels B. (2003) FXR induces the UGT2B4 enzyme in hepatocytes: a potential mechanism of negative feedback control of FXR activity. Gastroenterology. 124(7):1926-40.

Blom N, Gammeltoft S, Brunak S. (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. J Mol Biol. 294(5):1351-62.

Brown MT, Cooper JA. (1996) Regulation, substrates and functions of src. Biochim Biophys Acta. 1287:121–49.

Degirolamo, C., Sabba, C. & Moschetta, A. (2016) Therapeutic potential of the endocrine fibroblast growth factors FGF19, FGF21 and FGF23. *Nat Rev Drug Discov* 15,51-69.

Desnoyers LR, Pai R, Ferrando RE, Hotzel K, Le T, Ross J, Carano R, D'Souza A, Qing J, Mohtashemi I, Ashkenazi A, French DM. (2008) Targeting FGF19 inhibits tumor growth in colon cancer xenograft and FGF19 transgenic hepatocellular carcinoma models. Oncogene 27,85-97

Frame MC. (2002) Src in cancer: deregulation and consequences for cell behavior. Biochim. Biophys. Acta. 1602:114–130.

Frankenberg T, Miloh T, Chen FY, Ananthanarayanan M, Sun AQ, Balasubramaniyan N, Arias I, Setchell KD, Suchy FJ, Shneider BL. (2008) The membrane protein ATPase class I type 8B member 1 signals through protein kinase C zeta to activate the farnesoid X receptor. Hepatology. 48(6):1896-905.

Fu T, Kim YC, Byun S, Kim DH, Seok S, Suino-Powell K, Xu HE, Kemper B, Kemper JK. (2016) FXR Primes the Liver for Intestinal FGF15 Signaling by Transient Induction of β-Klotho. Mol Endocrinol. 30(1):92-103.

Gineste R, Sirvent A, Paumelle R, Helleboid S, Aquilina A, Darteil R, Hum DW, Fruchart JC, Staels B. (2008) Phosphorylation of farnesoid X receptor by protein kinase C promotes its transcriptional activity. Mol Endocrinol. 22(11):2433-47.

Gnerre C, Blättler S, Kaufmann MR, Looser R, Meyer UA. (2004) Regulation of CYP3A4 by the bile acid receptor FXR: evidence for functional binding sites in the CYP3A4 gene. Pharmacogenetics. 14(10):635-45.

Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi C, Wilson JG, Lewis MC, Roth ME, Maloney PR, Wilson TM, Kliewer SA. (2000) A regulatory cascade of the nuclear receptors FXR, SHP-1 and LRH-1 represses bile acid biosynthesis. Mol Cell. 6(3):517-26.

Harrison SC. (2003) Variation on an Src-like theme. Cell. 112(6):737.

Kemper JK. (2011) Regulation of FXR transcriptional activity in health and disease: Emerging roles of FXR cofactors and post-translational modifications. Biochim Biophys Acta. 1812(8):842-50

Kemper JK, Xiao Z, Ponugoti B, Miao J, Fang S, Kanamaluru D, Tsang S, Wu SY, Chiang CM, Veenstra TD. (2009) FXR acetylation is normally dynamically regulated by p300 and SIRT1 but constitutively elevated in metabolic disease states. Cell Metab. 10(5):392-404.

Keshava Prasad TS, Goel R, Kandasamy K, Keerthikumar S, Kumar S, Mathivanan S, Telikicherla D, Raju R, Shafreen B, Venugopal A, Balakrishnan L, Marimuthu A, Banerjee S, Somanathan DS, Sebastian A, Rani S, Ray S, Harrys Kishore CJ, Kanth S, Ahmed M, Kashyap MK, Mohmood R, Ramachandra YL, Krishna V, Rahiman BA, Mohan S, Ranganathan P, Ramabadran S, Chaerkady R, Pandey A. (2009) Human Protein Reference Database--2009 update. Nucleic Acids Res. D767-72.

Kliewer SA, Mangelsdorf DJ. (2015) Bile Acids as Hormones: The FXR-FGF15/19 Pathway. *Dig. Dis.* 33:327-331

Kim DH, Xiao Z, Kwon S, Sun X, Ryerson D, Tkac D, Ma P, Wu SY, Chiang CM, Zhou E, Xu HE, Palvimo JJ, Chen LF, Kemper B, Kemper JK. (2015) A dysregulated acetyl/SUMO switch of FXR promotes hepatic inflammation in obesity. EMBO J. 34(2):184-99.

Lee J, Seok S, Yu P, Kim K, Smith Z, Rivas-Astroza M, Zhong S, Kemper JK. (2012) Genomic analysis of hepatic farnesoid X receptor binding sites reveals altered binding in obesity and direct gene repression by farnesoid X receptor in mice. Hepatology. 56(1):108-17.

Levin VA. (2004) Basis and importance of Src as a target in cancer. Cancer Treat. Res. 119:89–119.

Li S, Hsu DD, Li B, Luo X, Alderson N, Qiao L, Ma L, Zhu HH, He Z, Suino-Powell K, Ji K, Li J, Shao J, Xu HE, Li T, Feng GS. (2014) Cytoplasmic tyrosine phosphatase Shp2

coordinates hepatic regulation of bile acid and FGF15/19 signaling to repress bile acid synthesis. Cell Metab. 20(2):320-32.

Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV Lustig KD, Mangelsdorf DJ, Shan B. (1999) Identification of a nuclear receptor for bile acids. Science. 284(5418):1362-5.

Miao J, Xiao Z, Kanamaluru D, Min G, Yau PM, Veenstra TD, Ellis E, Strom S, Suino-Powell K, Xu HE, Kemper JK. (2009) Bile acid signaling pathways increase stability of Small Heterodimer Partner (SHP) by inhibiting ubiquitin-proteasomal degradation. Genes Dev. 23(8):986-96.

Nicholes K, Guillet S, Tomlinson E, Hillan K, Wright B, Frantz GD, Pham TA, Dillard-Telm L, Tsai SP, Stephan JP, Stinson J, Stewart T, French DM. (2008) A mouse model of hepatocellular carcinoma: ectopic expression of fibroblast growth factor 19 in skeletal muscle of transgenic mice. Am. J. Pathol. 160,2295-2307

Owen BM, Mangelsdorf DJ, Kliewer SA. (2015) Tissue-specific actions of the metabolic hormones FGF15/19 and FGF21. *Trends Endocrinol. Metab.* 26,22-29 PMC4277911

Peng Z, Raufman JO, Xie G. (2012) Src-mediated cross-talk between farnesoid X and epidermal growth factor receptors inhibits human intestinal cell proliferation and tumorigenesis. PLos One. 7(10):e48461.

Russell DW. (2003) The enzymes, regulation, and genetics of bile acid synthesis. Annu Rev Biochem. 72:137-74.

Seok S, Kanamaluru D, Xiao Z, Ryerson D, Choi SE, Suino-Powell K, Xu HE, Veenstra TD, Kemper JK. (2013) Bile acid signal-induced phosphorylation of small heterodimer partner by protein kinase Cζ is critical for epigenomic regulation of liver metabolic genes. J Biol Chem. 288(32):23252-63.

Song CS, Echchgadda I, Baek BS, Ahn SC, Oh T, Roy AK, Chatterjee B. (2001) Dehydroepiandrosterone sulfotransferase gene induction by bile acid activated farnesoid X receptor. J Biol Chem. 276(45):42549-56.

Thomas SM, Brugge JS. (1997) Cellular functions regulated by Src family kinases. Annu. Rev. Cell Dev. Biol. 13:513–609.
Trauner M, Boyer JL. (2003) Bile salt transporters: molecular characterization, function, and regulation. Physiol Rev. 83(2):633-71.

Zhang Y, Edwards PA. (2008) FXR signaling in metabolic disease. FEBS Lett. 582(1):10-8.

Zhao Y, Sudol M, Hanafusa H, Krueger I. (1992) Increased tyrosine kinase activity of c-Src during calcium-induced keratinocyte differentiation. Proc. Natl. Acad. Sci. USA. 89:8298–8302.

Zheng XM, Resnick RJ, Shalloway D. (2000) A phosphortyrosine displacement mechanism for activation of Src by PTPalpha. EMBO J. 19(5):964-78.

Chapter Three

In vivo functional role of FXR phosphorylation in physiology and disease

3.1 Abstract

FXR plays important roles in diverse physiological processes involved in multiple organ systems. Loss of FXR in mouse models leads to several distinct deficiencies. $FXR^{-/-}$ mice display elevated bile acid pool size as well as elevated serum bile acid levels. They also show misregulation of many metabolic processes resulting in impaired glucose tolerance and insulin sensitivity, elevated cholesterol, LDL, HDL, and triglyceride levels. Additionally, $FXR^{-/-}$ mice show signs of liver damage and develop spontaneous tumors as they age. The diverse roles FXR plays highlight the importance of studying the activity and regulation of FXR in whole animal settings. In this study, we utilized adenoviral vectors to express wild type and tyrosine-67 site specific phosphorylation deficient mutant FXR proteins in the livers of C57BL/6J and $FXR^{-/-}$ mice. This adenoviral mediated expression allowed us to examine the role that phosphorylation of hepatic FXR at tyrosine-67 has on the expression of FXR target genes as well as the effects on the physiological roles of FXR in the body. We demonstrated that disruption of the tyrosine-67 phosphorylation site led to a decrease in expression of FXR targets. Additionally, bile acid levels as well as the levels of aspartate transaminase (AST), alanine transaminase (ALT), and bilirubin, three markers of liver damage, were elevated. The livers of $FXR^{-/-}$ mice expressing Y67F-FXR showed increased levels of macrophage infiltration, another sign of liver damage. These effects were exacerbated when the mice were challenged in models of cholestasis. Overall, we have shown that disruption of FXR phosphorylation at tyrosine-67 drastically

impairs FXR's ability to regulate bile acid responsive target genes and demonstrates the important physiological role FXR tyrosine-67 phosphorylation plays in bile acid homeostasis.

3.2 Introduction

Cholestatic liver diseases are progressive and can lead to liver failure. Currently, there is not an effective treatment for most cholestatic liver diseases. Cholestasis is defined as an impairment of bile secretion and flow. As a result of cholestasis, bile acids accumulate in the liver potentially causing fibrosis, inflammation, and cirrhosis. Liver cirrhosis dramatically increases the risk of developing hepatocellular carcinoma (HCC) which accounts for 80% of all liver tumors and is now the second leading cause of cancer deaths worldwide (El-Serag and Rudolph 2007). HCC is the fastest growing cause of cancer mortality in the United States. Mice lacking the key regulator of bile acids, FXR, have been shown to spontaneously develop liver tumors including hepatocellular adenoma and carcinoma between 13-15 months of age (Young et al., 2007). Conversely, increasing FXR expression has been shown to prevent tumor development (Degirolamo et al., 2015). Therefore, understanding the regulation of FXR in mouse models may help shed light on the development of HCC and possibly open new treatment avenues. The exact mechanism through which FXR prevents tumor growth is still unknown although there are several proposed mechanisms. FXR activity prevents accumulation of hydrophobic bile acids in the liver which at high levels can lead to cell damage. FXR can also directly influence inflammatory signaling and apoptosis through its interactions with NFκB (Gadaleta et al., 2011). Additionally, FXR has been shown to play important roles in regulating liver regeneration. Irregular liver regeneration has been suggested to promote a tumor prone environment.

In addition to their tumorigenic phenotype, disruption of FXR *in vivo* results in a host of deleterious metabolic effects. $FXR^{-/-}$ mice have increased bile acids pool size and increased serum bile acid levels (Kok et al., 2003). $FXR^{-/-}$ mice also have an impaired lipid profile with elevated serum levels of cholesterol, HDL, LDL, and triglycerides (Sinal et al., 2000). Additionally, $FXR^{-/-}$ mice have impaired glucose tolerance and insulin sensitivity. FXR regulates many physiological processes involving multiple organ systems, which highlights the importance of utilizing available mouse models and examining effects on FXR activity *in vivo*.

One tool that has proven very useful for studying the role of FXR in the liver has been adenoviral overexpression. When using adenovirus to overexpress a protein, the gene of interest is cloned into a plasmid containing the adenoviral background. This adenovirus can be injected directly into the tail vein of the mouse. Upon tail vein injection the adenoviral vector quickly and efficiently selectively targets hepatic tissue leading to expression of the gene of interest, in our case FXR. This allows isolation the role of FXR in the liver and investigation of how hepatic FXR affects the rest of the body. In this study, we utilized adenoviral reconstitution of wild type or Y67F-FXR in $FXR^{-/-}$ mice. Mutation of the site of a post-translational modification has proven a valuable tool when examining the role of the post-translational modification *in vivo* (Seok et al., 2013; Kim et al., 2015; Kim et al., 2016).

A key factor when studying *in vivo* functions is the conditions utilized. In our studies we utilized several models under different sets of conditions. To explore normal physiological conditions we utilized mice fasted and refed either normal chow or a diet supplemented with 0.5% CA briefly for 3-6 hours. This model allowed for observation of the general effects of FXR tyrosine-67 phosphorylation. However, major phenotypic changes are often not observable unless the models are challenged. When exploring bile acid physiology one common method of

challenge is bile acid overload. α-Naphthylisothiocyanate (ANIT) is a well characterized cholestatic agent which prevents normal bile flow, (Plaa and Priestly 1976) leading to hepatic bile acid overload. Administration of ANIT allows for better analysis of bile acid regulation. Here, we investigated the role that phosphorylation of tyrosine-67 has on these models.

3.3 Materials and Methods

In vivo experiments

$FXR^{-/-}$ mice were infected with adenoviral vectors expressing an empty vector, wild type flag-tagged FXR, or the flag-tagged Y67F mutant of FXR at an MOI leading to physiological levels of expression in the liver. Two weeks post infection, food was removed from the cages for 10 hours (7:00AM-5:00PM) after which mice were refed with either normal chow or chow supplemented with 0.5% cholic acid (Harlan Teklad) for 3 hours. After refeeding, mice were sacrificed and metabolic tissues were collected, snap frozen in liquid nitrogen, and stored at -80^{o}C for analysis. FXR immunoprecipitated from liver nuclear extracts was measured by western blot. All animal use was approved by the Institutional Animal Care and Use and Biosafety Committees at the University of Illinois at Urbana-Champaign and was in accordance with National Institutes of Health guidelines.

In vivo cholestatic challenge experiments

$FXR^{-/-}$ mice were infected with adenoviral vectors expressing an empty vector, wild type flag-tagged FXR, or flag-tagged Y67F mutant FXR at an MOI leading to physiological levels of expression in the liver. Twelve days post infection mice were treated with ANIT (75mg/kg), a chemical which causes intrahepatic cholestasis. Forty-eight hours post ANIT treatment mice were sacrificed and metabolic tissues were collected, snap frozen in liquid nitrogen, and stored at

-80^{o}C for analysis. FXR immunoprecipitated from liver nuclear extracts was measured by western blot. All animal use was approved by the Institutional Animal Care and Use and Biosafety Committees at the University of Illinois at Urbana-Champaign and was in accordance with National Institutes of Health guidelines.

RNA isolation

A small portion of the liver from mice infected with empty vector or adenoviral vectors expressing wild type or Y67F-FXR for 2 weeks was collected. RNA was isolated in Trizol reagent (Qiagen) according to the manufacturer's instructions. Relative gene expression levels were measured by qRTPCR analysis with SYBR Green (Roche).

Measurement of bile acid levels

A small portion (~100mg) of the liver from mice infected with adenoviral vectors overexpressing wild type or Y67F-FXR for 2 weeks was collected. One mL of 100% ethanol was added to the liver fragment. Fragment was then sonicated and stored at 55^{o}C overnight. The sample was then centrifuged at 13000 rpm for 10 minutes. The supernatant was analyzed with a bile acid measurement kit (Trinity Biotech, plc) according to the manufacturer's directions.

Measurements of ALT/AST levels

Blood was collected from mice infected with adenoviral vectors expressing either wild type FXR or Y67F-FXR. Heparin was added to the samples that were then centrifuged and the serum was analyzed with an ALT/AST/bilirubin measurement kit (Sigma) according to the manufacturer's instructions.

Development of a site specific FXR phosphorylation antibody

A commercial custom produced antibody specific for FXR phosphorylated at tyrosine-67 was designed (Abmart). A peptide containing a phosphorylated tyrosine with 8 amino acids upstream and downstream of tyrosine-67 was synthesized (PQISSSSY*Y*SNLGFYPQ). This peptide was then injected into rabbits. The FXR phospho-Y67 antibody was then purified from the serum of these rabbits. The purified antibody was reconstituted in PBS with 0.02% sodium azide.

Analysis of site specific FXR phosphorylation antibody specificity

COS-1 cells were co-transfected with Src and either wild type FXR plasmid, or a FXR plasmid with a tyrosine phosphorylation deficient mutation at tyrosine-66 (Y66F), tyrosine-67 (Y67F), or both tyrosine-66 and 67 (Y66/67F). Cells were then treated with CDCA for 15 minutes and FXR was immunoprecipitated. The levels of FXR tyrosine-67 phosphorylation were measured by western blotting using the site specific antibody developed by Abmart.

3.4 Results

Mutation of tyrosine-67 impairs the ability of FXR to regulate genes involved in bile acid homeostasis *in vivo*.

Our previous studies demonstrated that disruption of the FXR tyrosine-67 phosphorylation site impaired the ability of FXR to regulate its target genes in PMH. To explore the effect of FXR tyrosine-67 phosphorylation on target gene expression *in vivo*, $FXR^{-/-}$ mice were infected with adenoviral constructs expressing either empty vector, wild type FXR, or Y67F mutant FXR to endogenous levels and expression of FXR target genes was measured via

qPCR. As expected, wild type FXR increased expression of FXR target genes, however Y67F-FXR was unable to induce expression of several key genes in bile acid homeostasis, including *SHP* and *BSEP* (Figure 3.1). These data are consistent with that seen in cell culture models (Figure 2.12) and reinforce the conclusion that phosphorylation of FXR at tyrosine-67 plays an important role in FXR's ability to regulate gene transcription

Expression of Y67F-FXR is unable to rescue elevated levels of bile acids, AST, ALT and signs of liver damage observed in $FXR^{-/-}$ mice.

A key role of FXR is the maintenance of bile acid homeostasis. To assess the effect of tyrosine-67 phosphorylation on physiological bile acid levels, $FXR^{-/-}$ mice were infected with either empty vector, wild type FXR, or Y67F-FXR. Bile acids were then extracted from the liver, serum, and gallbladder and levels were measured. Mice reconstituted with Y67F-FXR had significantly higher levels of bile acids both in the serum and gallbladder when compared to mice reconstituted with wild type FXR. Hepatic bile acid levels appeared elevated, however levels did not reach statistically significant differences (Figure 3.2A). Bile acid levels in mice expressing Y67F-FXR were often equal to, or in some cases above, those in mice infected with empty vector, suggesting an impairment of bile acid regulation. H/E staining demonstrated that mice expressing Y67F-FXR also showed signs of fibrosis and liver damage similar to mice infected with the empty vector virus (Figure 3.2B). These data show that mutation of FXR tyrosine-67 disrupts the ability of FXR to regulate the levels of bile acids in the body leading to liver damage, likely caused by the inability of Y67F-FXR to regulate gene transcription.

Mice expressing Y67F-FXR show impaired ability to protect the liver from cholestatic challenge.

While under physiological conditions loss of tyrosine-67 phosphorylation led to a small but significant increase in bile acid levels (Figure 3.2) to further understand the effects this phosphorylation site has *in vivo*, adenoviral constructs were used to express wild type or Y67F-FXR in a mouse model of cholestasis. $FXR^{-/-}$ mice were infected with either empty vector, wild type FXR, or Y67F mutant FXR. Infected mice were then treated with ANIT for 48 hours to induce a cholestatic state. After treatment, tissues important for bile acid regulation were collected and bile acid levels were measured. During collection, several morphological changes were observed. It was seen that the gallbladders of mice expressing empty vector or Y67F-FXR were significantly swollen and had a dark green color whereas those of mice expressing wild type FXR were significantly smaller and had a yellow color to them. Additionally, serum collected from mice expressing empty vector and Y67F-FXR had a strong yellow tint to it (Figure 3.3A). Mice infected with empty vector or Y67F-FXR both showed higher levels of bile acids in the serum, gallbladder, and liver tissues (Figure 3.3B). Elevated hepatic bile acids levels are known to damage the liver. Consistent with these results, mice infected with empty vector or Y67F-FXR both had elevated levels of liver enzymes AST, ALT, and bilirubin, increased levels of scarring, and increased macrophage infiltration; all signs of liver damage (Figure 3.3C and D). These studies show that Y67F-FXR is less able to maintain bile acid homeostasis and protect the liver from the damage associated with elevated bile acid levels, demonstrating the importance of this phosphorylation site in the proper function of FXR *in vivo*.

To verify that partial reconstitution of FXR, only in the liver, does not have unknown consequences, the adenoviral empty vector, wild type, or Y67F-FXR constructs were overexpressed in C57BL/6J mice. When challenged with ANIT these mice showed similar trends as the infected $FXR^{-/-}$ mice, although some effects were dampened by the presence of

endogenous FXR (Figure 3.4). C57BL/6J mice infected with empty vector or Y67F-FXR showed significantly larger gallbladders, although the dark green color change observed in figure 3.3A was not seen. Bile acid levels in the serum, liver, and gallbladders were again elevated in C57BL/6J mice infected with control vector or Y67F-FXR, along with signs of liver damage observed in figure 3.3. These data show that in both models of partial reconstitution and hepatic overexpression disruption of the FXR tyrosine-67 phosphorylation site impairs the ability of FXR to protect the liver from the effects of cholestasis.

Measuring the specificity of the phosphorylated tyrosine-67 site-specific FXR antibody.

To better study how phosphorylation at tyrosine-67 affects FXR activity, an antibody specific for FXR phosphorylated at tyrosine-67 was commissioned from Abmart. To determine the specificity of the antibody, COS-1 cells were co-transfected with Src and either wild type FXR plasmid, or a FXR plasmid with a tyrosine phosphorylation deficient mutation at tyrosine 66, tyrosine-67, or both tyrosine-66 and 67. Cells were then treated with CDCA for 15 minutes and FXR was immunoprecipitated. The levels of FXR tyrosine-67 phosphorylation were measured by western blotting using the site specific antibody developed by Abmart. Cells cotransfected with Src and either wild type FXR or tyrosine-66 deficient FXR showed high levels of phosphorylation, whereas cells transfected with tyrosine-67 deficient or tyrosine-66 and 67 deficient FXR showed no phosphorylation (Figure 3.5). It is worth noting that the phosphorylation bands appeared at a higher molecular weight than FXR is normally seen. This shift was observed with both site specific and pan phosphotyrosine antibodies. This change in mobility is likely due to the addition of the phosphate group or some other post-translational modifications occurring in response to phosphorylation. Nonetheless, the site specific antibody was show to specifically recognize phosphorylation at tyrosine-67 of FXR.

Measurement of FXR phosphorylation with the tyrosine-67 site specific phosphorylation antibody *in vivo*.

Utilizing this new tool, we wanted to visualize the effect of treatment with cholic acid or FGF19 on FXR tyrosine-67 phosphorylation levels *in vivo*. To accomplish this, C57BL/6J mice were treated with signaling molecules previously shown to increase FXR phosphorylation (CA or FGF19) or control treatments. Liver sections were prepared using a Vibratome (University of Illinois at Urbana-Champaign, Institute for Genomic Biology Tissue Processing Core). FXR tyrosine-67 phosphorylation levels were then analyzed via immunohistochemistry or sections were stained using a macrophage infiltration kit (Millipore, Inc) according to the manufacturer's instructions. Significant increases in the levels of FXR tyrosine-67 phosphorylation were observed in samples treated with either CA or FGF19 (Figure 3.6). This agrees with previous data showing that these signaling molecules lead to FXR tyrosine-67 phosphorylation in PMHs (Figure 2.2). Going forward we hope to utilize this antibody to examine the levels of FXR tyrosine-67 in liver samples of human patients with different stages of liver disease. These types of experiments will give insight into the role FXR phosphorylation plays in human disease.

3.5 Discussion

In this study, we demonstrated that disruption of the tyrosine-67 phosphorylation site *in vivo* led to a decrease in expression of FXR targets genes, increased levels of bile acids, and signs of liver damage including elevated macrophage infiltration, AST, ALT, and bilirubin levels when compared to mice expressing wild type FXR. These effects were exacerbated when the mice were challenged in models of cholestasis. Overall, we have shown that disruption of FXR phosphorylation at tyrosine-67 drastically impairs FXR's ability to regulate bile acid responsive

target genes, which demonstrates the important physiological role FXR tyrosine-67 phosphorylation plays in bile acid homeostasis.

A key role of FXR *in vivo* is to protect the liver from bile acid toxicity. FXR accomplishes this by inhibiting the synthesis of bile acids through SHP, downregulating bile acid importers, upregulating bile acid exporters, and upregulating genes involved in conjugation and detoxification of bile acids. Loss of FXR leads to many deleterious effects including disruption of bile acid homeostasis, problems with lipid metabolism, and spontaneous tumor generation (Kok et al., 2003; Sinal et al., 2000; Young et al., 2007; and Degirolamo et al., 2015). Additionally, several recent studies have shown that certain mutations in the FXR gene are associated with human disease (Hu et al., 2016; Heni et al., 2013; Gomez-Ospina et al., 2016; and Nijmeijer et al., 2014). These factors demonstrate that understanding how FXR is regulated, particularly *in vivo* where the complex multi-organ bile acid signaling pathways are intact, may be key to understanding the pathology of human disease.

In this study, when the tyrosine-67 phosphorylation site of FXR was mutated, expression of key FXR target genes necessary for bile acid transport and synthesis were no longer regulated by FXR, with the greatest effect seen in the expression of *BSEP*. This misregulation led to increased bile acid levels in mice expressing Y67F-FXR when compared to mice expressing wild type FXR. With FXR's ability to protect the liver impaired, the accumulation of bile acids caused increased scarring and signs of liver damage. The effects seen in mice expressing Y67F-FXR are exacerbated when they were challenged in a model of cholestasis. When challenged, mice expressing Y67F-FXR showed not only elevated levels of bile acid and scarring, but also more severe signs of liver damage, including elevated liver enzyme levels along with macrophage infiltration when compared to mice expressing wild type FXR. When challenged

with ANIT, significant morphological changes including swelling and darkening of the gallbladder as well as a yellowish tint to the serum in mice expressing Y67F-FXR were also observed. These signs demonstrate that loss of FXR phosphorylation at tyrosine-67 dramatically impairs the ability of FXR to maintain homeostasis and protect the liver. Interestingly, these findings are similar to results seen in human mutations of FXR. In Gomez-Ospina et al., 2016, symptoms of four human patients born with mutations in the FXR gene were characterized. These patients showed elevated bile acid and liver enzyme levels along with other signs of liver damage. Additionally, these patients displayed decreased expression of FXR target genes, most notably *BSEP* which was also downregulated in our studies. Future studies exploring what connection FXR tyrosine-67 phosphorylation has with human FXR mutations will be required.

Recently, several studies have come out demonstrating the important role post-translational modifications and key amino acids play in the activities of FXR and other NRs *in vivo*. Seok et al., 2013 showed that mutation of the SHP threonine-55 phosphorylation site resulted in decreased recruitment of histone deacetylase 1(HDAC1), euchromatic histone-lysine N-methyltransferase 2, and BRM, leading to significant changes to bile acid, lipid, and glucose metabolism *in vivo*. Kim et al., 2016 demonstrated that loss of SHP sumoylation at lysine-68 led to impaired recruitment of the key SHP cofactors lysine specific demethylase 1, HDAC1 and mSin3a resulting in dramatic elevation of bile acid levels *in vivo*. Kim et al., 2015 examined the interplay between acetylation at lysine-217 and sumoylation at lysine-277. They found that sumoylation of FXR increases its interaction with NFκB while preventing heterodimerization with RXR. Disruption of these sites led to changes in glucose and lipid levels along with inflammatory responses. Lien et al., 2014 demonstrated that FXR is phosphorylated by AMPK at serine-250 with mutation of this site preventing FXR recruitment of transcriptional

coactivators to target genes. Interestingly they also showed that metformin, an AMPK activator which is often used in the treatment of diabetes, increased liver damage in mouse models of cholestasis. The current study shows that, consistent with results discussed in chapter two, disruption of the FXR tyrosine-67 phosphorylation site led to misregulation of FXR target gene expression in a gene specific manner. FXR regulation of bile acid synthetic genes and certain bile acid transporters were significantly decreased with the mutant Y67F-FXR when compared to wild type FXR, while the regulation of other bile acid transporters or genes involved in lipid metabolism were changed. This gene specific regulation may be due to differential cofactor complex recruitment by phosphorylated and unphosphorylated, FXR however the exact makeup of these cofactor complexes has not yet been identified.

In this study we showed that Src mediates the phosphorylation of FXR at tyrosine-67 in the liver. Several tyrosine kinase inhibitors which alter Src activity, including bosutinib, dasatinib, and ponatinib, have been used in the treatment of chronic myelogenous leukemia (Roskoski, 2015; Bauer et al., 2016). However, unintended side effects have been found with these drugs. Consistent with our findings several studies have been published demonstrating that some patients taking these drugs have developed symptoms including elevated AST/ALT levels, liver injury, and in some cases acute liver failure (Bonvin et al., 2008; Doan et al., 2015; Shamroe and Comeau, 2013). Future studies examining the effect pharmaceutical tyrosine kinase inhibitors have on FXR tyrosine-67 phosphorylation and how this relates to their hepatotoxic effects may be warranted.

For this study we utilized a FXR phosphotyrosine-67 site specific antibody. With this antibody, we were able to visualize the effects of bile acid and FGF19 signaling on the phosphorylation of FXR at tyrosine-67 in mouse livers. This site specific antibody will serve as

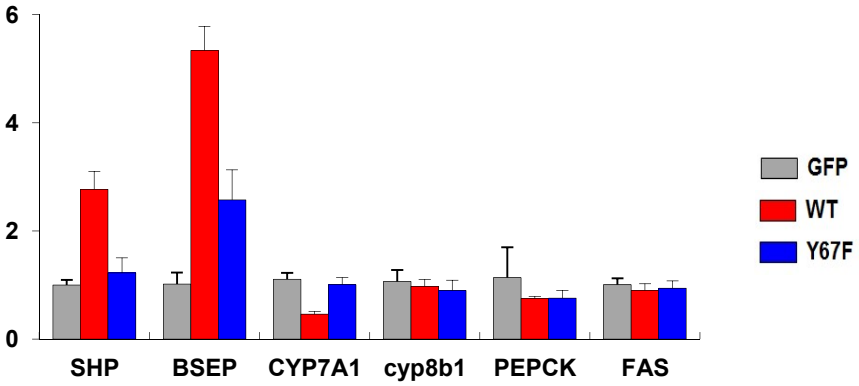
a useful tool in future studies to explore the levels of FXR tyrosine-67 phosphorylation in mouse models as well as samples from human patients with liver disease.

In this study we utilized two main mouse models to explore the effect of FXR tyrosine-67 phosphorylation on bile acid regulation *in vivo*. The first model we utilized was $FXR^{-/-}$ mice in which no endogenous FXR is expressed with reconstituted expression of wildtype, or Y67F-FXR in the liver through adenoviral expression. Using adenoviral constructs to compare the expression of wild type and Y67F-FXR in $FXR^{-/-}$ mice allows us to examine the effects of these proteins at physiological levels, however adenoviral expression targets the liver so it does not achieve a full body reconstitution of FXR. Therefore, systemic signaling effects of FXR from other tissues may not be seen in the $FXR^{-/-}$ mice. To verify that partial reconstitution of FXR, only in the liver, does not have unknown consequences, a second model in which wild type or Y67F-FXR constructs were adenovirally overexpressed in C57BL/6J mice was utilized. These mice have normal endogenous FXR expression and signaling preserved, but upon infection now also have wild type or Y67F-FXR overexpressed in the liver. In both of these models, infected mice were challenged with ANIT for 48 hours to induce a cholestatic state. Both models showed similar trends with mice expressing wildtype FXR showing lower levels of bile acids in serum, liver, and gallbladder, as well as decreased signs of liver damage while mice expressing Y67F-FXR had levels near control empty vector mice. In general $FXR^{-/-}$ mice showed slightly higher levels of the measured signs of liver damage compared to the C57BL/6J model, likely due to the lack of intestinal FXR signaling, but the change in levels were not very significant. This suggests that the major role of FXR tyrosine-67 phosphorylation may be in the liver, although an intestinal specific reconstitution would be required to prove this.

3.6 Figures

Figure 3.1

A)



B)

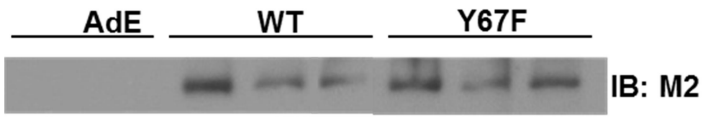
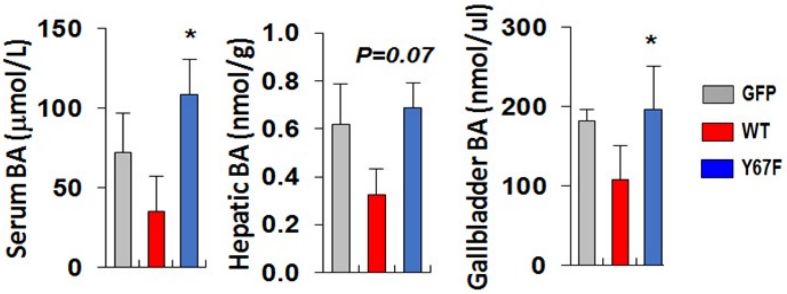


Figure 3.1: Disruption of the FXR tyrosine-67 phosphorylation site decreases expression of FXR target genes *in vivo*. A) FXR $^{-/-}$ mice were infected with either empty adenoviral vector (GFP), or adenoviral vectors expressing wild type FXR or the phosphorylation deficient Y67F-FXR. Mice were then fed CA supplemented diet for 3 hours. RNA was then isolated from the liver of these mice and cDNA was made. Expression of various FXR target genes was measured by qPCR analysis. B) Expression levels of FXR were measured via western blot. N=3. This work was done by Daniel Ryerson.

Figure 3.2

A)



B)

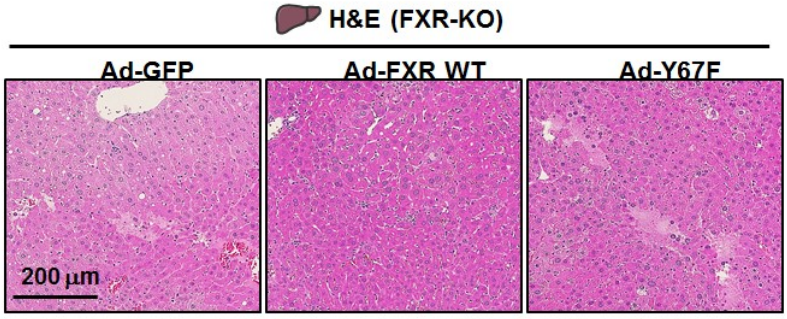
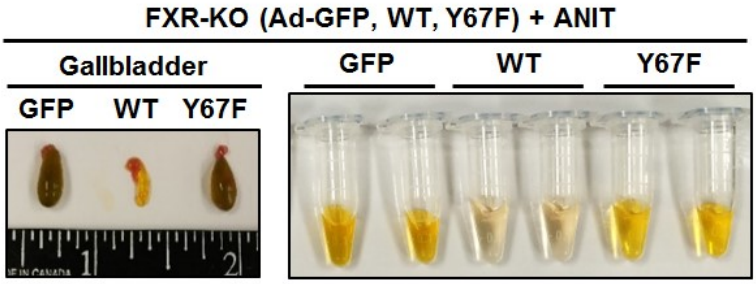


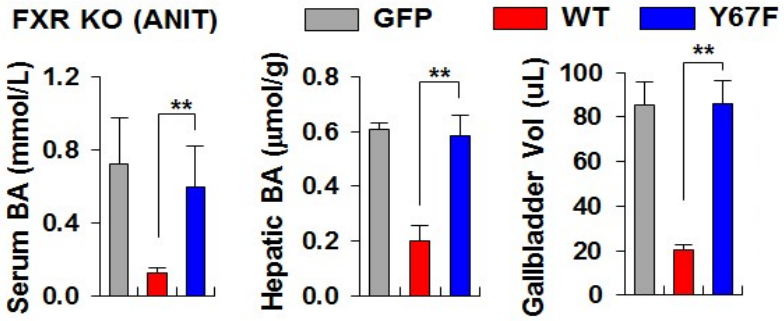
Figure 3.2: Expression of Y67F-FXR is unable to rescue elevated levels of bile acids, AST, ALT and signs of liver damage observed in FXR$^{-/-}$ mice. FXR$^{-/-}$ mice were infected with either empty vector, wild type FXR or Y67F-FXR for 12 days. Mice were then fasted for 10 hours and fed a diet supplemented with CA for 3 hours. A) Bile acids were then extracted from several tissues as described in Methods and bile acids were measured with a bile acid kit (Trinity Biotech, plc) according to the manufacturer's directions. Gallbladder contents were measured directly with the kit. B) Liver sections were stained with H&E. N=3 This work was done by Daniel Ryerson with help from Dong Hyun Kim.

Figure 3.3

A)



B)



C)

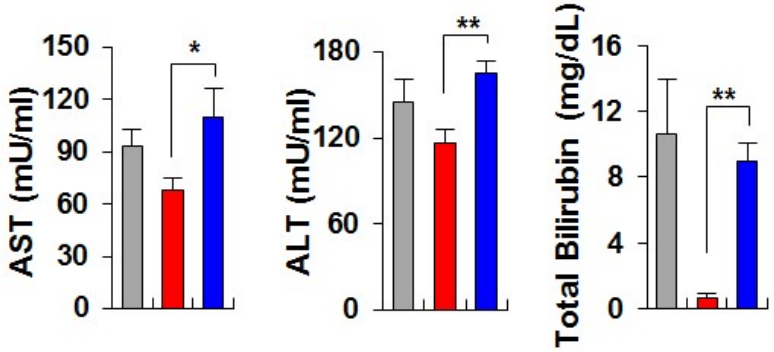


Figure 3.3 (cont.)

D)

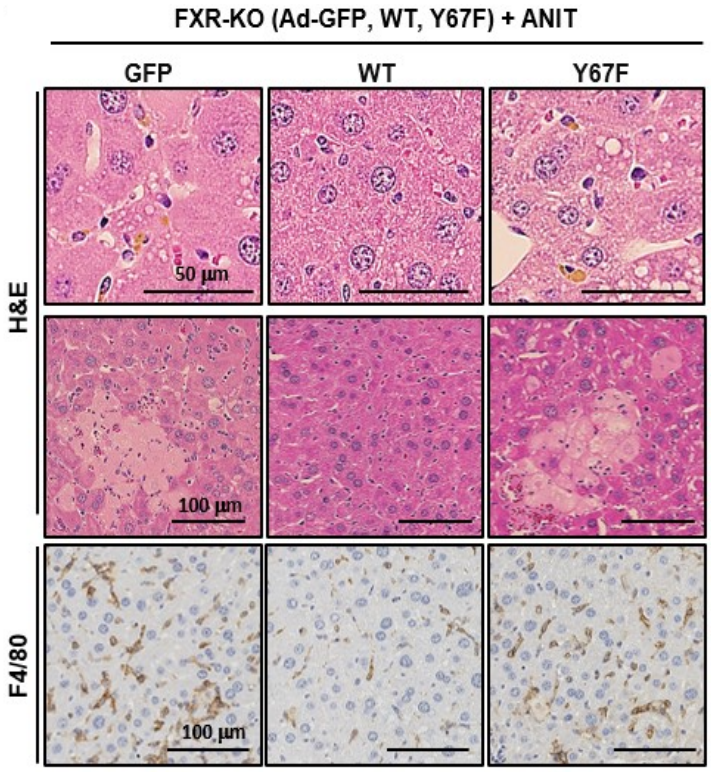
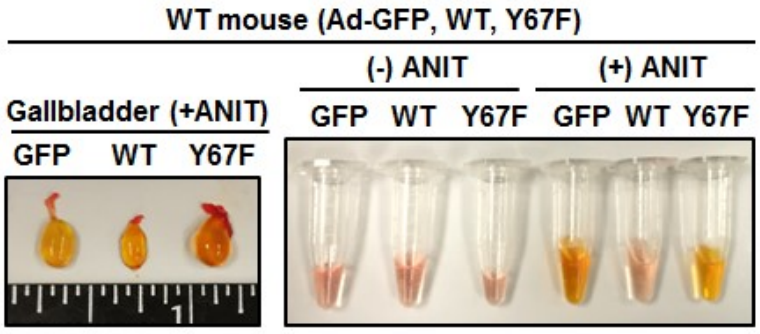


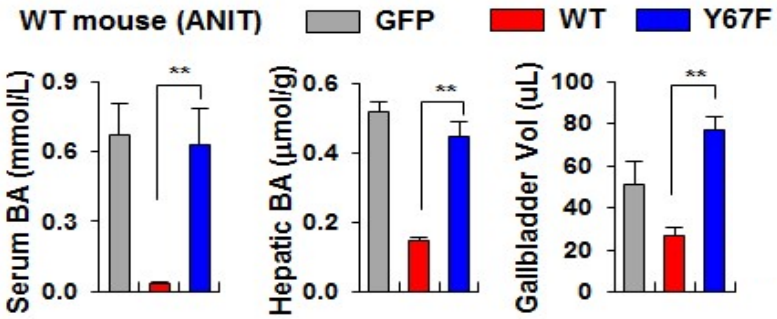
Figure 3.3: Mice expressing Y67F-FXR show impaired ability to protect the liver from cholestatic challenge. $FXR^{-/-}$ mice were infected with either empty vector (GFP) wild type FXR or Y67F-FXR for 12 days. These mice were then treated with ANIT for 2 days. A) Gallbladder and serum were collected and pictures were taken showing observable differences in size and color. B) Bile acids levels were measured as described in Methods using a bile acid kit (Trinity Biotech, plc) according to manufacturer's directions. C) ALT/AST/Bilirubin levels were measured using a kit (Sigma, Corp.) according to the manufacturer's instructions. D) Macrophage infiltration in the liver detected with a kit (Millipore, Inc). This work was done by Dong Hyun Kim with help from Daniel Ryerson.

Figure 3.4

A)



B)



C)

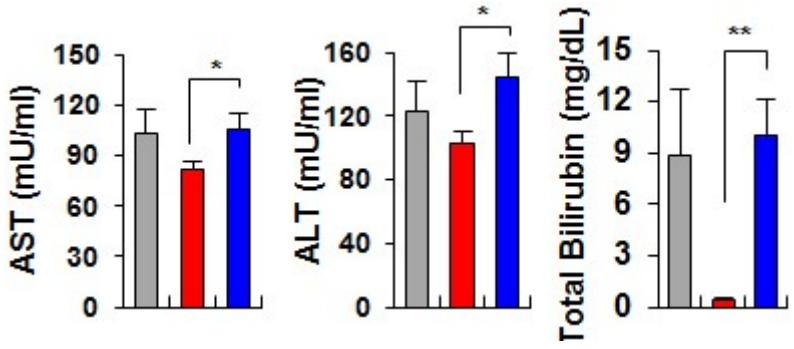


Figure 3.4 (cont.)

D)

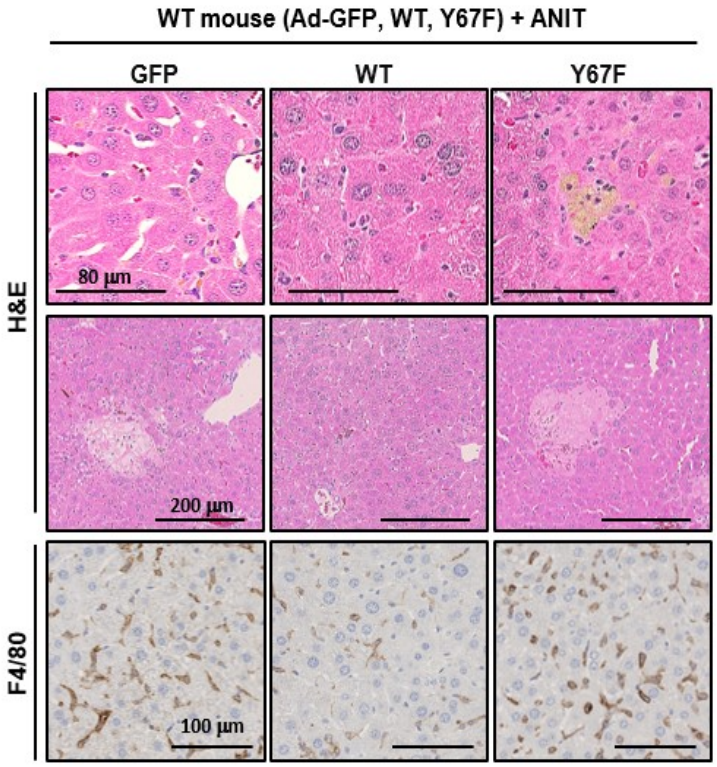
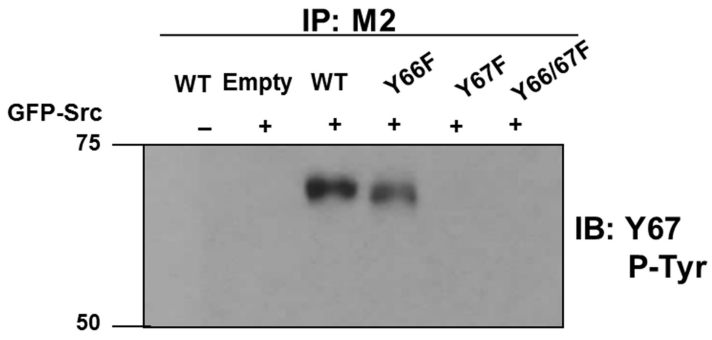


Figure 3.4: Wild type mice overexpressing expressing Y67F-FXR show impaired ability to protect the liver from cholestatic challenge. C57BL6/J mice were infected with either empty vector (GFP), wild type FXR or Y67F-FXR for 12 days. These mice were then treated with ANIT from 2 days. A) Gallbladder and serum were collected and pictures were taken showing observable differences in size and color. B) Bile acid levels measured as described in Methods using a bile acid kit (Trinity Biotech, plc). C) ALT/AST/Bilirubin levels measured using a kit (Sigma, Corp) according to the manufacturer's instructions. D) Macrophage infiltration in the liver detected with a kit (Millipore, Inc). This work was done by Dong Hyun Kim with help from Daniel Ryerson.

Figure 3.5

A)



B)

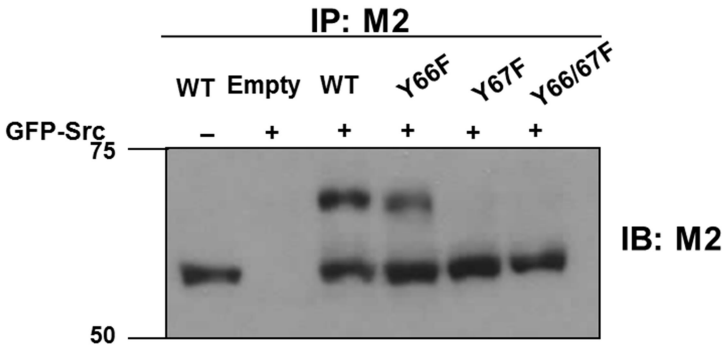


Figure 3.5: Measuring the specificity of the FXR tyrosine-67 site specific phosphorylation antibody. COS-1 cells were co-transfected with Src and either an empty vector, a plasmid expressing wild type FXR, or a plasmid expressing a FXR mutant in which tyrosine-66, tyrosine-67 or both tyrosine-66 and 67 have been mutated to a phenylalanine. A) FXR was then immunoprecipitated and tyrosine-67 phosphorylation levels were measured via western blot with site specific phosphorylation antibody. B) The membrane was then stripped and FXR levels were then measured via western blot. This work was completed by Dong Hyun Kim with help from Daniel Ryerson.

Figure 3.6

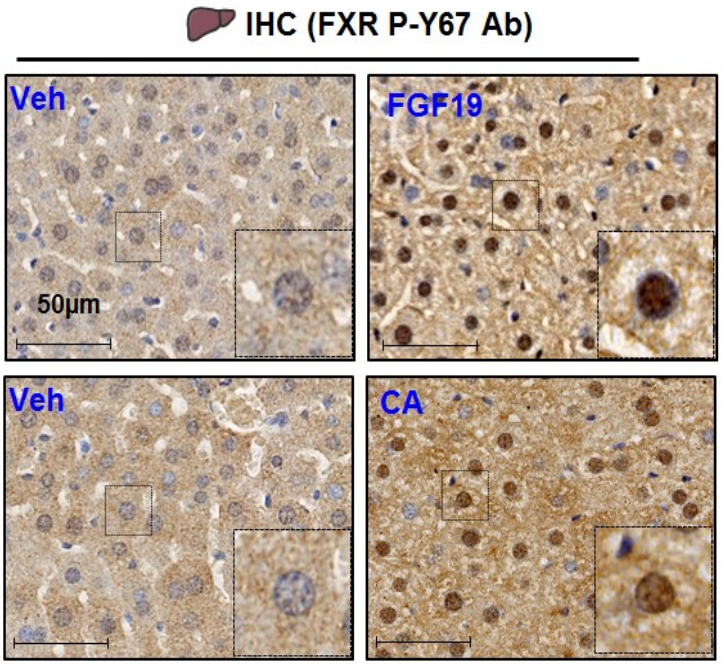


Figure 3.6: Measurement of FXR phosphorylation with tyrosine-67 site specific phosphorylation antibody *in vivo*. C57BL/6J mice were fed a diet supplemented with CA or treated with FGF19. Liver sections were obtained from the University of Illinois at Urbana-Champaign core facility Vibratome. Tyrosine phosphorylation levels of FXR in liver sections were determined through immunohistochemistry utilizing a FXR tyrosine-67 phosphorylation antibody. This work was completed by Sangwon Byun with help from Daniel Ryerson and Dong Hyun Kim.

3.7 References

Bauer S, Buchanan S, Ryan I. (2016) Tyrosine Kinase Inhibitors for the Treatment of Chronic-Phase Chronic Myeloid Leukemia: Long-Term Patient Care and Management. J Adv Pract Oncol. 7(1):42-54.

Bonvin A, Mesnil A, Nicolini FE, Cotte L, Michallet M, Descotes J, Vial T. (2008) Dasatinib-induced acute hepatitis. Leuk Lymphoma. 49(8):1630-2.

Degirolamo C, Modica S, Vacca M, Di Tullio G, Morgano A, D'Orazio A, Kannisto K, Parini P, Moschetta A. (2015) Prevention of spontaneous hepatocarcinogenesis in farnesoid X receptor-null mice by intestinal-specific farnesoid X receptor reactivation. Hepatology 61:161-70.

Doan V, Wang A, Prescott H. (2015) Bosutinib for the treatment of chronic myeloid leukemia. Am J Health Syst Pharm. 72(6):439-47.

Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, Zimmermann J, Lydon NB. (1996) Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med 2:561–566.

El-Serag HB and Rudolph KL. (2007) Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology. 132(7):2557–76.

Gadaleta RM, Oldenburg B, Willemsen EC, Spit M, Murzilli S, Salvatore L, Klomp LW, Siersema PD, van Erpecum KJ, van Mil SW. (2011)
Activation of bile salt nuclear receptor FXR is repressed by pro-inflammatory cytokines activating NF-κB signaling in the intestine. Biochim Biophys Acta. 1812(8):851-8.

Gomez-Ospina N, Potter CJ, Xiao R, Manickam K, Kim MS, Kim KH, Shneider BL, Picarsic JL, Jacobson TA, Zhang J, He W, Liu P, Knisely AS, Finegold MJ, Muzny DM, Boerwinkle E, Lupski JR, Plon SE, Gibbs RA, Eng CM, Yang Y, Washington GC, Porteus MH, Berquist WE, Kambham N, Singh RJ, Xia F, Enns GM, Moore DD. (2016) Mutations in the nuclear bile acid receptor FXR cause progressive familial intrahepatic cholestasis. Nat Commun. 7:10713.

Han MS, Chung KW, Cheon HG, Rhee SD, Yoon CH, Lee MK, Kim KW, Lee MS. (2009) Imatinib mesylate reduces endoplasmic reticulum stress and induces remission of diabetes in db/db mice. Diabetes. 58(2):329-36.

Harrison SA, Torgerson S, Hayashi PH. (2003) The natural history of nonalcoholic fatty liver disease: a clinical histopathological study. Am J Gastroenterol. 98(9):
2042–7.

Heni M, Wagner R, Ketterer C, Böhm A, Linder K, Machicao F, Machann J, Schick F, Hennige AM, Stefan N, Häring HU, Fritsche A, Staiger H. (2013) Genetic variation in

NR1H4 encoding the bile acid receptor FXR determines fasting glucose and free fatty acid levels in humans. J Clin Endocrinol Metab 98(7):E1224-9.

Hu M, Fok BS, Wo SK, Lee VH, Zuo Z, Tomlinson B. (2016) Effect of common polymorphisms of the farnesoid X receptor and bile acid transporters on the pharmacokinetics of ursodeoxycholic acid. Clin Exp Pharmacol Physiol 43(1):34-40.

Kim DH, Xiao Z, Kwon S, Sun X, Ryerson D, Tkac D, Ma P, Wu SY, Chiang CM, Zhou E, Xu HE, Palvimo JJ, Chen LF, Kemper B, Kemper JK. (2015) A dysregulated acetyl/SUMO switch of FXR promotes hepatic inflammation in obesity. EMBO J. 34(2):184-99.

Kim DH, Kwon S, Byun S, Xiao Z, Park S, Wu SY, Chiang CM, Kemper B, Kemper JK. (2016) Critical role of RanBP2-mediated SUMOylation of Small Heterodimer Partner in maintaining bile acid homeostasis. Nat Commun. 7:12179.

Kok T, Hulzebos CV, Wolters H, Havinga R, Agellon LB, Stellaard F, Shan B, Schwarz M, Kuipers F. (2003) Enterohepatic circulation of bile salts in farnesoid X receptor-deficient mice: efficient intestinal bile salt absorption in the absence of ileal bile acid-binding protein. J Biol Chem. 278(43):41930-7.

Kuo WL, Yu MC, Lee JF, Tsai CN, Chen TC, Chen MF. (2012) Imatinib mesylate improves liver regeneration and attenuates liver fibrogenesis in CCL4-treated mice. J Gastrointest Surg. 16(2):361-9.

Lien F, Berthier A, Bouchaert E, Gheeraert C, Alexandre J, Porez G, Prawitt J, Dehondt H, Ploton M, Colin S, Lucas A, Patrice A, Pattou F, Diemer H, Van Dorsselaer A, Rachez C, Kamilic J, Groen AK, Staels B, Lefebvre P. (2014) Metformin interferes with bile acid homeostasis through AMPK-FXR crosstalk. J Clin Invest.124(3):1037-51.

Nijmeijer RM, Schaap FG, Smits AJ, Kremer AE, Akkermans LM, Kroese AB, Rijkers GT, Schipper ME, Verheem A, Wijmenga C, Gooszen HG, van Erpecum KJ. (2014) Impact of global Fxr deficiency on experimental acute pancreatitis and genetic variation in the FXR locus in human acute pancreatitis. PLoS One 9(12):e114393.

Ono K, Suzushima H, Watanabe Y, Kikukawa Y, Shimomura T, Furukawa N, Kawaguchi T, Araki E. (2012) Rapid amelioration of hyperglycemia facilitated by dasatinib in a chronic myeloid leukemia patient with type 2 diabetes mellitus. Intern Med. 51(19):2763-6.

Plaa GL, Priestly BG. (1976) Intrahepatic cholestasis induced by drugs and chemicals. Pharmacol Rev. 28(3):207-73.

Ridruejo E, Cacchione R, Villamil AG, Marciano S, Gadano AC, Mandó OG. (2007) Imatinib-induced fatal acute liver failure. World J Gastroenterol. 13(48):6608-111.

Rinella ME, Loomba R, Caldwell SH, Kowdley K, Charlton M, Tetri B, Harrison SA. (2014) Controversies in the diagnosis and management of NAFLD and NASH. Gastroenterol Hepatol. 10(4):219–27.

Roskoski R Jr. (2015) Src protein-tyrosine kinase structure, mechanism, and small molecule inhibitors. Pharmacol Res. 94:9-25.

Shamroe CL, Comeau JM. (2013) Ponatinib: A new tyrosine kinase inhibitor for the treatment of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia. Ann Pharmacother. 47(11):1540-6.

Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. (2000) Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. Cell. 102(6):731-44.

Tonyali O, Coskun U, Yildiz R, Karakan T, Demirci U, Akyurek N, Benekli M, Buyukberber S. (2010) Imatinib mesylate-induced acute liver failure in a patient with gastrointestinal stromal tumors. Med Oncol. 27(3):768-73.

Williams CD, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M, Landt CL, Harrison SA. (2011) Prevalence of nonalcoholic fatty liver disease and nonalcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. Gastroenterology. 140(1):124–31.

Yang F, Huang X, Yi T, Yen Y, Moore DD, Huang W. (2007) Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. Cancer Res 67:863- 867

Chapter Four

Future Directions

These studies have identified an exciting new post-translational modification of FXR, tyrosine-67 phosphorylation, which plays a critical role in FXR regulation of bile acid homeostasis and liver protection. We have identified the site, the kinase, and the physiological signaling responsible for this phosphorylation. Additionally, we have demonstrated that loss of FXR tyrosine-67 phosphorylation results in impaired bile acid homeostasis and liver damage *in vivo*. Going forward there are several experiments that may be done to continue pushing this project forward.

First, our lab has obtained a FXR-floxed mouse model generously provided by Dr. Sayeepriyadarshini Anakk from the Department of Molecular and Integrative Physiology at University of Illinois at Urbana-Champaign. This model was originally generated by Johan Auwerx and Kristina Schoonjans at Ecole Polytechnique. Utilizing this model, work is currently underway utilizing a coinjection of adenoviral associated virus (AAV) expressing thyroxine-binding globulin (TBG) promoter driven Cre and AAV expressing TBG promoter driven wildtype or Y67F-FXR. TBG is a liver specific promoter allowing AAV TBG driven Cre to induce a knockout of endogenous FXR only in the liver. AAV TBG driven wildtype or Y67F-FXR then allows liver specific reconstitution. We will utilize this model to replicate our previous *in vivo* data. This model will improve upon our current models as it does not have the confounding endogenous FXR expression in the liver that our overexpression model does, while still maintaining normal FXR function in the intestine and other tissues which the $FXR^{-/-}$ mice were lacking. Additionally, utilizing AAV containing other tissue specific promotors it is possible to further explore the roles of FXR tyrosine-67 phosphorylation in many diverse tissues.

Our lab has recently obtained human liver samples from patients with a variety of hepatobiliary diseases including primary biliary cirrhosis, nonalcoholic steatohepatitis, and nonalcoholic fatty liver disease. These samples obtained from the Liver Tissue Cell Procurement and Distribution System of NIH will be analyzed through immunohistochemistry for total FXR levels, FXR tyrosine-67 phosphorylation levels, total Src levels and phospho-Src. These finding will allow us to better understand the roles FXR tyrosine-67 phosphorylation and Src signaling play in human disease progression.

Finally, several studies have shown that Src inhibitors can be used *in vivo* to treat a number of metabolic disorders. In the future it will be interesting to utilize these inhibitors along with FXR tyrosine-67 mutants to determine if the beneficial effects seen are related to FXR tyrosine-67 phosphorylation by Src.

In summary, these studies have identified a previously unknown type of post-translational modification of FXR, in tyrosine-67 phosphorylation. Utilizing a combination of bioinformatic tools and biochemical assays, we identified likely kinase motifs on FXR and demonstrated that one of the predicted kinases, Src, can interact with FXR both *in vitro* and *in vivo* in response to bile acid signaling. We have shown that Src is both necessary and sufficient to phosphorylate FXR. We have also demonstrated that when the tyrosine-67 phosphorylation site is mutated FXR can no longer regulate a number of key target genes important for maintaining bile acid homeostasis. This disruption of FXR regulation leads to observable physiological changes *in vivo,* demonstrated by increased levels of bile acids and liver enzymes, inflammation, and tissue damage of the liver. We have shown that the tyrosine-67 phosphorylation site plays a major role in regulating FXR function and understanding how this modification occurs and how it regulates FXR may prove key to understanding and developing treatments for human liver diseases.