ROLE OF BILE ACIDS AND FARNESOID X RECEPTOR IN HEPATIC AUTOPHAGY AND ITS IMPLICATIONS IN ETHANOL-INDUCED HEPATOTOXICITY

BY

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Submitted to the graduate degree program in Pharmacology, Toxicology & Therapeutics and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Retention of bile acids (BAs) in the liver during cholestasis plays an important role in the development of cholestatic liver injury. Several studies have reported that high concentrations of certain BAs induce cell death and inflammatory response in the liver, and BAs may promote liver tumorigenesis. Macroautophagy (hereafter referred to as autophagy) is a lysosomal degradation process that regulates organelle and protein homeostasis and serves as a cell survival mechanism under a variety of stress conditions. However, it is not known if BAs modulate autophagy in hepatocytes. In the present study, we determined autophagic flux in livers of farnesoid X receptor (FXR) knockout (KO) mice that have increased concentrations of hepatic BAs and in primary cultured mouse hepatocytes that were treated with BAs. The results showed that autophagic flux was impaired in livers of FXR KO mice and in BA-treated primary mouse hepatocytes. Mechanistically, BAs did not affect the activities of cathepsin or the proteasome, but impaired autophagosomal-lysosomal fusion likely due to reduction of Rab7 protein expression and targeting to autophagosomes. In conclusion, BAs suppress autophagic flux in hepatocytes by impairing autophagosomal-lysosomal fusion, which may be implicated in bile acid-induced liver tumor promotion observed in FXR KO mice.

Alcoholic liver disease encompasses a wide spectrum of pathogenesis including steatosis, fibrosis, cirrhosis, and alcoholic steatohepatitis. Acute alcohol treatment induces autophagy via FoxO3a-mediated autophagy related gene expression and protects against alcohol-induced steatosis and liver injury in mice. Moreover, inhibition of autophagy by pharmacological approach or deletion of autophagy genes exacerbates

alcohol-induced steatosis and hepatotoxicity. Because we found that FXR KO mice had impaired hepatic autophagy and the role of FXR in ethanol hepatotoxicity is not known, we thus determined the hepatotoxicity and its mechanisms induced by acute ethanol treatment in FXR KO mice. In the present study, wild type and FXR KO mice were treated with acute ethanol for 16 hours. We found that ethanol treated-FXR KO mice had exacerbated hepatotoxicity and steatosis compared to wild type mice. Furthermore, we found that ethanol treatment had decreased expression of various essential autophagy genes and several other FoxO3a target genes in FXR KO mice compared with wild type mice. Mechanistically, we did not find a direct interaction between FXR and FoxO3a. Ethanol-treated FXR KO mice had increased Akt activation, increased phosphorylation of FoxO3a resulting in decreased FoxO3a nuclear retention and DNA binding. Furthermore, ethanol treatment induced hepatic mitochondrial spheroid formation in FXR KO mice, but not in wild type mice, which may serve as a compensatory alternative pathway to remove ethanol-induced damaged mitochondria in FXR KO mice. Moreover, induction of FXR with WAY-362450 protected against acute ethanol-induced steatosis, but not hepatotoxicity. These results suggest that lack of FXR impaired FoxO3a-mediated autophagy and in turn exacerbated alcohol-induced liver injury.

In conclusion, this dissertation provided novel insights in how two different liver pathologies may cross talk. We demonstrated that the increased hepatic bile acid levels lead to impaired autophagy through the inhibition of autophagosomal-lysosomal fusion. Moreover, FXR deficiency exacerbated alcohol induced hepatotoxicity and steatosis likely by two mechanisms: bile acid-mediated inhibition of autophagy and Akt-mediated

repression of ethanol-induced FoxO3a activation. This dissertation presented novel therapeutic targets for both cholestasic liver injury and alcoholic liver disease. Autophagy and FXR are possible therapeutic targets for cholestasis, whereas, FXR and FoxO3a emerge as novel therapeutic targets for alcoholic liver disease.

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To Dapple and Smoke, woof, woof, woof.

Dedicated to

My loved ones who departed from life too soon...

Sister, Ruth Maria Manteufel
March 7, 1986 – October 18, 1995

Father-in-law, Larry Edwin Werth
October 7, 1947 – January 26, 2008

TABLE OF CONTENTS

List of Abbreviations:

ABST: apical sodium-dependent bile acid transporter; ADH1: class I alcohol dehydrogenase; AF-1: ligand-independent transcriptional activation function; AF-2: ligand-dependent activation function; AICAR: adenosine, 5-amino-4-imidazole carboxamide riboside; ALT: alanine aminotransferase; ALD: alcoholic liver disease; Apo: apolipoprotein; AMPK: AMP-activated protein kinase; ANIT: α-naphthylisothiocyanate; aPKC: atypical protein kinase C; AST: aspartate aminotransferase; ATF6: activating transcription factor 6; BAAT: bile acid CoA amino acid N-acyltransferase; BACS: bile acid CoA synthase; BAs: bile acids; BDL: bile duct ligation; Bip: binding immunoglobulin protein; BSA: bovine serum albumin; Bsep: bile salt exporting pump; CA: cholic acid; CAR: constitutive androgen receptor; CBP: cAMP response element-binding-binding protein; CDCA: chenodeoxycholic acid; CHOP: CCAAT-enhancer-binding protein homologous protein; CK1: casein kinase 1; CQ: chloroquine; CYP: cytochrome P450; DCA: deoxycholic acid; DQ-BSA: DQ- bovine serum albumin; E1: ubiquitin-activating enzyme; E2: ubiquitin carrier; E3: ubiquitin ligase; 4EBP1: translational initiation factor 4E binding protein-1; EBSS: Earle's Balanced Salt Solution; 6ECDCA: 6α-ethyl-chenodeoxycholic acid; eIF2-α: eukaryotic initiation factor 2-alpha; ER: endoplasmic reticulum; ERK: extracellular-signal-regulated kinase; FAS: fatty acid synthase; FFA: free fatty acids; FGF 15/19: fibroblast growth factor 15/19; Fgfr4: fibroblast growth factor receptor 4; FoxO3a: forkhead box O3a; FXR: farnesoid X receptor; GAP: GTPase-activating protein; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GFP: green fluorescent protein; GSH: glutathione; GSK3β: glycogen synthase kinase beta; HCC: hepatocellular carcinoma; HEK: human embryonic kidney; HNF4: hepatocyte nuclear factor-4; HOPS: homotypic fusion and

vacuole protein sorting; IKKβ: inhibitor of nuclear factor kappa B kinase subunit beta; IL-22: interleukin-22; IP_3: inositol 1,4,5-triphosphate; IR-1: inverted repeat-1; K: lysine; Keap1: kelch-like ECH-associated protein 1; KIR: Keap1 interacting domain; KO: knockout; LC3: microtubule associated protein light chain 3; LAMP-1*: lysosome* associated membrane protein-1; LAMP-2: lysosome associated membrane protein-2; LIR: LC3 interacting region; LRH-1: liver receptor homolog-1; 3-MA: 3-methyladenine; Map1lc3b: microtubule associated protein 1 light chain 3 beta; MDB: Mallory-Denk body; MDM2: murine double minute 2; Mfn1: mitofusin-1; Mfn2: mitofusin-2; MnSOD: manganese superoxide dismutase; mTOR: mammalian target of rapamycin; mTORC: mTOR complex; NES: nuclear export sequence; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NLS: nuclear localization sequence; Nrf2: nuclear factor (erythroid-derived 2)-like 2; NTCP: sodium-taurocholate cotransporter protein; OATP: organic anion transporting polypeptide; OST: organic solute transporter; p70S6K: 70 kDa ribosomal protein S6 kinase; PAS: phagophore assembly site; PBC: primary biliary cirrhosis; PCAF: p300/CBP-associated factor; PI-3-P: phosphatidylinositol-3-phosphate; PE: -phosphatidylethanolamine; PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PI3-K: phosphoinositide 3-kinase complex; PPA2: protein phosphatase 2; PPAR: peroxisome proliferator-activated receptor; PSC: primary sclerosing cholangitis; PTEN: phosphatase and tensin homolog; PXR: pregnane X receptor; RAR: retinoic acid receptor; RFP: red fluorescent protein; RIP: receptor interacting protein; Rubicon: run domain protein as Beclin-1 interacting and cysteine rich containing; RXRα: retinoid X receptor alpha; S: serine; Sirt1: sirtuin-1; SHP: small heterodimer partner; Skp2: S-phase kinase-associated protein 2; SNARE:

soluble n-ethylmaleimide-sensitive factor attachment protein receptor; SQSTM1: sequestosome 1; SREBP1: sterol regulatory element-binding protein 1; T: threonine; TCA: Taurocholic acid; TRAF6: tumor necrosis factor associated receptor-6; TSC: tuberous sclerosis protein; UBA: ubiquitin associated domain; UDCA: ursodeoxycholic acid; ULK1: UNC-51 like kinase; UVRAG: UV radiation resistance-associated gene protein; VAMP7: vesicle-associated membrane protein 7; VPS34: vacuolar protein sorting 34; Vti1B: vesicle t-SNARE-interacting protein homologous 1B; WAY: WAY-362450; WT: wild type; XBP1: x-box binding protein 1.

CHAPTER 1:

INTRODUCTION

The portions of this section are reproduced from following publication with permission of the publisher:

1. Manley S, Williams JA, Ding WX. Role of p62/SQSTM1 in liver physiology and pathogenesis. Exp Biol Med (Maywood). 2013;238(5):525-38.

2. Ding WX, Manley S, Ni HM. The emerging role of autophagy in alcoholic liver disease. Exp Biol Med (Maywood). 2011;236(5):546-56.

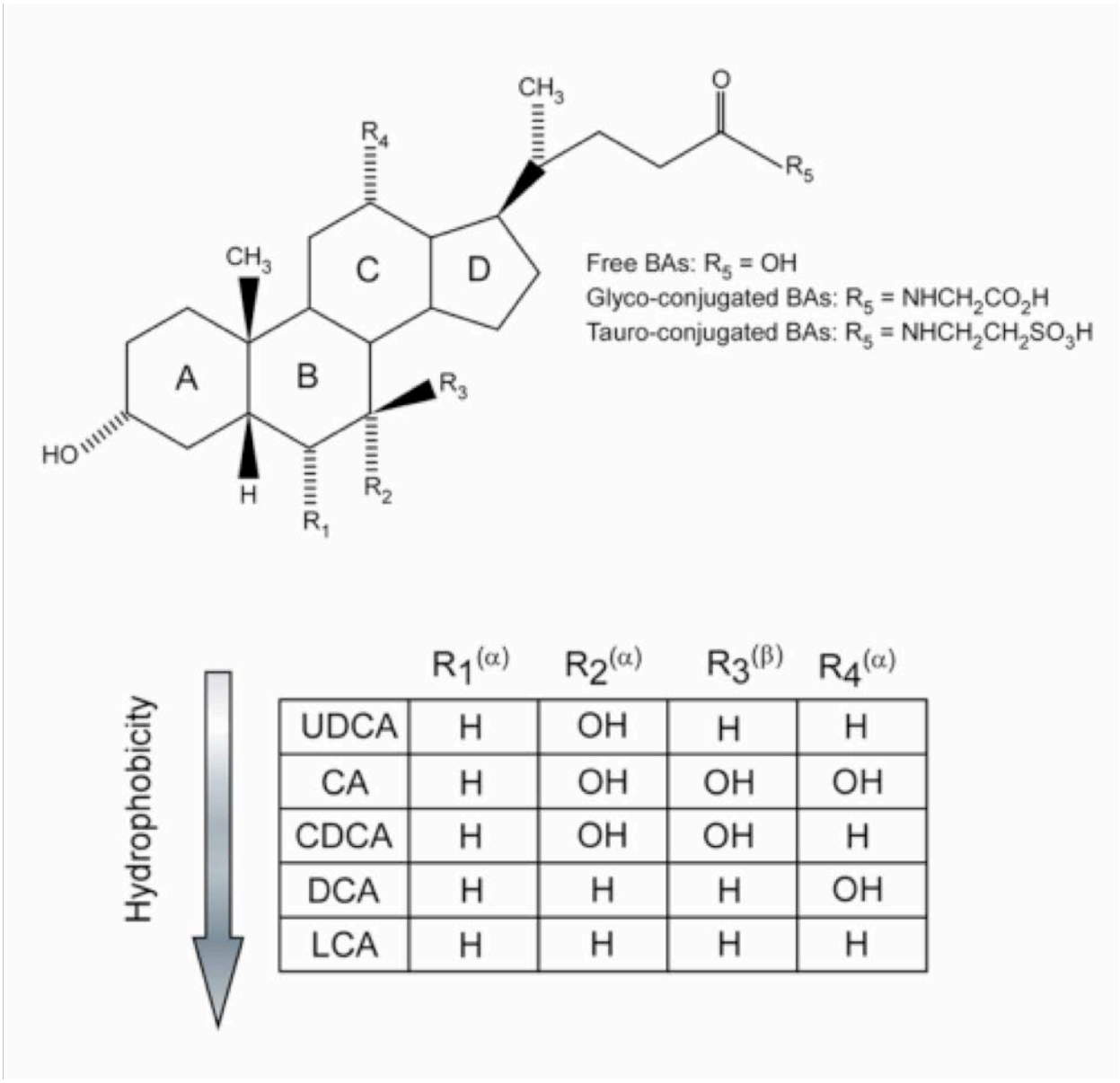
1.1 Physiological and pathological roles of bile acids

Bile acids are amphipathic detergent-like molecules synthesized as end products of cholesterol catabolism in hepatocytes through multiple enzymatic steps, and are classified as either primary bile acids, synthesized in the liver, or secondary bile acids, synthesized in the intestines (Modica, Gadaleta et al. 2010). The majority of the circulating bile acids are glycine or taurine conjugated. Bile acids are planar amphipathic molecules with hydrophilic alpha face containing hydroxyl groups and hydrophobic beta face containing no substituents, and the modifications and conjugates on the carbon backbone determine the hydrophobicity and subsequent toxicity **(Figure 1.1)** (Modica, Gadaleta et al. 2010). Moreover, the hydrophobicity index of bile acids is determined using high pressure liquid chromatography (HPLC), but it should be cautioned that the HPLC method is imperfect (Hofmann and Hagey 2008). Nevertheless it is generally accepted that ursodeoxycholic acid (UDCA) is one of the most hydrophilic bile acids and deoxycholic acid (DCA) is one of the most hydrophobic bile acids (Modica, Gadaleta et al. 2010).

Furthermore, the well-known physiological functions of bile acids include hepatic bile formation and absorption of dietary lipids and fat-soluble vitamins (Lefebvre, Cariou et al. 2009). Moreover, bile acids participate in various physiological processes including regulation of their own homeostasis, adaptive responses to cholestasis and other insults to the liver, lipid metabolism, and glucose metabolism (Monte, Marin et al. 2009). Bile acids are synthesized in the liver, stored in the gallbladder, and secreted into the small intestines to facilitate the absorption of lipids and vitamins. When the flow of bile acids from the liver to the duodenum is obstructed, resulting in accumulation of bile

acids in the liver, this condition is know as cholestasis. Recent research and discoveries show that bile acids are not passive players, but are involved with multiple physiological functions, especially glucose and lipid metabolism inside and outside of the liver.

Figure 1.1



	$R_1^{(\alpha)}$	$R_2^{(\alpha)}$	$R_3^{(\beta)}$	$R_4^{(\alpha)}$
UDCA	H	OH	H	H
CA	H	OH	OH	OH
CDCA	H	OH	OH	H
DCA	H	H	H	OH
LCA	H	H	H	H

Bile Acid Structure

Bile acids are synthesized from cholesterol backbone, and hydroxylation of bile acids alters hydrophobicity. UDCA is a hydrophilic primary bear bile acid. CA and CDCA are primary human bile acids. DCA is a hydrophobic secondary bile acid. Bile acids are conjugated prior to transport out of the hepatocytes into the bile canaliculi. Figure is reprinted with permission from Deciphering the Nuclear Bile Acid Receptor FXR Paradigm, Volume 8, Modica S, Gadaleta RM, and Moschetta A, Nuclear Receptor Signaling, e005, Copyright (2010), with permission from the publisher.

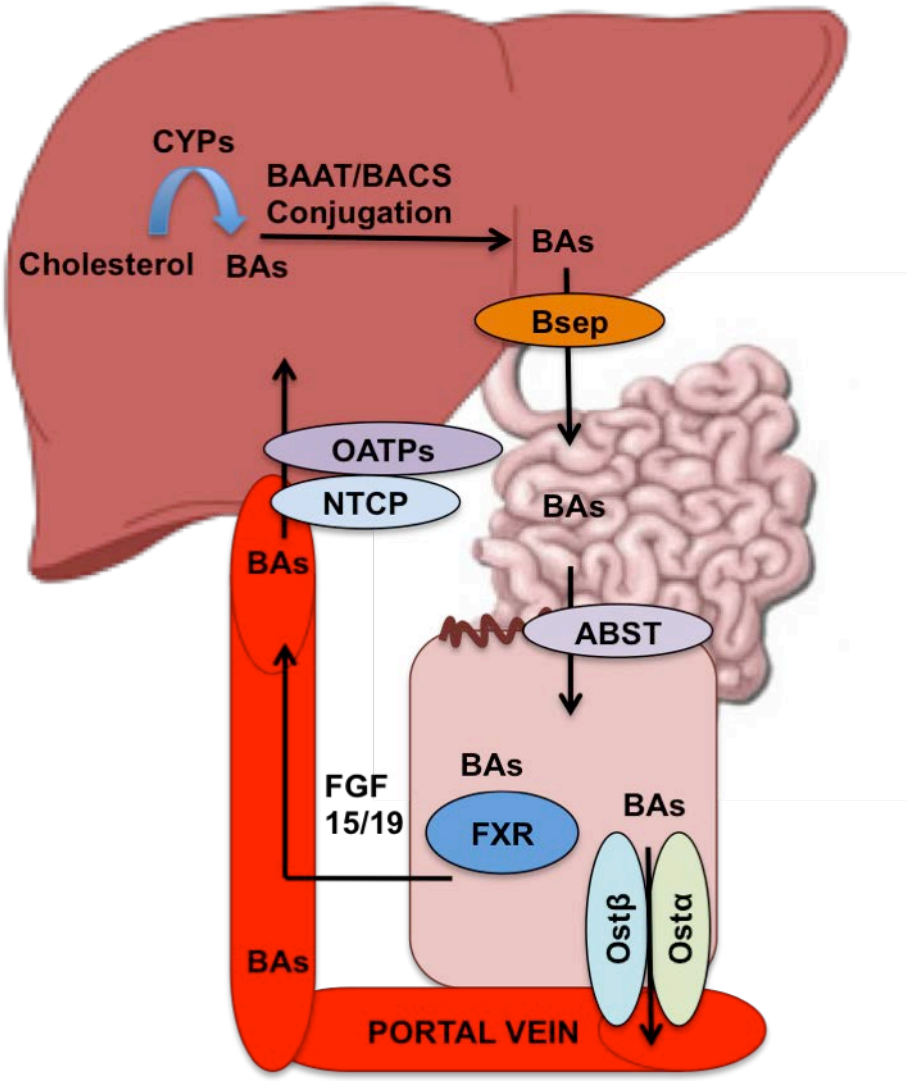
1.2 Bile acids synthesis and enterohepatic circulation

Primary bile acids are the result of a multistep cholesterol catabolism performed by various enzymes including several cytochrome P450s (CYPs). In the classic pathway, cholesterol is first converted into 7-α-hydroxycholesterol by CYP7A1 in the endoplasmic reticulum (ER). 7-α-hydroxycholesterol is then primarily converted to cholic acid (CA) in multistep enzymatic reactions involving CYP8B1 and CYP27A1 (Chiang 1998; Lefebvre, Cariou et al. 2009). In the alternative or acidic pathway when the CYP7A1 level is low, CYP27A1 in the mitochondria converts cholesterol into 27-hydroxycholesterol. Multistep enzymatic reactions involving CYP7B1 modify 27-hydroxycholesterol into chenodeoxycholic acid (CDCA). It has been suggested that classic pathway is the pathway under normal physiological conditions whereas the alternate pathway becomes the major pathway to compensate for low bile acids synthesis in certain liver diseases. CA and CDCA then undergo taurine or glycine conjugation mediated by bile acid CoA amino acid N-acyltransferase (BAAT) and bile acid CoA synthase (BACS) respectively to increase water solubility.

Conjugated bile acids are transported into the bile canaliculi and subsequent bile ducts via the ATP binding cassette transporter ABCB11, also commonly known as bile salt exporting pump (Bsep) (Gerloff, Stieger et al. 1998). Once in the bile ducts, bile acids travel toward the small intestines and are secreted to assist in absorption of fatty acids and water insoluble vitamins. The bile acids are reabsorbed from the ileum by the apical sodium-dependent bile acid transporter (ABST) (Wong, Oelkers et al. 1995). Bile acid receptor, Farnesoid X Receptor (FXR), is then activated in the enterocytes by bile acids and induces fibroblast growth factor 15/19 (FGF15/19) secretion, a hormone

responsible for negative regulation of bile acids synthesis (Schaap, van der Gaag et al. 2009; Gadaleta, van Mil et al. 2010). Organic solute transporters (Ost) that exist in its heterodimeric form containing Ostα and Ostβ transport the bile acids into the portal vein to return them to the liver for recycling (Dawson, Hubbert et al. 2005; Gadaleta, van Mil et al. 2010). Sodium-taurocholate cotransporter protein (NTCP), and organic anion transporting polypeptide (OATP) absorb the bile acids back into the hepatocytes. This cycle is known as enterohepatic circulation, which is responsible for the tight regulation of the total bile acid levels and feedback between the liver and the intestines **(Figure 1.2)**.

Figure 1.2



Enterohepatic Circulation

Bile acids are synthesized from cholesterol through multienzymatic synthesis involving CYPs and are glyco- or tauro-conjugated by BAAT or BACS. Conjugated bile acids are transported into bile canaliculi by Bsep, and are secreted into the small intestines. ABST transports bile acids into enterocytes in the ileum, and FXR is activated by bile acids to induce FGF15/19 production and secretion. Bile acids then are transported into portal vein by Ostα/β and returned to the liver. OATPs and NTCP facilitate the absorption of bile acids into the liver.

1.3 Impaired flow of bile acids results in liver injury

Disruption in the enterohepatic circulation, either inside or outside of the liver results in cholestasis, one of the frequent indicators for liver transplantation (Murray and Carithers 2005). Currently, UDCA, a hydrophilic bile acid, is the only drug approved to treat cholestasis (Paumgartner 2006). Intrahepatic cholestasis occurs when the bile flow is disrupted inside the liver, and extrahepatic cholestasis occurs when disruption of the bile flow occurs outside of the liver. Known etiologies of cholestasis include gallstones, biliary trauma, cystic fibrosis, primary biliary cirrhosis, primary sclerosing cholangitis, tumors, and drugs (e.g. erythromycin and flucloxacillin) (Hirschfield, Heathcote et al. 2010; Padda, Sanchez et al. 2011). Manifestations of cholestasis include hepatic bile acids accumulation, hepatic inflammation, fibrosis and inevitably, cirrhosis (Gadaleta, van Mil et al. 2010). Excessive amount of bile acids is clearly cytotoxic.

At higher concentrations, bile acids exert cytotoxicity due to their detergent-like properties (Lefebvre, Cariou et al. 2009), and cholestasis is accompanied by liver injury including destruction of bile ducts and hepatocyte death. Plasma membrane damage, oxidative stress, mitochondrial dysfunction, apoptosis, and inflammation all are cell injury mechanisms exerted by the cytotoxic nature of bile acids (Perez and Briz 2009; Allen, Jaeschke et al. 2011). Moreover, the cytotoxicity induced by bile acids relies on their hydrophobicity. Hydrophobic bile acids including chenodeoxycholic acid have the ability to enter the lipid membrane and disrupt the structure and function of the membrane and cells leading to the inevitable apoptosis (Billington, Evans et al. 1980; Perez and Briz 2009). However, the mechanism of bile acid-mediated cell death in cholestatic liver disease and whether it is mediated by apoptosis or necrosis is a

controversial issue (Woolbright and Jaeschke 2012). Obstructive cholestasis in bile duct-ligated mice demonstrated increased focal necrosis, increased plasma necrosis biomarkers and a lack of apoptosis plasma biomarkers (Woolbright, Antoine et al. 2013). Nevertheless, it is well established that direct bile acid toxicity *in vitro* result in apoptosis. However, the mechanism of bile acid toxicity *in vivo* needs to be further studied.

Interestingly, hydrophilic bile acids, in particular UDCA, have been shown to be cytoprotective. Indeed, UDCA is used as therapeutic agent for cholestasis and has been suggested to exert three major protective mechanisms including protecting cholangiocytes against hydrophobic bile acids, stimulating hepatobiliary secretion and protecting hepatocytes from bile acids induced apoptosis (Paumgartner and Beuers 2002).

1.4 Farnesoid X Receptor is the master regulator of bile acid homeostasis

FXR, a former orphan nuclear receptor, is the master regulator of bile acid homeostasis, and bile acids, in particular, CDCA, are the endogenous ligands for FXR (Forman, Goode et al. 1995; Seol, Choi et al. 1995; Makishima, Okamoto et al. 1999; Parks, Blanchard et al. 1999; Wang, Chen et al. 1999). Synthetic ligands such as GW4064 also can activate FXR (Maloney, Parks et al. 2000). The n-terminus of FXR contains a ligand-independent transcriptional activation function (AF-1), a DNA-binding domain consisting of two highly conserved zinc finger motifs, and a hinge region that allows simultaneous receptor dimerization and DNA binding (Chawla, Repa et al. 2001). The c-terminus contains a ligand binding domain, a dimerization interface and a ligand-

dependent activation function (AF-2) **(Figure 1.3)** (Chawla, Repa et al. 2001). FXR binds to an inverted repeat-1 (IR-1) response element, an inverted AGGTCA sequence separated by one base pair, as either a monomer or a heterodimer with Retinoid X Receptor α (RXRα) (Lefebvre, Cariou et al. 2009). Furthermore, ligand binding induces FXR-RXR heterodimer to dissociate from co-repressors and recruit co-activators to initiate target gene transcription (Modica, Gadaleta et al. 2010). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is one known co-activator of FXR and is shown to induce FXR activation in a ligand dependent manner by interacting with FXR-RXR heterodimer (Kanaya, Shiraki et al. 2004; Savkur, Thomas et al. 2005; Kanaya and Jingami 2006). Moreover FXR can be regulated by post-translational modifications. Acetylation of FXR by p300 stabilizes the molecule but inactivates FXR interaction with RXR and DNA binding. FXR acetylation can be reversed by sirtuin-1 (Sirt1), a NAD-dependent deacetylase (Kemper, Xiao et al. 2009). FXR is highly expressed in the liver and intestines and regulates the enterohepatic circulation of the bile acids (Forman, Goode et al. 1995).

Figure 1.3

Structure of Farnesoid X Receptor.

FXR contains five regions: a ligand-independent activation factor (AF1), a DNA-binding domain, a hinge domain, a ligand-binding domain, and a ligand-dependent activation factor (AF2).

FXR is involved with bile acids homeostasis essentially through transcriptional regulation of genes involved with bile acids synthesis and transport. In the liver, FXR regulates bile acid levels by activating small heterodimer partner (SHP), a co-repressor, which then interacts with liver receptor homolog-1 (LRH-1) to inhibit gene transcription of bile acid synthesis enzymes, CYP7A1 and CYP8B1. The decrease in bile acid synthesis enzymes subsequently down regulates bile acid synthesis (Goodwin, Jones et al. 2000; Lee, Lee et al. 2006; Sanyal, Bavner et al. 2007). FXR also regulates bile acids synthesis through a second mechanism in the intestines. Activation of FXR by bile acids induces FGF15/19 secretion from the intestines (Inagaki, Choi et al. 2005). Activated and released FGF15/19 then travels from the intestines to the liver by portal circulation and binds to fibroblast growth factor receptor 4 (Fgfr4) in liver to suppress the transcription of *Cyp7a1* and subsequent inhibition of bile acid synthesis (Lefebvre, Cariou et al. 2009). Indeed, FGF15/19 is the key regulator of bile acid synthesis through intestinal FXR activation (Kong, Wang et al. 2012). Whole body FXR knockout (KO) mice displayed elevated bile acids due to increased synthesis (Sinal, Tohkin et al. 2000; Kim, Ahn et al. 2007), whereas hepatocyte specific FXR KO mice displayed normal levels of bile acids (Kim, Ahn et al. 2007; Borude, Edwards et al. 2012).

FXR deficiency leads to spontaneous liver tumorigenesis including hepatocellular carcinoma (HCC), adenoma and hepatocholangiocellular carcinoma (Kim, Morimura et al. 2007; Yang, Huang et al. 2007). Indeed, FXR and its target gene, SHP, are downregulated in human HCC samples (Takahara, Takahashi et al. 2008; Wolfe, Thomas et al. 2011; Liu, Meng et al. 2012; Su, Ma et al. 2012). Therefore, FXR is

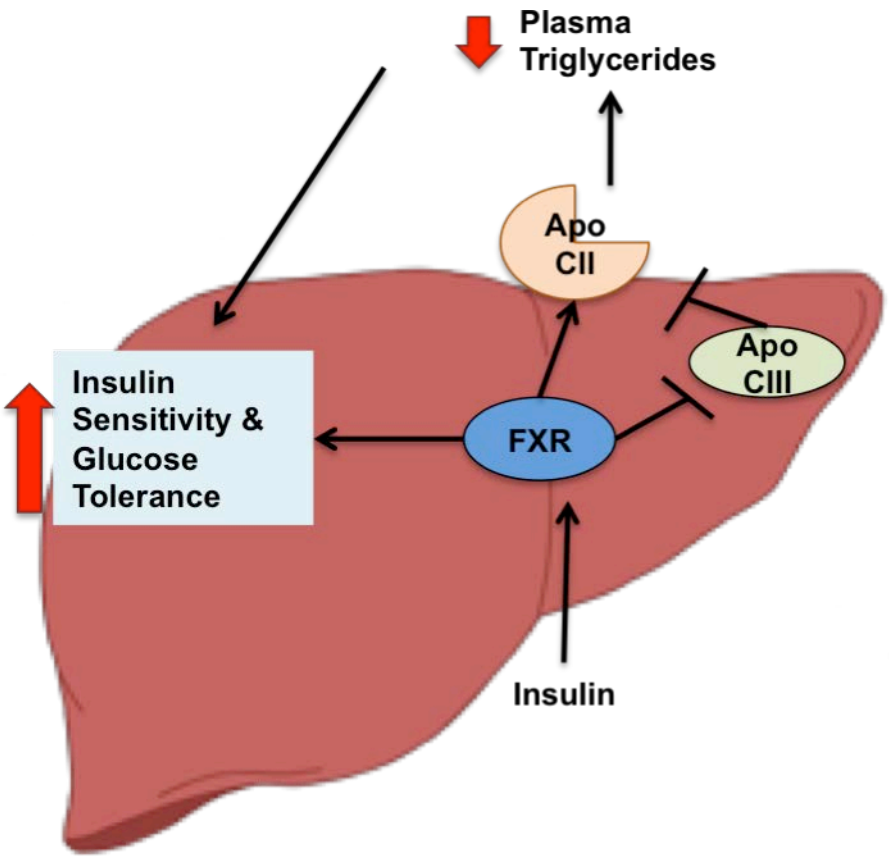
suspected to be a tumor suppressor in the liver. However, it is currently not clear how elevated bile acid levels and FXR deficiency leads to liver tumorigenesis.

1.5 FXR is a modulator of lipid and glucose metabolism

FXR has been reported to regulate pathways involved with lipid, glucose and energy metabolism. FXR lowers hepatic triglycerides via SHP-mediated inhibition of sterol regulatory binding protein 1 (SREBP1)-mediated hepatic lipogenesis (Watanabe, Houten et al. 2004). Additionally, FXR decreases plasma triglycerides by increasing apolipoprotein CII (apoCII), a co-activator of lipoprotein lipase (Kast, Nguyen et al. 2001), and inhibiting apoCIII, a co-inhibitor of lipase, thus FXR activation promotes lipase-mediated plasma triglyceride clearance (Claudel, Inoue et al. 2003).

FXR also plays a role in glucose metabolism by cross-talking with insulin. Insulin has been shown to regulate FXR, however, FXR is also involved in improving insulin sensitivity. FXR deficient mice exhibited decreased insulin sensitivity and impaired glucose tolerance (Cariou, van Harmelen et al. 2006; Ma, Saha et al. 2006; Zhang, Lee et al. 2006), whereas, pharmacological activation or constitutive activation of FXR improved insulin sensitivity and glucose tolerance. The mechanism of how FXR improves insulin sensitivity and glucose tolerance is not completely understood because the expression of FXR is non-existent in skeletal muscles, and very low in adipose tissues. However, a phenomenon known as lipotoxicity may explain how FXR is involved with regulation of insulin sensitivity and glucose levels (Modica, Gadaleta et al. 2010). Activation of FXR reduces lipotoxicity by reducing circulating triglycerides and free fatty acids (FFA), which may improve insulin sensitivity **(Figure 1.4)**.

Figure 1.4



FXR Improves Insulin Sensitivity and Glucose Tolerance.

Insulin promotes FXR activation. Activated FXR then increases expression of ApoCII, a co-activator of lipoprotein lipase, and decreases ApoCIII, an inhibitor of the lipase. Increased lipase activity clears plasma triglycerides and decreases lipotoxicity. Decreased lipotoxicity coupled with FXR activation improves insulin sensitivity and glucose tolerance.

1.6 Autophagy

Macroautophagy, hereafter called autophagy, is a cellular protective mechanism responsible for disposing and recycling damaged organelles and proteins, and thus promotes cell survival. The autophagy process is characterized by development of the isolation membrane into the autophagosome; in which, the autophagosome will fuse with the lysosome to degrade the engulfed substances. Currently there are more than 30 Atg genes identified to be involved with autophagy or autophagy like processes in yeast, and most of them have mammalian homolog counterparts (Klionsky and Emr 2000). Moreover, there is accumulating evidence revealing that autophagy is a tumor suppressing process.

1.7 Multiple signaling pathways regulate autophagy

Autophagy is usually induced under stress conditions including intracellular pathogens, hypoxia, increase in reactive oxygen species, endoplasmic reticulum, starvation, amino acids deprivation, radiation, proteasome inhibition and protein aggregates (Ding, Manley et al. 2011). Rapamycin, an inhibitor of mammalian target of rapamycin (mTOR) complex (mTORC), induces autophagy, which reveals that mTORC is a regulator of autophagy (Blommaart, Luiken et al. 1995; Kamada, Sekito et al. 2004). mTOR is a serine/threonine kinase complex composed of two heteromeric complexes, mTORC1 and mTORC2 (Yang and Guan 2007). In addition to regulation of autophagy, mTOR has multiple functions including translation regulation by phosphorylating two proteins: 70 kDa ribosomal protein S6 kinase-1 (p70S6K) and translational initiation factor 4E binding protein-1 (4EBP1), which activates the protein synthesis (Inoki, Zhu et

al. 2003). In addition to translation regulation, mTOR is also responsible for sensing the nutrient levels from the upstream signaling pathways.

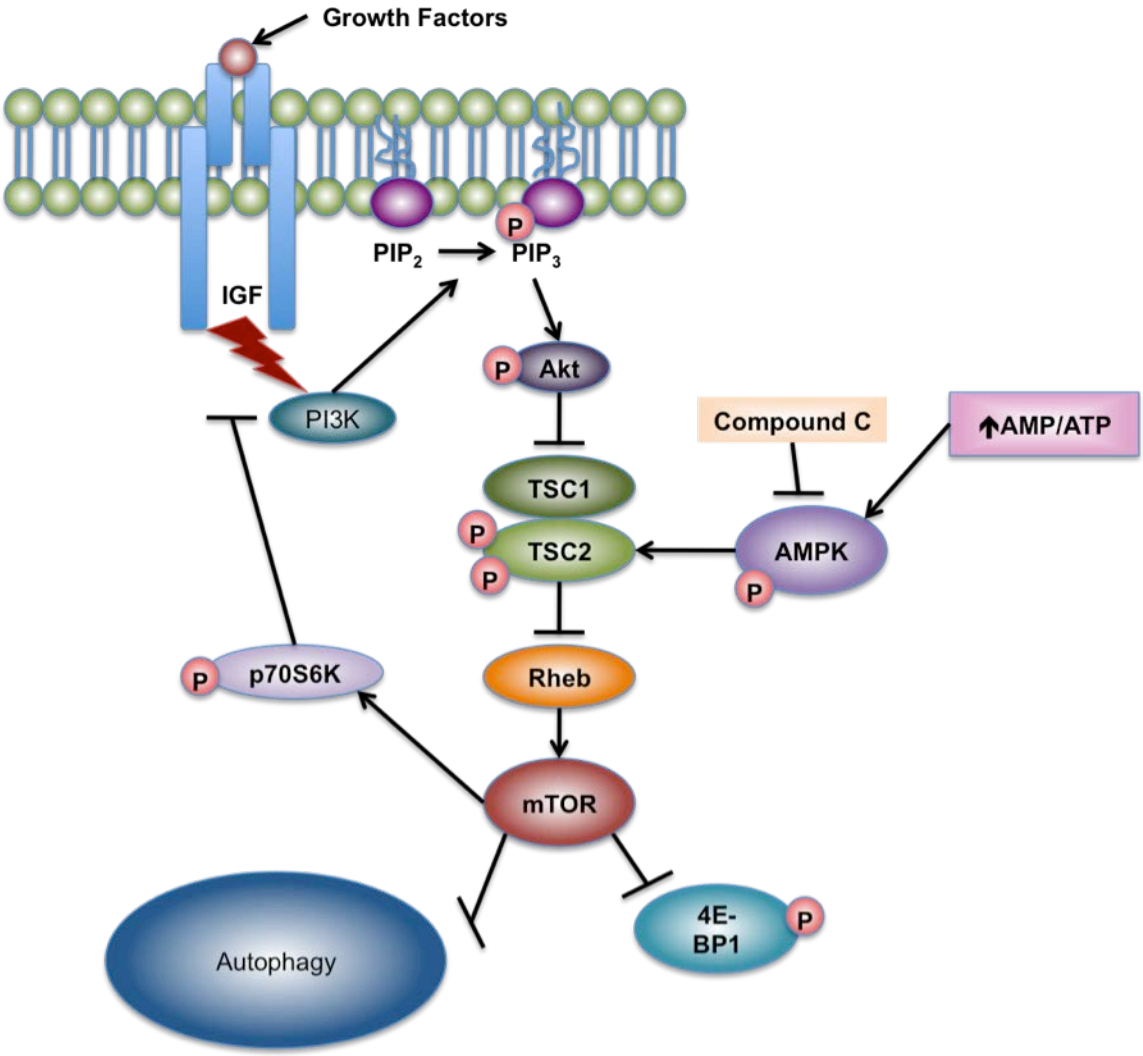
Class 1 phosphatidylinosital-3 kinase (PI3K) signaling pathway is the canonical signaling pathway that regulates mTOR. When PI3K signaling pathway is activated by the growth factors, PI3K phosphorylates Akt, which activates mTOR through the phosphorylation of tuberous sclerosis protein 2 (TSC2). TSC2 is part of a complex that is also composed of tuberous sclerosis protein 1 (TSC1) and is responsible for negative regulation of autophagy (Yang and Guan 2007). In a stable TSC complex, TSC2 induces GTPase-activating protein (GAP) activity, therefore, accelerating GTP hydrolysis of Rheb, a small GTPase, converting Rheb from active GTP bound form to inactive GDP bound form. Inactive Rheb subsequently inhibits mTOR and activates the autophagic process (Jung, Ro et al. 2010; Yang and Klionsky 2010). Therefore, when TSC2 is phosphorylated, the TSC complex is dissociated rendering TSC2 inactive resulting in activation of mTOR, and suppression of the autophagic process.

Moreover, AMP-activated protein kinase (AMPK) signaling pathway also regulates mTOR. The role of AMPK signaling pathway in autophagy regulation is still controversial. To reflect on energy stress condition, the AMP/ATP ratio increases, and the excess AMP binds to and activates AMPK. In turn, AMPK phosphorylates TSC2 and increases the TSC2 GAP activity toward Rheb in which inhibits mTOR (Inoki, Zhu et al. 2003). It has been show that the expression of the dominant negative form of AMPK completely inhibits autophagy in hepatocytes, HT-29 and Hela cells (Meley, Bauvy et al. 2006). However, the adenosine analog, adenosine 5-amino-4-imidazole carboxamide riboside (AICAR) activates AMPK and actually suppresses autophagy in hepatocytes

(Samari and Seglen 1998). In contrary, compound C inhibits AMPK and induces autophagy in cancer cells by down regulating the Akt-mTOR pathway (Vucicevic, Misirkic et al. 2011). In conclusion, the role of AMPK in autophagy needs to be clarified by studying possible off-target effects of activators and inhibitors of AMPK. In addition to the pharmacological approaches, it will be better to study autophagy by genetically up-regulating or knocking down AMPK.

mTOR is the primary regulator of autophagy, however, autophagy can be activated by mTOR-independent pathways. Agents that reduce the cAMP levels have been shown to induce mTOR-independent autophagy including calpain inhibitors and L-type Ca^{2+} channel agonists (Ravikumar, Sarkar et al. 2010). In addition to the decrease in cAMP levels, drugs that decrease the intracellular inositol and inositol 1,4,5-triphosphate (IP_3) concentrations also induce mTOR-independent autophagy. Examples of common drugs that reduce inositol include lithium, carbamazepine and valproic acid (Sarkar, Floto et al. 2005; Ravikumar, Sarkar et al. 2010). The general consensus is that autophagy is usually induced under stress conditions to act as a cell protective mechanism either regulated by mTOR or mTOR-independent pathways **(Figure 1.5)**.

Figure 1.5



Akt and AMPK signaling pathways regulate mTOR.

Activation of Akt pathway inhibits TSC complex, activates Rheb, and subsequently activates mTOR complex, which inhibits autophagy. AMPK activates TSC complex, therefore, inhibits mTOR and induces autophagy. mTOR complex activates protein translation by phosphorylation of 4E-BP1 and p70S6K.

1.8 From isolation membrane to autophagosome

Induction of autophagy requires several multimolecular complexes including the following: ULK1 (UNC-51 like kinase) protein kinase complex, vacuolar protein sorting 34 (VPS34)-Beclin-1 class III PI3-kinase complex, Atg9-Atg2-Atg18 complex, and Atg5-Atg12-Atg16 and Atg8/LC3 (microtubule-associated protein 1 light chain 3) conjugation systems **(Figure 1.6)** (Ding, Manley et al. 2011). Generally the ULK1 complex initiates the autophagy process and then two ubiquitin-like conjugation systems including Atg7, Atg3, and Atg10 and Atg5-Atg12-Atg16 complex promote the conjugation of light chain 3, a mammalian homolog of Atg8, with phosphatidylethanolamine (PE) to form the PE conjugated form of LC3 (LC3-II) (Kabeya, Mizushima et al. 2000; Ohsumi 2001; Chan, Longatti et al. 2009; Hosokawa, Hara et al. 2009). LC3-II then translocates to the early autophagosomal membrane to initiate membrane elongation and formation of double membrane structures (Kabeya, Mizushima et al. 2000). The autophagosome subsequently fuses with the lysosome to become a mature autolysosome to complete the degradation process (Kimura, Noda et al. 2007; Saftig, Beertsen et al. 2008).

The ULK complex consists of ULK1 (Atg1 homolog), FIP200 (Atg 17-like molecule), Atg13 and Atg101, and is localized in the cytosol (Ganley, Lam du et al. 2009; Hosokawa, Hara et al. 2009; Mizushima 2010). Upon autophagy induction, the complex initiates the formation of the autophagic isolation membrane. Further on, the mTOR regulates the activity of the ULK complex through phosphorylation, and the ULK complex is activated by dephosphorylation (Ding, Manley et al. 2011). Furthermore, ULK is a serine/threonine protein kinase responsible for phosphorylating Atg13 and FIP200 and recruiting downstream autophagy proteins for the autophagosome

formation (Chan, Longatti et al. 2009). Moreover, ULK complex is involved in cross-talking with the VPS34-Beclin-1 class III PI3-kinase complex as a regulatory mechanism in early autophagy induction (Jung, Ro et al. 2010).

Beclin-1, an Atg6 homolog, is imperative for initiation and regulation of the autophagy process by establishing a complex with VPS34, VPS15, and Atg14 (Itakura, Kishi et al. 2008). The Bcl family proteins, Bcl-2 and Bcl-x, which dissociate Beclin-1 from the VPS34, by binding with Beclin-1, negatively regulate the activity of Beclin-1 (Pattingre, Tassa et al. 2005; He and Levine 2010). One of the major functions of Beclin-1 complex is to promote the activation of VPS34, a class III PI3-kinase responsible for producing phosphatidylinositol-3-phosphate (PI-3-P) (Kihara, Kabeya et al. 2001; Kihara, Noda et al. 2001; Zeng, Overmeyer et al. 2006; Ding, Manley et al. 2011). Moreover, Beclin-1 also interacts with other proteins responsible for inducing autophagy, including UV radiation resistance-associated gene protein (UVRAG) and Ambra-1 (Liang, Feng et al. 2006; Fimia, Stoykova et al. 2007). In addition, Bif-1 positively regulates the Beclin-1 complex by interacting with UVRAG, and Rubicon interacts directly with VPS34 to negatively regulate the Beclin-1 complex (Zhong, Wang et al. 2009; Sun, Zhang et al. 2011). 3-methyladenine (3-MA) is a class III PI3-kinase inhibitor and acts on VPS34, which inhibits autophagosome formation (Seglen and Gordon 1982).

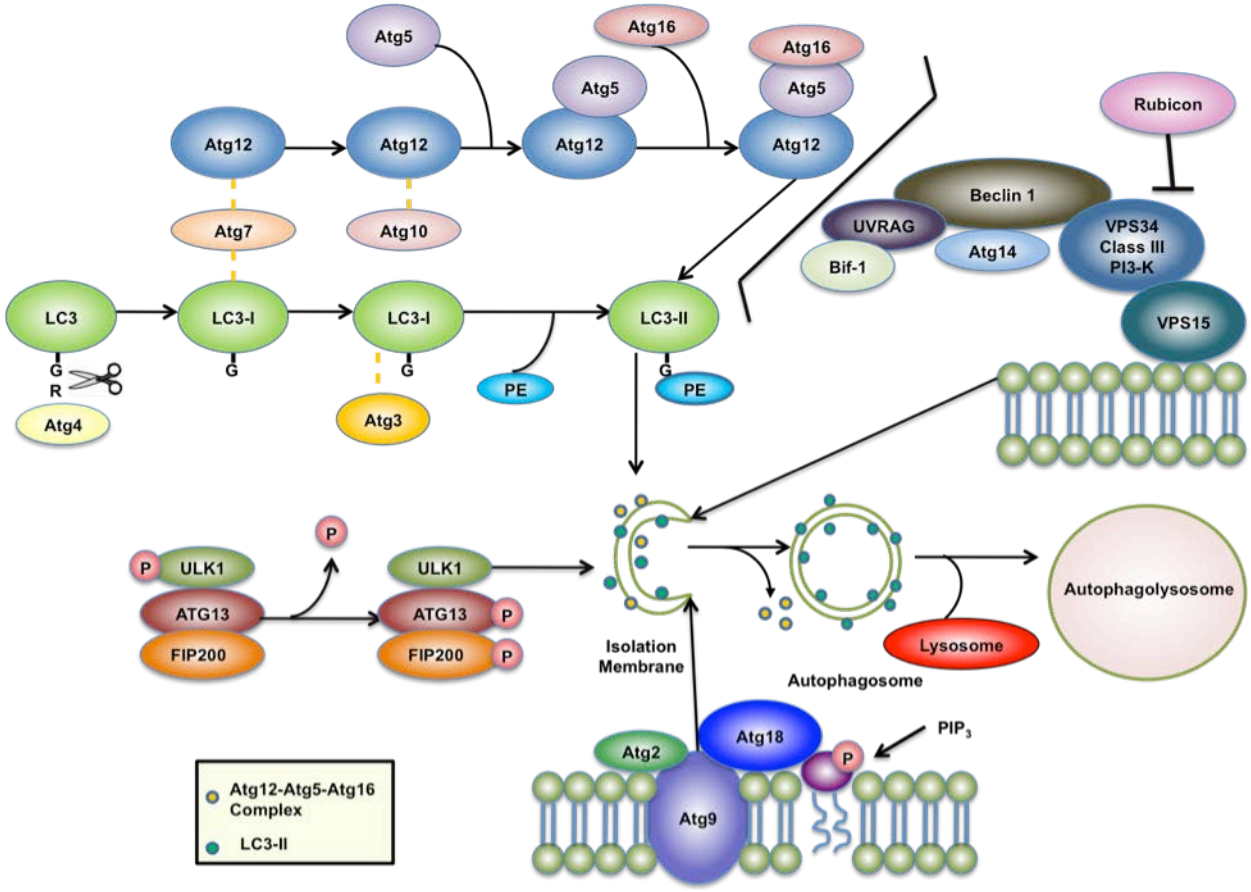
A bilayered lipid membrane is required for autophagosome formation. Atg9 is a transmembrane protein with six transmembrane domains with its carboxyl termini in the cytosol and is conserved in all species. Atg9 is responsible for recruiting the lipid membrane to a pre-autophagosomal structure known as phagophore assembly site

(PAS) (Young, Chan et al. 2006; Webber and Tooze 2010). In mammalian cells, Atg9 exists as two functional orthologs, Atg9L1 and Atg9L2. Atg9L2 is only expressed in the placenta and pituitary gland whereas Atg9L1 is ubiquitously expressed (Yamada, Carson et al. 2005). Atg9 interacts with Atg18, a PI-3-P binding protein, and Atg2, a peripheral membrane protein, in the PAS (Wang, Kim et al. 2001; Reggiori, Tucker et al. 2004; Obara, Sekito et al. 2008). The source of the membrane is unknown, but it has been proposed that Atg9 is cycled between the trans-Golgi network, late endosomes and the PAS to recruit additional membranes for autophagosomes (Young, Chan et al. 2006; Webber and Tooze 2010). Other possible membrane sources that have been proposed for the origin of autophagosomes include the plasma membrane, ER and mitochondrial contact site, endosomal membrane, and mitochondrial membrane (Tooze and Yoshimori 2010; Lamb, Yoshimori et al. 2013). Finally, the de novo synthesis model is feasible since it appears that the phagophore expands rather than being formed in a single step from the existing membrane (Chen and Klionsky 2011).

Finally, two ubiquitin-like conjugation systems play a role as the engine in autophagy machinery by regulating the elongation of the isolation membrane that engulfs the substances to form the autophagosome (Ohsumi 2001). Without this core complex, autophagy cannot occur. In the first system, Atg12, a ubiquitin-like protein, is activated by Atg7, a ubiquitin-activating enzyme (E1)-like protein, which enables Atg 10, a ubiquitin carrier (E2)-like protein, to transfer Atg12 to establish a covalent bond with Atg5 (Mizushima, Noda et al. 1998; Ichimura, Kirisako et al. 2000; Suzuki, Kirisako et al. 2001). The Atg5-Atg12 complex then interacts with Atg16 to establish ubiquitin ligase (E3)-like Atg5-Atg12-Atg16 complex that is required for autophagy activation and

localization of LC3 to the autophagosomal membrane (Hanada, Noda et al. 2007). In the second ubiquitin-like conjugation system, Atg4 cleaves LC3 to expose the conserved Gly^{120} residue in the c-terminus. PE then is conjugated to the unconjugated form of LC3 (LC3-I) at Gly^{120} via Atg7 and Atg3, an E2-like protein, to form the conjugated form of LC3 (LC3-II) (Kabeya, Mizushima et al. 2000; Ohsumi 2001). LC3-I is found in cytosol whereas LC3-II is localized on the autophagosomal membrane. LC3-II is required for completion of elongation and closure of the membrane into a mature autophagosome (Kabeya, Mizushima et al. 2000; Kirisako, Ichimura et al. 2000). Unlike other Atg proteins such as Atg5, Atg7 or Atg16 that are found only transiently associated with autophagosomal membrane, LC3-II is relatively stable on the autophagosomal membrane (Rubinsztein, Cuervo et al. 2009; Mizushima, Yoshimori et al. 2010; Klionsky, Abdalla et al. 2012). Therefore, LC3 is commonly used as a marker for autophagy both *in vivo* and *in vitro*.

Figure 1.6



Autophagy machinery.

Autophagy involves the formation of double-membrane autophagosomes that fuse with lysosomes to form autolysosomes for the degradation of intracellular proteins and organelles. At least four important functional groups of Atg proteins are required for autophagy: (1) ULK1 protein- kinase complex and (2) VPS34 – Beclin 1 class III PI3-kinase complex regulate autophagy initiation; (3) Atg9 – Atg2 – Atg18 complex regulates expansion of phagophore assembly site (PAS) by carrying lipids; and (4) the Atg5 – Atg12 – Atg16 and LC3 conjugation systems regulate the elongation of autophagosome membranes. Phosphatidylethanolamine (PE)-conjugated LC3 (called LC3-II) remains on the isolation membranes and autophagosome membranes, whereas the Atg12 – Atg5 – Atg16 complex transiently associates with the isolation membranes and dissociates from the autophagosome membranes. Once autophagosomes fuse with lysosomes to form autolysosomes, the inner membrane LC3-II is degraded by lysosomal enzymes whereas the outer membrane LC3-II is de-conjugated and recycled. Pharmacological autophagy inhibitors such as 3-methyladenine (3-MA) and chloroquine (CQ) are also highlighted**.**

1.9 Autophagosomal-lysosomal fusion is required for completion of autophagy degradation process

Degradation of engulfed substance in the autophagosome is dependent on the autophagosomal-lysosomal fusion and also the lysosomal functions. It has been shown that microtubule dependent lysosomal positioning is critical for regulation of the autophagosomal-lysosomal fusion process (Korolchuk, Saiki et al. 2011). Newly formed autophagosomes are randomly scattered across the cytoplasm; however, during maturation, the autophagosomes use microtubules to move toward the microtubule organizing center where the lysosomes are present (Yamamoto, Tagawa et al. 1998). At the microtubule organizing center, an autophagosome fuses with a lysosome via interaction of assorted fusion proteins found on both the autophagosome and the lysosome. Chemicals such as vinblastine and nocodazole that disrupt the microtubule stability and structure thus can inhibit autophagy by impairing the fusion process (Kovacs, Reith et al. 1982; Webb, Ravikumar et al. 2004).

Furthermore, multiple proteins including lysosomal associated membrane proteins 1 and 2 (LAMP-1, LAMP-2), Rab proteins and soluble n-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) mediate the fusion of autophagosomes to lysosomes (Jager, Bucci et al. 2004; Kimura, Noda et al. 2007; Saftig, Beertsen et al. 2008). LAMP-1 and LAMP-2 are well known lysosomal membrane markers, and LAMP-2 depletion decreases fusion (Gonzalez-Polo, Boya et al. 2005). This suggests that LAMP-1 and LAMP-2 are responsible for coordinating the fusion of the lysosome with the autophagosome, however the precise mechanism is not yet fully understood. Rab proteins are members of small GTP-binding proteins and are

responsible for binding to the membranes as well as recruiting effectors of fusion. Rab7 is a required component for a complete autophagosomal-lysosomal fusion (Ganley, Wong et al. 2011). Along with Rab7, post-Golgi SNAREs including vesicle-associated membrane protein 7 (VAMP7), vesicle t-SNARE-interacting protein homologous 1B (Vti1b) and syntaxin-7 also are required for the autophagosomal-lysosomal fusion (Pryor, Mullock et al. 2004; Fraldi, Annunziata et al. 2010; Furuta, Fujita et al. 2010). When autophagosomal-lysosomal fusion is completed, the newly formed autolysosome degrades the substrate through the enzymatic activity inside the autolysosome.

1.10 Cathepsins, hydrolases and lysosomal pH drive lysosomal degradation process

The completion of the autophagy process is dependent on the lysosomal degradation process. Lysosomes were first identified and described by Christian de Duve in 1955 as a cytosolic membrane structure containing at least 50 acid hydrolases that are pH sensitive including phosphatases, nucleases, glycosidases, proteases, peptidases, sulphatases and lipases (de Duve 1983). Due to the wide range of enzymes contained in lysosomes, lysosomes are capable of degrading all macromolecules found in the cell in order to recycle.

Cathepsins are the best-studied lysosomal hydrolases and can be divided into three subgroups according to their active amino acid sites including cysteine, aspartate and serine (Rawlings, Tolle et al. 2004). Additionally, cathepsins are proteases with pKa values of approximately 3.5 and 8.0, which are imperative for its enzymatic function (Turk and Turk 2009). Aspartic protease cathepsin D is the most common lysosomal

protease along with various cysteine cathepsins including cathepsins B and C (Turk, Stoka et al. 2002). The cathepsins do not have high substrate specificity, which contributes to their ability to degrade a wide range of proteins (Turk and Turk 2009). The enzymatic activity of cathepsins relies on the relatively low pH level (3.8-5) well characterized in the lysosome. Nevertheless, most of the cathepsins still can function at neutral pH with either decreased stability or altered specificity (Turk, Turk et al. 2001).

Further on, lysosomal degradation is attributed to the cooperative efforts between pH sensitive enzymes and proteins responsible for pH maintenance. Proton pumping vacuolar ATPases, chloride transporters and cAMP-regulated chloride channels regulate the pH level in the lysosomes (Kornak, Kasper et al. 2001; Forgac 2007; Graves, Curran et al. 2008; DiCiccio and Steinberg 2011). Furthermore, when the lysosome pH is raised, the lysosome function is impaired by inactivation of lysosomal pH sensitive enzymes including the acid hydrolases and cathepsins. Interestingly, when the lysosomal membrane permeabilization event occurs, the contents and cathepsins are released from the lysosome and trigger apoptosis. In addition, the rapid rupture of lysosomes may result in necrosis (Kroemer and Jaattela 2005).

1.11 Autophagy is required for cell survival and suppresses tumorigenesis

Autophagy is a cellular protective mechanism and the suppression of autophagy has serious consequences. Beclin-1 knockout mice die in utero whereas Atg3, Atg5, Atg7, Atg9, and Atg16L knockout mice die within a day after birth (Qu, Yu et al. 2003; Yue, Jin et al. 2003; Kuma, Hatano et al. 2004; Komatsu, Waguri et al. 2005; Saitoh, Fujita et al. 2008; Sou, Waguri et al. 2008; Saitoh, Fujita et al. 2009; Mizushima and

Levine 2010). Moreover, FIP200 and Ambra1 deletions are also embryonic lethal in mice (Gan, Peng et al. 2006; Fimia, Stoykova et al. 2007). Therefore, autophagy is required for viability, especially during the embryonic development.

Because of the autophagy gene knockout mice are either embryonic lethal or die at neonatal stage, tissue specific genetic knockout mice have been developed. Liver-specific Atg5 or Atg7 knockout mice develop liver injury, hepatomegaly, inflammation, fibrosis and spontaneous liver tumors (Mizushima and Levine 2010; Inami, Waguri et al. 2011; Takamura, Komatsu et al. 2011; Ni, Boggess et al. 2012). Furthermore, Beclin-1 heterozygous mice are more susceptible to liver tumorigenesis (Qu, Yu et al. 2003). Suppression of autophagy also results in the accumulation of ubiquitinated protein aggregates and damaged organelles, including mitochondria, leading to cellular dysfunction (Komatsu, Waguri et al. 2005; Hara, Nakamura et al. 2006; Komatsu, Waguri et al. 2006; Kim, Rodriguez-Enriquez et al. 2007; Mizushima 2007; Nakai, Yamaguchi et al. 2007; Raben, Hill et al. 2008). Mechanistically, it appears that the persistent activation of Nrf2 due to the accumulation of p62 in autophagy-deficient liver is responsible for the pathogenesis observed in autophagy-deficient livers. The mechanisms by which p62 activates Nrf2 is discussed below. Further deletion of p62 reduced hepatomegaly, liver injury and the number of tumors in liver-specific Atg7 knockout mice (Takamura, Komatsu et al. 2011). We recently also reported that further deletion of Nrf2 completely rescued hepatomegaly, liver injury and liver tumors in liver-specific Atg5 knockout mice (Ni, Woolbright et al. 2014). Why persistent Nrf2 activation would trigger liver injury in liver-specific Atg5 knockout mice is currently still not clear.

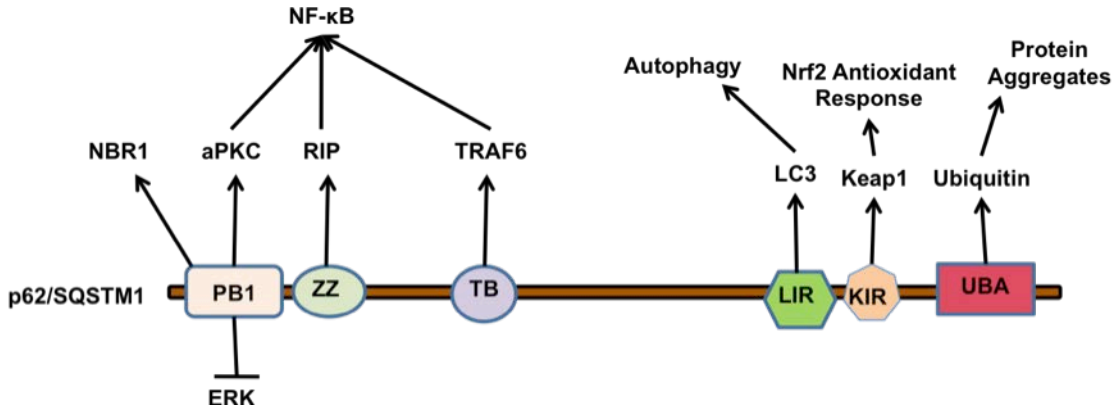
1.12 p62 is an autophagy substrate with multiple roles

p62 is a scaffold protein with multiple domains known for signaling transduction modulations and also plays a role in mediating the balance between the cell survival and death **(Figure 1.7)** (Moscat and Diaz-Meco 2009). The PB1 domain in the n-terminus is responsible for interacting with atypical protein kinase C (aPKC) and polarity protein, Par-6, which implicate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) function downstream (Sanz, Diaz-Meco et al. 2000; Duran, Serrano et al. 2004; Moscat and Diaz-Meco 2009). In addition, the PB1 domain is suspected to play a role in p62 oligmerization that allows p62 to establish a signal organizing center in order to interact with other signaling molecules (Moscat, Diaz-Meco et al. 2006; Moscat, Diaz-Meco et al. 2007). Located next to the PB1 domain is the ZZ zinc finger region responsible for binding to receptor interacting protein (RIP), a TNFα signaling adaptor (Sanz, Sanchez et al. 1999; Moscat, Diaz-Meco et al. 2007). Along with PB1 domain, the TB domain is responsible for interacting with tumor necrosis factor associated receptor-6 (TRAF6), a lysine (K) 63 E3 ligase. p62 self-oligmerization promotes TRAF6 interaction and K63 polyubiquitination, which leads to NF-κB activation and subsequent inflammation response (Sanz, Diaz-Meco et al. 2000; Moscat, Diaz-Meco et al. 2006; Duran, Linares et al. 2008; Moscat and Diaz-Meco 2009). The Kelch-like ECH-associated protein 1 (Keap1) interacting domain (KIR) in the c-terminus mediates the stress response in p62 accumulation by interacting with the Kelch-repeat domain of Keap1, dissociating it from nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor and allowing Nrf2 to translocate to the nucleus to activate stress response gene transcription (Jain, Lamark et al. 2010). Interestingly,

Nrf2 has been implicated in tumorigenesis via its antioxidant program (DeNicola, Karreth et al. 2011).Disruption in the autophagy degradation process promotes p62 accumulation and p62 positive protein aggregates.

p62 is an autophagy substrate and also serves as an autophagy receptor to bind to ubiquitinated proteins or organelles to promote their degradation by autophagy (Bjorkoy, Lamark et al. 2005; Komatsu, Waguri et al. 2007). p62 binds with ubiquitinated proteins through its ubiquitin associated domain (UBA) in the c-terminus (Donaldson, Li et al. 2003). p62 also interacts with LC3 through its LC3 interacting region (LIR) in the c-terminus (Bjorkoy, Lamark et al. 2005; Pankiv, Clausen et al. 2007; Ichimura, Kumanomidou et al. 2008). Therefore, the LIR enables p62 to tether ubiquitinated proteins to the autophagosomal membrane via its direct interaction with LC3 (Ichimura, Kumanomidou et al. 2008; Komatsu and Ichimura 2010). Accumulation of p62 has been implicated in tumorigenesis and hepatocellular carcinoma but the mechanism is not clear (Duran, Linares et al. 2008; Mathew, Karp et al. 2009; Inami, Waguri et al. 2011; Takamura, Komatsu et al. 2011). Furthermore, a couple of groups have shown that knockout of p62 attenuates liver inflammation, hepatomegaly, and the rate of tumor growth in liver-specific Atg7 knockout mice (Komatsu, Waguri et al. 2007; Takamura, Komatsu et al. 2011). This suggests that p62 promotes tumorigenesis when autophagy is suppressed. Mechanistically, the persistent activation of Nrf2 due to p62 accumulation is most likely the key mechanism to promote liver tumorigenesis because further deletion of Nrf2 completely abolished liver tumorigenesis in liver-specific Atg5 knockout mice (Ni, Woolbright et al. 2014).

Figure 1.7



p62 structure, binding partners and functions.

p62 has six distinct functional domains: PB1, ZZ, TB, LIR, KIR and UBA. PB1 domain self- and hetero-oligomerizes with other PB1 containing proteins, such as NBR1, ERK and aPKC. p62 binds with RIP at ZZ zinc finger region, and TRAF6 at TB domain, which regulates NF-kB activation. p62 interacts with LC3 through the LIR, and Keap1 through the KIR. The c-terminal UBA domain of p62 binds to ubiquitin.

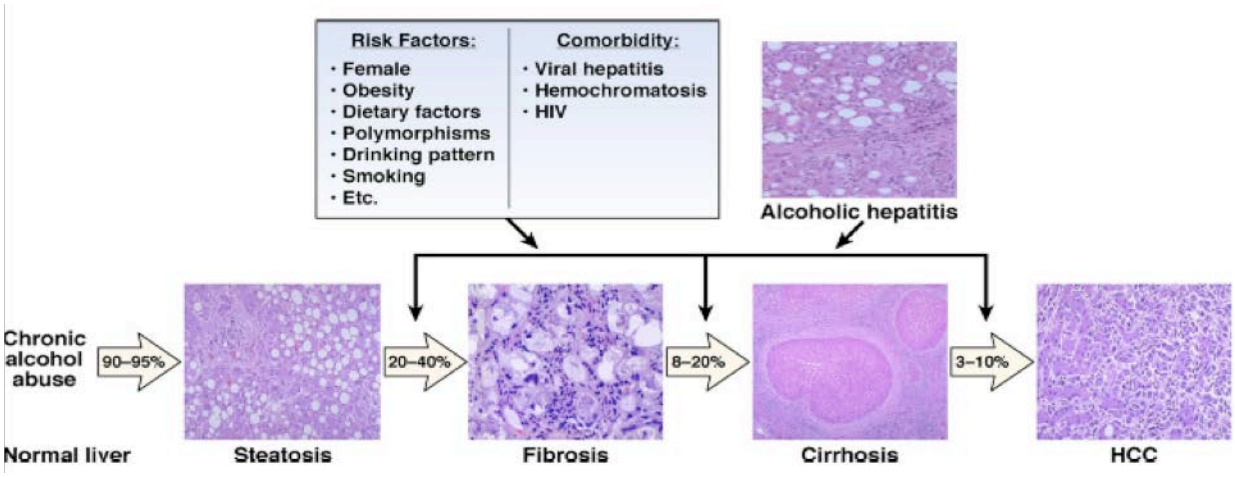
1.13 Alcoholic liver disease is a major contributor of liver diseases worldwide

Alcohol consumption and abuse lead to alcoholic liver disease (ALD), a major contributor of liver diseases and mortality both in the United States and worldwide (Rehm, Mathers et al. 2009; Gao and Bataller 2011). ALD is characterized by the development of steatosis in early stages and could progress to fibrosis, alcoholic hepatitis, cirrhosis, and hepatocellular carcinoma **(Figure 1.8)** (Ding, Manley et al. 2011; Gao and Bataller 2011). ALD also induces hepatic metabolic changes, increases oxidative stress, and alters lipid metabolism resulting in the accumulation of lipid droplets (Ding, Manley et al. 2011). Importantly, 95% of alcoholic patients develop steatosis; however, only 20 to 40% of patients progress to fibrosis and only a very small portion of patients exhibit a more severe form of ALD such as cirrhosis and HCC (Gao and Bataller 2011). This brings on the question of why most of alcoholics are protected from severe forms of ALD? While it is still not clear, several mechanisms have been suggested that may be responsible for the variations observed among different drinkers. Indeed, ALD usually manifests in synergy with other risk factors such as sex, obesity, dietary factors, genetic factors, smoking and viral hepatitis (Wilfred de Alwis and Day 2007; Tsukamoto, Machida et al. 2009; O'Shea, Dasarathy et al. 2010).

Women are twice as sensitive to ethanol-induced hepatotoxicity and may develop ALD in shorter duration and lesser doses of alcohol than men (Sato, Lindros et al. 2001; O'Shea, Dasarathy et al. 2010). Women and men both displayed gender differences in the pharmacokinetics of alcohol mediated by several possible causes including the differences in relative amount of gastric alcohol dehydrogenase, higher rate of body fat in women, and change in the absorption of alcohol with menstrual cycle

(Frezza, di Padova et al. 1990; Sato, Lindros et al. 2001; O'Shea, Dasarathy et al. 2010). Race and ethnicity also may influence the outcome of alcohol-mediated liver injury (Stewart 2002). Alcoholic cirrhosis rates are higher in African-American and Hispanic males than Caucasian males. Moreover, the mortality rate from ALD is highest in Hispanic males (Stinson, Grant et al. 2001). The differences in alcohol consumption appear not to be related to the racial and ethnic differences in mortality (Wickramasinghe, Corridan et al. 1995). Therefore, genetic factors play a role in alcoholism and ALD. Children of alcoholics raised in adopted families are more predisposed to alcoholism in comparison to adopted children of nonalcoholics (Goodwin, Schulsinger et al. 1973). Furthermore, twin studies showed that monozygotic twins are twice as likely to drink as dizygotic twins and alcoholic cirrhosis prevalence is higher in monozygotic twins than dizygotic twins (Kaprio, Koskenvuo et al. 1987; Reed, Page et al. 1996). Genetic polymorphisms of alcohol metabolizing genes including alcohol dehydrogenase, acetaldehyde dehydrogenase, and cytochrome P450s also have been associated with ALD (O'Shea, Dasarathy et al. 2010). Moreover, genes involved with the regulation of endotoxin-mediated release of cytokines have been associated with ALD (O'Shea, Dasarathy et al. 2010). Another possible explanation is that ethanol may also trigger the cellular defense mechanisms such as autophagy and increased expression of interleukin-22 (IL-22) in addition to its detrimental effects (Ki, Park et al. 2010; Ren, Hu et al. 2010; Ding, Manley et al. 2011).

Figure 1.8



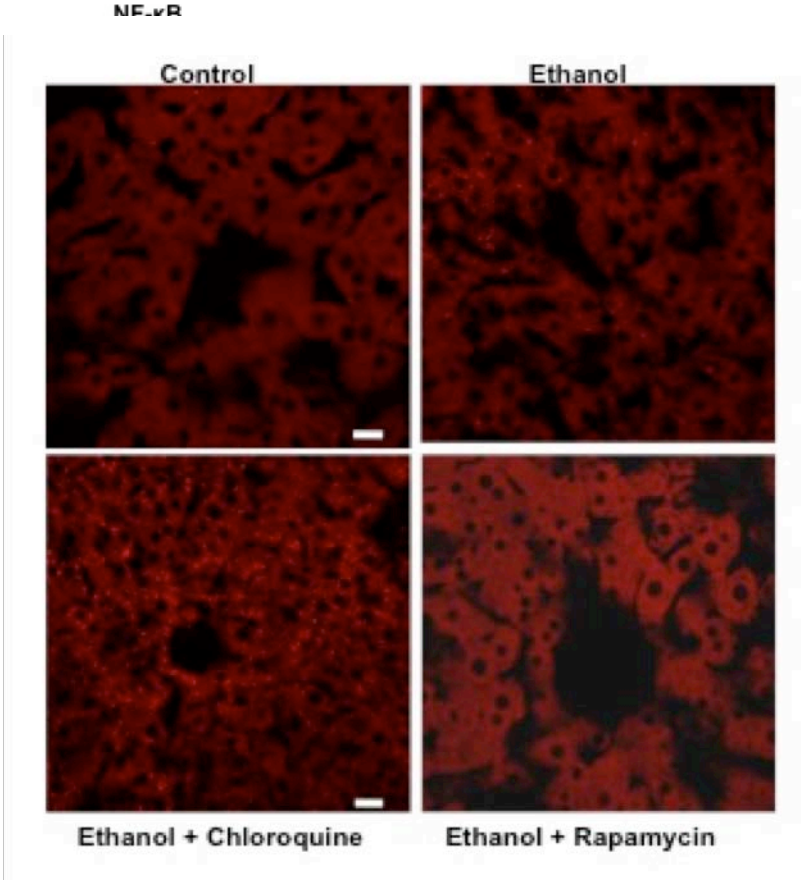
Pathogenesis of ALD.

95% of alcoholics develop steatosis, however, only 35% of drinkers develops more severe forms of ALD including fibrosis, cirrhosis, alcoholic hepatitis, and HCC. (Gao and Bataller 2011). Reprinted from Alcoholic Liver Disease: Pathogenesis and New Therapeutic Targets, Volume 141 Issue 5, Gao and Bataller, Gastroenterology, 1572-1585., Copyright (2011), with permission from Elsevier.

1.14 Activation of autophagy alleviates alcohol-induced liver injury

As previously discussed, autophagy is an evolutionarily conserved catabolic process responsible for the degradation of cellular proteins and damaged organelles, and is a cellular protective mechanism in response to stress (Ding, Manley et al. 2011). Liver enlargement known as hepatomegaly is a common response to alcohol toxicity in both alcohol-fed animals and alcoholics. Alcohol-induced hepatomegaly is a result of protein and lipid accumulation (Baraona, Leo et al. 1975). Chronically alcohol-fed rat livers display normal protein synthesis activity; however, the rate of protein degradation is decreased by around 40% in the livers of alcohol-fed animals (Donohue, Sorrell et al. 1987; Donohue, Zetterman et al. 1989; Dolganiuc, Thomes et al. 2012). Moreover, alcohol administration disrupts lysosomal proteolytic activity by alkalinizing the lysosomal interior and disrupting the trafficking of cathepsins to lysosomes (Kharbanda, McVicker et al. 1996; Kharbanda, McVicker et al. 1997). These findings may suggest that autophagy could be impaired in chronic alcohol consumption conditions although this is still controversial. However, acute alcohol exposure induces autophagy to selectively promote the degradation of lipid droplets and damaged mitochondria (Ding, Li et al. 2011). Indeed, pharmacologic activation of autophagy has been shown to alleviate alcohol-induced steatosis and hepatotoxicity, whereas pharmacologic inhibition of autophagy exacerbates alcohol-induced liver injury **(Figure 1.9)** (Ding, Li et al. 2010; Lin, Zhang et al. 2013).

Figure 1.9



Pharmacological Activation of Autophagy Alleviates Ethanol-Induced Steatosis.

Ethanol induces steatosis, and pharmacological inhibition of autophagy by chloroquine exacerbates ethanol-induced steatosis, whereas activation of autophagy by rapamycin alleviates ethanol-induced steatosis (Ding *et. al.* 2010).

1.15 FXR may have a protective role against alcohol-induced liver injury

Alcohol consumption induces hepatic metabolic changes, increases oxidative stress, and alters lipid metabolism, which result in liver injury. Moreover, alcohol may induce cholestasis in all stages of ALD (Tung and Carithers 1999). Indeed, chronic alcohol consumption in rats increases total and unconjugated serum bile acids and alters the bile acid profiles in the intestines (Xie, Zhong et al. 2013). Chronic alcohol consumption induces acetylation of FXR resulting in inactivation of FXR and subsequent increased hepatic bile acid levels in mouse. Indeed, pharmacological activation of FXR by a specific FXR agonist, WAY-362450, attentuates ethanol-induced liver injury, steatosis and inflammation (Wu, Zhu et al. 2014). Furthermore, alcohol consumption induces the expression of gene encoding bile acid synthesis enzyme (CYP7A1) and basolateral bile acid transporters (Ostα and β) in rat livers (Xie, Zhong et al. 2013). Bile acids also regulate alcohol metabolism by inducing class I alcohol dehydrogenase (ADH1) gene expression through activation of FXR in human hepatocytes, but not in rodent hepatocytes. Human *ADH1A and ADH1B* proximal promoter regions contain putative FXR response elements (Langhi, Pedraz-Cuesta et al. 2013). These recent studies reveal that bile acid metabolism and ethanol metabolism cross-talk by regulating each other.

Altered hydrophobicity in bile acid pools by CDCA feeding has been shown to aggravate liver injury induced by chronic alcohol feeding. CDCA feeding plus chronic ethanol drinking in mice leads to increased hepatic triglycerides and elevated serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), markers of liver

injury (Montet, Oliva et al. 2002). The mechanism of liver injury in CDCA feeding plus chronic ethanol drinking is not known.

1.16 FoxO3a, a transcription factor, upregulates autophagy related gene transcription in response to ethanol

Forkhead box O3a (FoxO3a), a member of FoxO family of transcription factors, is involved with a variety of cellular processes including proliferation, apoptosis, stress resistance, metabolic responses, and autophagy (Birkenkamp and Coffer 2003; van der Horst and Burgering 2007; van der Vos and Coffer 2008). The FoxO3a structure contains a Forkhead DNA binding domain, two nuclear localization sequences (NLS) and a nuclear export sequence (NES) in the c-terminus **(Figure 1.10)** (Calnan and Brunet 2008). Activation and repression of FoxO3a is regulated by an intricate system involving multiple signaling pathways, co-factors, and multiple binding partners. Furthermore, FoxO3a activation is regulated by multiple posttranslational modifications including phosphorylation, acetylation, and ubiquitination **(Figure 1.10)**. Akt is identified to be the key regulator of FoxO3a activation, in which Akt phosphorylation enhances FoxO3a binding to the 14-3-3 protein resulting in cytoplasmic accumulation and subsequent inactivation of FoxO3a (Tzivion, Dobson et al. 2011). Conversely, dephosphorylation at sites threonine 32 and serine 253 by protein phosphatase 2A (PPA2) promotes nuclear FoxO3a accumulation and subsequent activation of Foxo3a (Singh, Ye et al. 2010). Casein kinase 1 (CK1), inhibitor of nuclear factor kappa B kinase subunit beta (IKKβ) and extracellular-signal-regulated kinase (ERK) all phosphorylate FoxO3a resulting in the inhibition of FoxO3a activation (Hu, Lee et al. 2004; Calnan and Brunet 2008; Yang, Zong et al. 2008). Conversely, the

phosphorylation of FoxO3a by AMPK at serine residues 413 and 588 induces FoxO3a activation (Greer, Oskoui et al. 2007). Acetylation of FoxO3a by cAMP response element binding-binding protein (CBP), p300, and p300/CBP-associated factor (PCAF) results in inactivation of FoxO3a. Interestingly, deacetylation of FoxO3a by Sirt1, a NAD-dependent deacetylase, results in FoxO3a ubiquitination and degradation (Wang, Chan et al. 2012). In addition, it has been found that Sirt1 has dual effects on FoxO3a-mediated gene expression. Sirt1 enhances FoxO3a-induced expression of cell cycle arrest and oxidative stress resistance genes, but inhibits FoxO3a-induced expression of apoptotic genes (Brunet, Sweeney et al. 2004). These results suggest that Sirt1-mediated acetylation of FoxO3a may determine the specificity of FoxO3a-induced gene expression. Finally, ubiquitination of FoxO3a by E3 ligases, S-phase kinase-associated protein 2 (Skp2), and murine double minute 2 (MDM2), targets FoxO3a toward the ubiquitin-proteasome system for degradation (Huang and Tindall 2011; Wang, Chan et al. 2012).

Multiple binding partners also regulate FoxO3a activation and enable FoxO3a to confer a wide range of transcriptional responses (van der Vos and Coffer 2008). FoxO family members have been shown to bind with the following nuclear receptors: constitutive androgen receptor (CAR) (Kodama, Koike et al. 2004), hepatic nuclear factor-4 (HNF4) (Hirota, Daitoku et al. 2003), peroxisome proliferator-activated receptors alpha and gamma (PPARα and PPARγ) (Dowell, Otto et al. 2003; Qu, Su et al. 2007), pregnane X receptor (PXR) (Kodama, Koike et al. 2004), and retinoic acid receptor (RAR) (Zhao, Herrera et al. 2001) and influence both nuclear receptor-mediated and FoxO-mediated gene transcription. The interaction of FoxO with HNF4

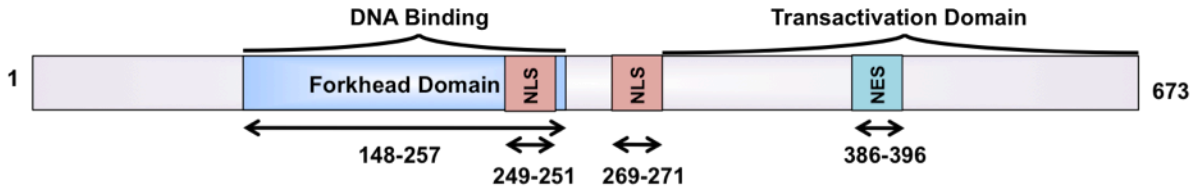
and PPARα suppresses nuclear receptor-mediated transcription. In contrast, FoxO interacts with CAR, PPARγ, PXR and RAR to induce nuclear receptor-mediated transcription. FoxO-mediated transcription is suppressed by the interaction with PPARα/γ and CAR (van der Vos and Coffer 2008). FoxO3a possesses a LxxLL motif in the c-terminal of the Forkhead DNA-binding domain and may interact with nuclear receptors through its LxxLL motif (Zhao, Herrera et al. 2001). Moreover, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) has been reported to interact with FoxO3 and inhibit the transcription of FoxO3a target genes in skeletal muscles (Sandri, Lin et al. 2006). In contrast, PGC-1α interacts with FoxO3a but increases the expression of FoxO3a-dependent oxidative stress protection genes in the vascular endothelium (Olmos, Valle et al. 2009). These data may suggest that the role of FoxO3a and PGC-1α interaction could be tissue-specific. The role of FoxO3a and PGC-1α interaction in FoxO3a-dependent gene transcription in liver has not been studied.

The activation of autophagy is another cellular protective function of FoxO3a. FoxO3a has been shown to activate both autophagic-lysosomal pathway and ubiquitin-proteasomal system in skeletal muscles. In addition to Akt pathway-mediated induction of autophagy, FoxO3a induces the expression of autophagy-related genes and subsequent induction of autophagy independent of mTOR (Mammucari, Milan et al. 2007; Zhao, Brault et al. 2007) Activation of autophagy by FoxO3a also has been verified in cardiomyocytes (Sengupta, Molkentin et al. 2009) and hematopoietic stem cells (Warr, Binnewies et al. 2013). Furthermore, our lab recently demonstrated that acute ethanol-induced autophagy in mouse livers is mediated by the activation of

FoxO3a (Ni, Du et al. 2013). Acute ethanol treatment promotes nuclear FoxO3a translocation by attenuating Akt-mediated phosphorylation of FoxO3a at serine 253 (Ni, Du et al. 2013). Serine 253 phosphorylation of FoxO3a enhances FoxO3a binding with the 14-3-3 protein and targets FoxO3a for degradation by the ubiquitin-proteasomal system (Tzivion, Dobson et al. 2011).

Sirt1 is responsible for deacetylating FoxO3a and tips the balance by shifting the FoxO3a response toward inducing cell cycle arrest and oxidative stress resistance and away from induced cell death (Brunet, Sweeney et al. 2004). Acute ethanol exposure decreases Sirt1 protein levels by increasing the NADH/NAD+ radio and subsequently increases the acetylation of FoxO3a. Interestingly, resveratrol, a Sirt1 agonist, further increases ethanol-induced autophagy related gene expressions (Ni, Du et al. 2013). Altogether, the data suggest that the acetylation of FoxO3a may determine the binding affinity to the binding sites in the promoter regions of autophagy-related genes. Furthermore, FoxO3a has been found to be hepatoprotective against ethanol-induced liver steatosis and injury, and FoxO3a deficient mice display enhanced liver injury, inflammation, and steatosis upon acute and chronic ethanol exposure (Ni, Du et al. 2013; Tumurbaatar, Tikhanovich et al. 2013).

Figure 1.10



Enzyme	Phosphorylation Sites	Effect
Akt	T32 S253 S315	Inhibition
CK1	S318 S321	Inhibition
IKKβ	S644	Inhibition
AMPK	T179 S399 S413 S555 S588 S626	Activation
ERK	S294 S344 S425	Inhibition

Enzyme	Acetylation Sites	Effect
CBP/p300	K242 K245 K259	Inhibition
PCAF	K290	Inhibition
?-Oxidative Stress	K271 K569	Inhibition
SIRT1-Deacetylase	K271 K290	Activation /Inhibition

Enzyme	Ubiquitination Sites	Effect
Skp2	K569 K242? K259? K290?	Inhibition

Structure of FoxO3a and Its Post-Translational Modification Sites.

FoxO3a has a DNA binding forkhead site at amino acids 148-257, two nuclear localization sequences at amino acids 249-251 and 269-271, and a nuclear export site at amino acids 386-396. The c-terminus of FoxO3a also acts as a transactivation domain. FoxO3a has multiple phosphorylation sites that can be phosphorylated by multiple enzymes including Akt, CK1, IKKβ, AMPK, and ERK pathways. Furthermore, FoxO3a also can be acetylated by CBP/p300, PCAF, and an unknown enzyme via oxidative stress. Sirt1 deacetylates FoxO3a. Skp2 ubiquitinates FoxO3a and targets FoxO3a for proteasomal degradation. MDM2 also ubiquitinates FoxO3a at undetermined sites. (Figure Adapted from (Calnan and Brunet 2008)).

CHAPTER 2:

SPECIFIC AIM 1

2.1 Specific Aim 1: Bile acids, in particular deoxycholic acid (DCA), have been reported to increase the GFP-LC3 puncta count in primary rodent hepatocytes (Zhang, Park et al. 2008). Our preliminary data showed that LC3-II and p62 protein expressions were consistently increased in bile acids-treated primary hepatocytes. Mice deficient in autophagy develop spontaneous liver tumors. FXR KO mice also develop spontaneous liver tumors not unlike autophagy deficient mice. Furthermore, my preliminary data have shown that FXR KO mice livers exhibit increased LC3-II and p62 protein levels. **I hypothesize that bile acids suppress autophagy in hepatocytes.** To determine the role and mechanisms by which bile acids regulate autophagy, I examined the following three sub aims.

Figure 2.1

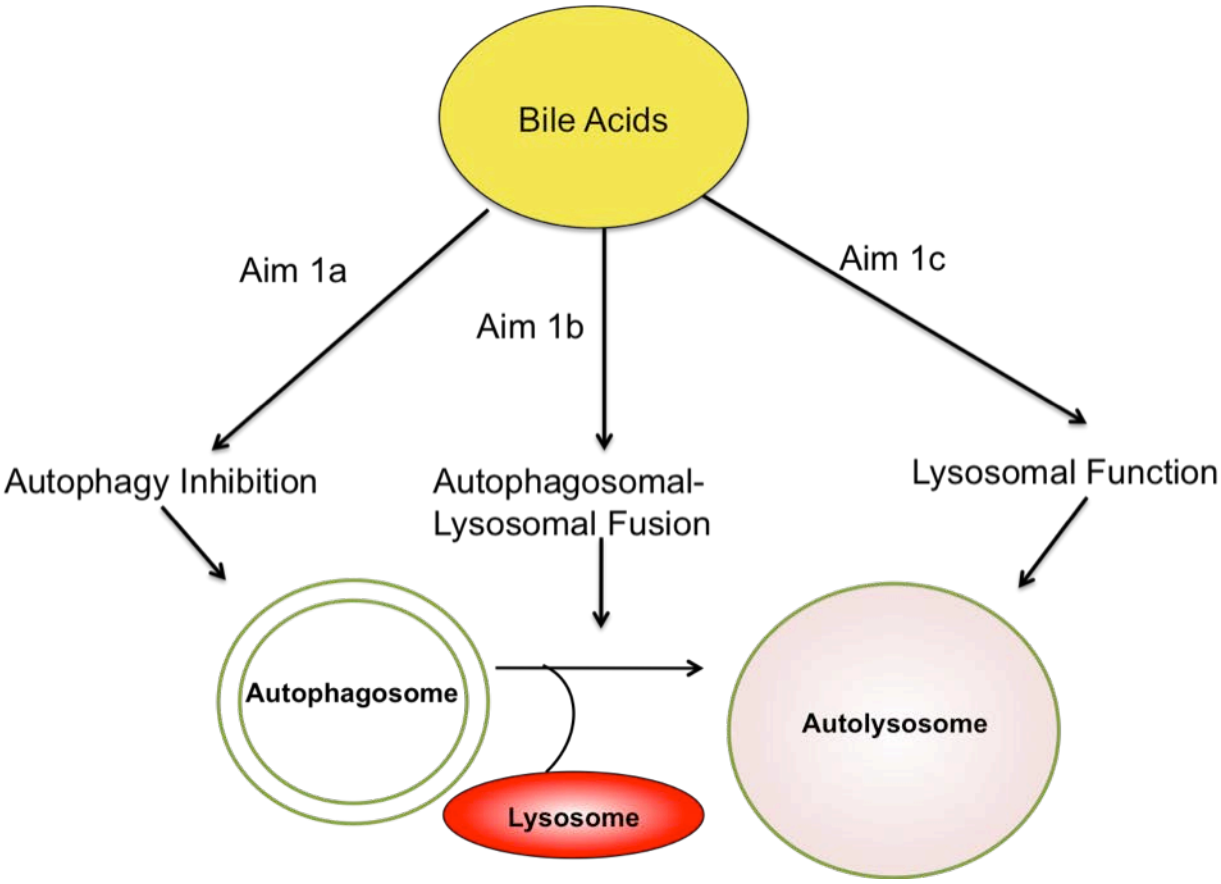


Diagram of the Specific Aim 1. Aim 1a is to determine if bile acids inhibits autophagy. Aim 1b is to investigate the effects of bile acids on autophagosomal-lysosomal fusion, a required event in autophagic degradation process. Aim 2c is to examine the effects of bile acids on lysosomal function and integrity. Bile acids are hypothesized to suppress autophagy in hepatocytes.

2.2 Aim 1a: To determine the effects of bile acids on autophagy.

In my preliminary studies, CA and CDCA both consistently increased the p62 and LC3-II protein levels, suggesting that bile acids may modulate autophagy. In addition, data from the autophagy flux assays suggested that bile acids suppress autophagy. **I hypothesize that bile acids impair autophagy flux.**

2.3 Aim 1b: To examine the effects of bile acids on fusion of autophagosome with lysosome.

Accumulation of LC3-II may occur from either inhibition in the degradation process or increase in the formation of autophagosomes as a result of autophagy induction. The autophagy flux assay from the preliminary data suggests that bile acids suppress autophagy in the late stage due to the accumulation of LC3-II proteins. Late stage inhibition of autophagy may occur from either the inhibition of the autophagosomal-lysosomal fusion process or impaired lysosomal function. **I hypothesize that bile acids impair the autophagosomal-lysosomal fusion process.**

2.4 Aim 1c: To determine the effects of bile acids on the lysosomal functions, the downstream process of autophagy.

Hydrophobic bile acids exert toxic effects and trigger apoptosis. It has been shown that lysosomal membrane permeabilization results in apoptosis. Bile acids possibly mediate the release of cathepsins from the lysosomes and induce the apoptotic pathway. Another possibility is that the bile acids increase pH inside the lysosomes by either lysosome membrane permeabilization or disrupting the pH homeostasis mediated by proton pumping ATPases, chloride channels, and cAMP regulated chloride channels. **I**

hypothesize that hydrophobic bile acids disrupt the lysosomal function by initiating the lysosomal membrane permeabilization event and the subsequent release of cathepsins.

CHAPTER 3:

BILE ACIDS SUPPRESS AUTOPHAGIC FLUX IN HEPATOCYTES

The portions of this section are reproduced from following publication with permission of the publisher:

1. Manley S, Ni HM, Kong B, Apte U, Guo G, and Ding WX. Suppression of autophagic flux by bile acids in hepatocytes. Toxicol Sci. 2014;137(2):478-90. PMCID: 3908720**.**

3.1 INTRODUCTION

Bile acids (BAs) are amphipathic molecules synthesized in the liver and are important for the intestinal absorption of dietary fats and fat-soluble vitamins (Chiang 2003). However, during cholestasis, BAs are accumulated in the liver, resulting in liver injury by inducing cell death (Schoemaker, Gommans et al. 2003). Hydrophobic BAs, such as cholic acid (CA) and chenodeoxycholic acid (CDCA), are known to be toxic in cultured hepatocytes. The mechanisms for BA-induced toxicity are not fully understood, but activation of Fas death receptor, oxidative stress and endoplasmic reticulum stress may be involved (Faubion, Guicciardi et al. 1999; Yerushalmi, Dahl et al. 2001; Tamaki, Hatano et al. 2008).

Autophagy is a cellular lysosomal degradation pathway that degrades cellular proteins and damaged organelles to promote cell survival in response to a variety of stresses. The autophagy process is characterized by the formation of double-membrane autophagosomes. Autophagosomes fuse with lysosomes to form autolysosomes where the autophagosome-enwrapped contents are degraded (Mizushima 2007). The mechanisms by which autophagosomes fuse with lysosomes are not clear, but soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, the small GTP binding protein Rab7, and the homotypic fusion and vacuole protein sorting (HOPS) complex may play a role in regulating the fusion of autophagosomes with lysosomes in mammalian cells (Jager, Bucci et al. 2004; Furuta, Fujita et al. 2010; Itakura, Kishi-Itakura et al. 2012).

Autophagy is a dynamic process, and the ubiquitin-like protein, microtubule-associated protein 1 light chain 3 (LC3), is thought to be important for isolation

membrane elongation and eventual closure of the autophagosomal membrane (Nakatogawa, Ichimura et al. 2007). LC3 is conjugated to phosphatidylethanolamine (PE) (called LC3-II) and targets the autophagosomal membrane (Kabeya, Mizushima et al. 2000). The LC3-II on the outer autolysosome membrane is de-conjugated and removed by the cysteine protease Atg4B and recycled, while the LC3-II on the inner membrane together with the enveloped cytosolic contents are degraded by the lysosome (Kirisako, Ichimura et al. 2000). Thus, the accumulation of LC3-II could be due to either the induction of autophagy or the inhibition of lysosomal functions and/or the defect of fusion of autophagosomes with lysosomes, which leads to impaired degradation of LC3-II (Klionsky, Abdalla et al. 2012). This dynamic process of autophagosome formation, delivery of autophagosomal cargo to lysosomes and completion of lysosomal degradation of cargo is known as autophagic flux. Therefore, autophagic flux is a more accurate indicator of autophagic process than simple measurement of the number of autophagosomes (Mizushima, Yoshimori et al. 2010). Autophagy also selectively degrades some specific autophagy substrates such as sequestosome 1 (SQSTM1)/p62, which accumulate in autophagy-deficient cells or mouse liver (Komatsu, Waguri et al. 2007; Kirkin, Lamark et al. 2009; Ni, Boggess et al. 2012). Thus, determination of LC3-II and p62 levels in the presence or absence of lysosomal inhibitors, such as chloroquine (CQ), has been widely used to monitor autophagic flux (Klionsky, Abdalla et al. 2012).

Farnesoid X receptor (FXR) is a member of the nuclear hormone receptor superfamily and highly expressed in the liver, intestine, kidney and adrenal gland (Forman, Goode et al. 1995; Lu, Repa et al. 2001). FXR plays a critical role in

maintaining cholesterol and BA homeostasis based on studies from FXR knockout (KO) mice and FXR agonists (Sinal, Tohkin et al. 2000; Kim, Morimura et al. 2007). BAs including CA and CDCA are very potent endogenous ligands of FXR. FXR KO mice have increased hepatic apoptosis, inflammation, compensatory proliferation and hepatomegaly, and spontaneous liver tumors in aging mice (Kim, Morimura et al. 2007; Yang, Huang et al. 2007). All these hepatic pathology in FXR KO mice echo those observed in liver-specific Atg7 or Atg5 KO mice, animals with impaired autophagy in the liver (Inami, Waguri et al. 2011; Takamura, Komatsu et al. 2011). While the exact mechanisms by which FXR KO mice develop these phenotypes in the liver are under diligent investigation, FXR KO mice have increased concentrations of BAs in liver and serum, which is suggested to partially responsible for promoting liver tumor formation in mice (Kim, Morimura et al. 2007; Yang, Huang et al. 2007). We thus hypothesized that FXR KO mice have impaired autophagy in the liver, which could be due to increased concentrations of BAs. Here, we report that whole body, but not liver-specific, FXR KO mice had increased accumulation of p62 and LC3-II proteins in the liver, suggesting autophagy is impaired in the livers of whole body FXR KO mice. Furthermore, we found that BAs decreased Rab7 expression and its recruitment to autophagosomes, resulting in impaired autophagosomal-lysosomal fusion and subsequent decreased autophagic flux in primary cultured mouse hepatocytes.

3.2 MATERIALS AND METHODS

Reagents and Antibodies. CA, CDCA, taurocholic acid (TCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA) were obtained from Sigma-Aldrich. All cell culture materials were obtained from Invitrogen. Antibodies used in the study were cleaved caspase-3 (Cell Signaling Cat. #9661), GFP (Santa Cruz Biotechnology, Cat. # sc-9996), p62 (Abnova, Cat. # H00008878-M01), β-Actin (Sigma, Cat. # a5541), Atg5 (MBL, Cat# PM050), GAPDH (Cell Signaling, Cat. # 2118), Rab7 (Cell Signaling Cat. # 9367), and LAMP-1 and LAMP-2 (Developmental Studies Hybridoma Bank, Iowa City, IA, Cat. # 1D4B and Cat. # ABL-93). The rabbit polyclonal anti-LC3B antibody was described previously (Ding, Ni et al. 2009). The secondary antibodies used in this study for immunoblotting analysis were HRP-conjugated goat anti-mouse (Jackson ImmunoResearch, Cat. #115-035-062), rabbit (Jackson ImmunoResearch, Cat. #111-035-045), and rat (Jackson ImmunoResearch Cat. # 111-035-143) antibodies. The secondary antibodies used for immunostaining were Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch Cat. # 111-165-144), Cy3-conjugated goat anti-rat (Jackson ImmunoResearch Cat. #112-165-143), DyLight^{TM} conjugated goat anti-mouse (Jackson ImmunoResearch Cat. #115-505-146), and AlexaFluor 488 goat anti-rabbit antibodies (Invitrogen).

Animals and Primary Mouse Hepatocyte Culture. Wild type (WT) C57BL/6J, FXR KO, FXR $^{flox}/^{flox}$ (Albumin Cre negative or positive) mice (FXR $^{fl}/^{fl}$, Alb Cre- or FXR $^{fl}/^{fl}$, Alb Cre+) were housed with free access to water and chow. All animals received humane care. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. Mouse hepatocytes isolated

from WT and FXR-/- mice were described previously (Ding, Ni et al. 2004), and cultured in William's medium E with 10% fetal bovine serum, 2 mM glutamine as well as routine antibiotic supplements for 2 h for attachment. Cells were then cultured in the same medium without serum overnight before treatment. All cells were maintained in a 37°C incubator with 5% CO_2.

Viability Assay.

Primary hepatocytes were seeded in a 12 well plate (1 x 10^5 per well) and cells were treated with various concentrations (50, 100, or 200 µM) of BAs for 6 or 24 h. Cells were stained with propidium iodide (1 µg/mL) and Hoechst 33342 (1 µg/mL) followed by fluorescence microscopy. Both apoptotic (condensed and fragmented nuclei) and PI positive cells were considered dead cells, and the percentage of viable cells was calculated. More than 300 cells were counted from each experiment and data are means± SD from 3 independent experiments

Fluorescence, Confocal and Electron Microscopy.

Adenovirus expressing GFP-LC3 was used as described previously (Gao, Ding et al. 2008). To examine autophagy, primary hepatocytes were seeded in a 12 well-plate (1 x 10^5 per well) on a cover slide and infected with adenovirus-GFP-LC3 (100 viral particles per cell) overnight. Cells were treated with CA (100 µM), CDCA (100 µM) and TCA (100 µM) in the presence or absence of chloroquine (CQ, 20 µM) for 6 h. For some experiments, hepatocytes were transfected with RFP-GFP-LC3 using TurboFect (Fermentas) for 24 h followed by designated treatments. After treatments, cells were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 2 h at

room temperature or kept at 4°C for microscopy. Fluorescence images were acquired under a Nikon Eclipse 200 fluorescence microscope with MetaMorph software. For the immunostaining assay, fixed primary hepatocytes were immunostained with anti-Lamp1 or anti-Rab7 antibodies followed by Cy3-conjugated secondary antibody and Hoechst 33342 staining as previously described. Cell images were then obtained using a Leica Confocal microscope (TCS SPE model). For electron microscopy (EM), hepatocytes were fixed with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4), followed by 1% OsO_4. After dehydration, thin sections were cut and stained with uranyl acetate and lead citrate. Digital images were obtained using a JEM 1016CX electron microscope.

DQ-bovine serum albumin (BSA) Assay. Primary cultured mouse hepatocytes were first loaded with DQ-BSA (10 µg/mL, Invitrogen) 1 hr prior to treatment with various BAs and CQ for another 6 h. DQ-BSA is a derivative of BSA that is labeled to such a high degree with BODIPY® dyes, BODIPY TR-X, that the dye is strongly self-quenched. Proteolysis of the BODIPY-BSA conjugate results in de-quenching and the released protein fragment that contains isolated red fluorophore is brightly fluorescent that has excitation and emission maxima of 590 nm and 620 nm. Following treatment, hepatocytes were washed with PBS to remove excessive DQ-BSA and lysed in 1% Triton X-100 in 50 mM Tris-HCL (pH 8.8) solution. Fluorescence intensity of the lysates was quantified using a Tecan Infinite M200 Pro plate reader (excitation: 590 and emission: 620).

RNA Isolation and Real-Time qPCR. RNA was isolated from cultured hepatocytes and livers using Qiagen RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed into

cDNA by RevertAid reverse transcriptase (Fermentas). Real-time PCR was performed on an Applied Biosystems Prism 7900HT real-time PCR instrument (ABI, Foster City, CA) using Maxima SYBR green/rox qPCR reagents (Fermentas). Primer sequences were as follows: *β-actin* forward: 5' – TGT TAC CAA CTG GGA CGA CA – 3'; *β-actin* reverse: 5' – GGG GTG TTG AAG GTC TCA AA – 3'; *Map1lc3b* forward: 5' – CCGAGAAGACCTTCAAGCAG – 3'; *Map1lc3b* reverse: 5' – ACACTTCGGAGATGGGAGTG – 3'; *Sqstm1/p62* forward: 5' – AGA ATG TGG GGG AGA GTG TG– 3'; *Sqstm1/p62* reverse: 5' – TCG TCT CCT CCT GAG CAG TT – 3'; *Shp* forward: 5' – CTG CAG GTC GTC CGA CTA TT – 3'; and *Shp* reverse: 5' – ACC TCG AAG GTC ACA GCA TC – 3'.

Cathepsin B and Proteasome Activity Assay. Specific fluorescence substrates were used to measure Cathepsin B and proteasome activities. Primary cultured mouse hepatocytes were treated with CA (100 μM), CDCA (100 μM), TCA (100 μM), CQ (20 μM) or E64D (10 μM) for 6 h. After treatment, cells were lysed in M2 buffer (50 mM Tris pH 7.5, 130 mM NaCl, 10% glycerol, 0.5% NP-40, 0.5 mM EDTA, and 0.5 mM EGTA in dH_2O) without protease inhibitors. To determine cathepsin B activity, protein lysates (15 μg) were incubated with 2 μM z-RR-AMC (Calbiochem # 219392) in assay buffer (10 mM HEPES-NaOH pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 mM PMSF, and 1 mM DTT in dH_2O) for 1 hr, and fluorescence intensity was determined using a Tecan Infinite M200 Pro plate reader (excitation: 380 and emission: 460). To determine proteasome activity, protein lysates (10 μg) were incubated with 0.65 μM Suc-LLVY-AMC

(Calbiochem #539142) in assay buffer (50 mM Tris pH 7.5, 25 mM KCl, 10 mM NaCl, and1mM $MgCl_2$) for 1 hr, and the fluorescence intensity was determined using a Tecan Infinite M200 Pro plate reader (excitation: 380 nm and emission: 460 nm).

Immunoblot Analysis. Cells were washed in PBS and lysed in RIPA buffer. Twenty micrograms of protein from each sample were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were stained with primary antibodies followed by secondary horseradish peroxidase-conjugated antibodies. The membranes were further developed with SuperSignal West Pico chemiluminescent substrate (Pierce). Densitometry analysis was performed using Un-Scan-It software and further normalized with β-actin.

Statistical Analysis. All experimental data were expressed as mean ± SE and subjected to a Student t-test or one-way analysis of variance with Holm-Sidak post hoc test where appropriate. *$p<0.05$ was considered significant.

3.3 RESULTS

Increased Accumulation of Hepatic p62 and LC3-II Proteins in FXR KO Mice

Since mice with autophagy-deficiency in the liver and FXR KO mice all develop spontaneous liver tumors, we hypothesized that FXR KO mice may have impaired autophagy in the liver. Indeed, similar to liver-specific Atg7 or Atg5 KO mice, both 3 and 6 month old FXR KO mice had increased accumulation of p62 proteins in the liver **(Figure 3.1A)**. Interestingly, hepatic LC3-II protein levels were also increased in 3 and 6 month old FXR KO mice compared to their age-matched WT controls. In contrast, there was no difference between WT and FXR KO mice for other autophagy proteins responsible for regulation of upstream events for the formation of autophagosomes, such as Atg5-Atg12 and Beclin-1. Moreover, there was no difference in hepatic mRNA levels of *Sqstm1/p62* or *Map1lc3b (Microtubule-associated protein 1 light chain 3 beta)* between WT and FXR KO mice, whereas the level of *Shp*, a FXR target gene, was dramatically diminished in FXR KO mice **(Figure 3.1B)**. It is known that FXR KO mice have increased hepatic and serum BAs (Sinal, Tohkin et al. 2000; Kim, Morimura et al. 2007), we next determined whether the increased levels of p62 and LC3-II was due to the increased BAs or the lack of FXR. We thus determined the levels of p62 and LC3-II in liver-specific FXR KO mice, which we previously showed that these mice had normal hepatic BAs (Borude, Edwards et al. 2012). No significant differences were found for p62, LC3-II, Atg5-Atg12 and Beclin-1 protein expression between liver-specific FXR KO mice and their WT littermates **(Figure 3.1C)**. Altogether, these data indicate that FXR KO mice had increased hepatic levels of p62 and LC3-II, which is less likely due to the direct effects of lack of FXR.

Figure 3.1

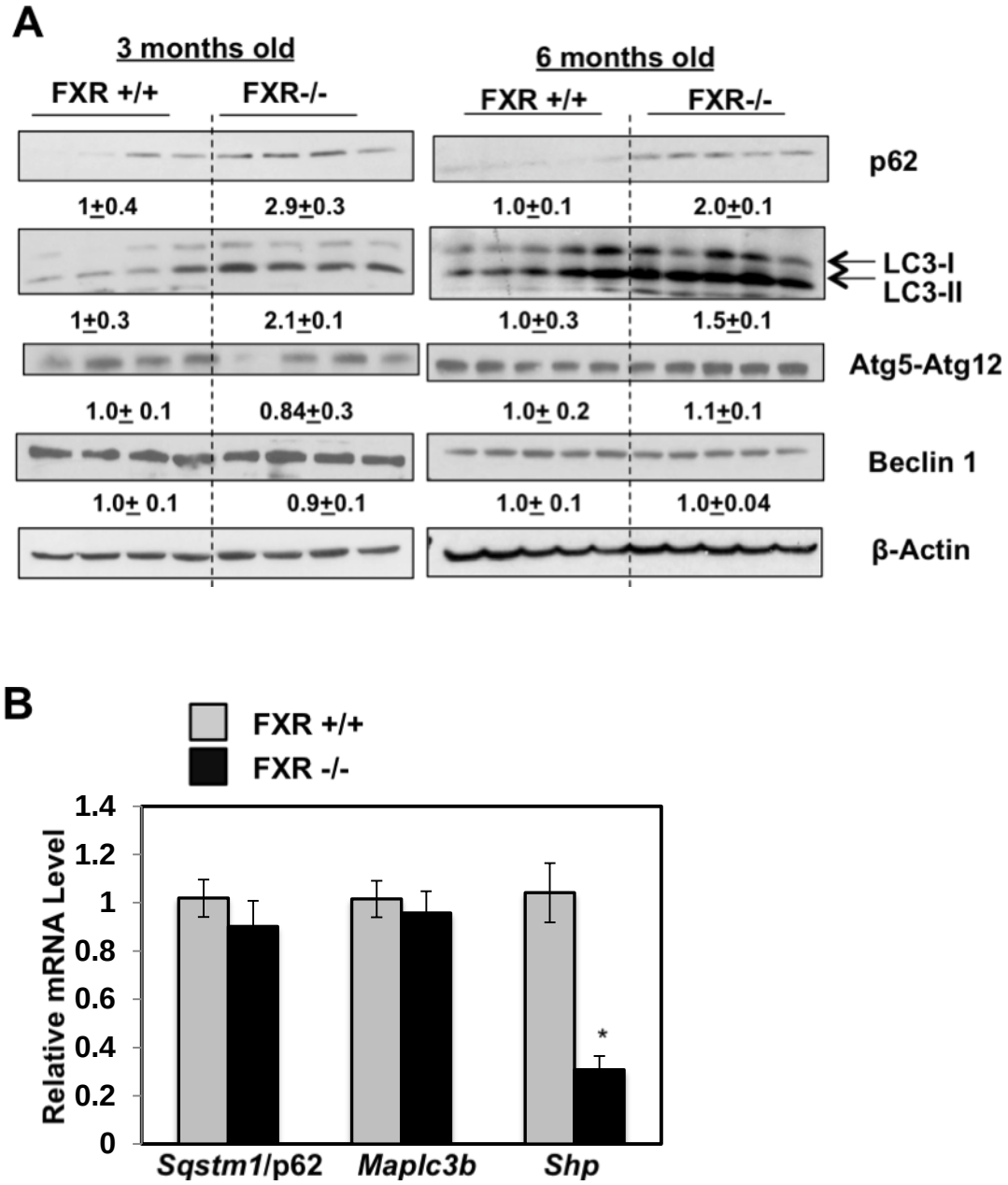
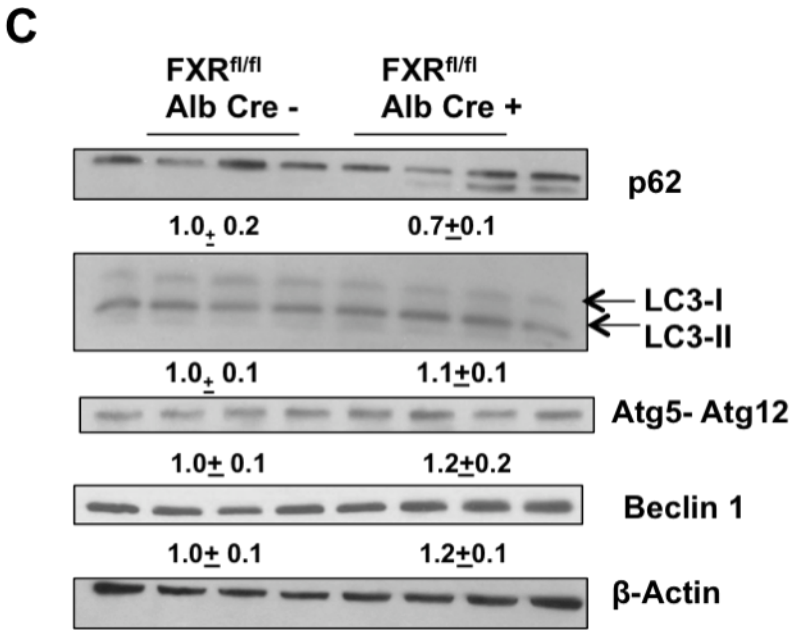

A
3 months old
6 months old
FXR +/+
FXR-/-
FXR +/+
FXR-/-
p62
1±0.4
2.9±0.3
1.0±0.1
2.0±0.1
LC3-I
LC3-II
1±0.3
2.1±0.1
1.0±0.3
1.5±0.1
Atg5-Atg12
1.0± 0.1
0.84±0.3
1.0± 0.2
1.1±0.1
Beclin 1
1.0± 0.1
0.9±0.1
1.0± 0.1
1.0±0.04
β-Actin
B
FXR +/+
FXR -/-
Relative mRNA Level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Sqstm1/p62
Maplc3b
Shp

Figure 3.1 (cont'd)



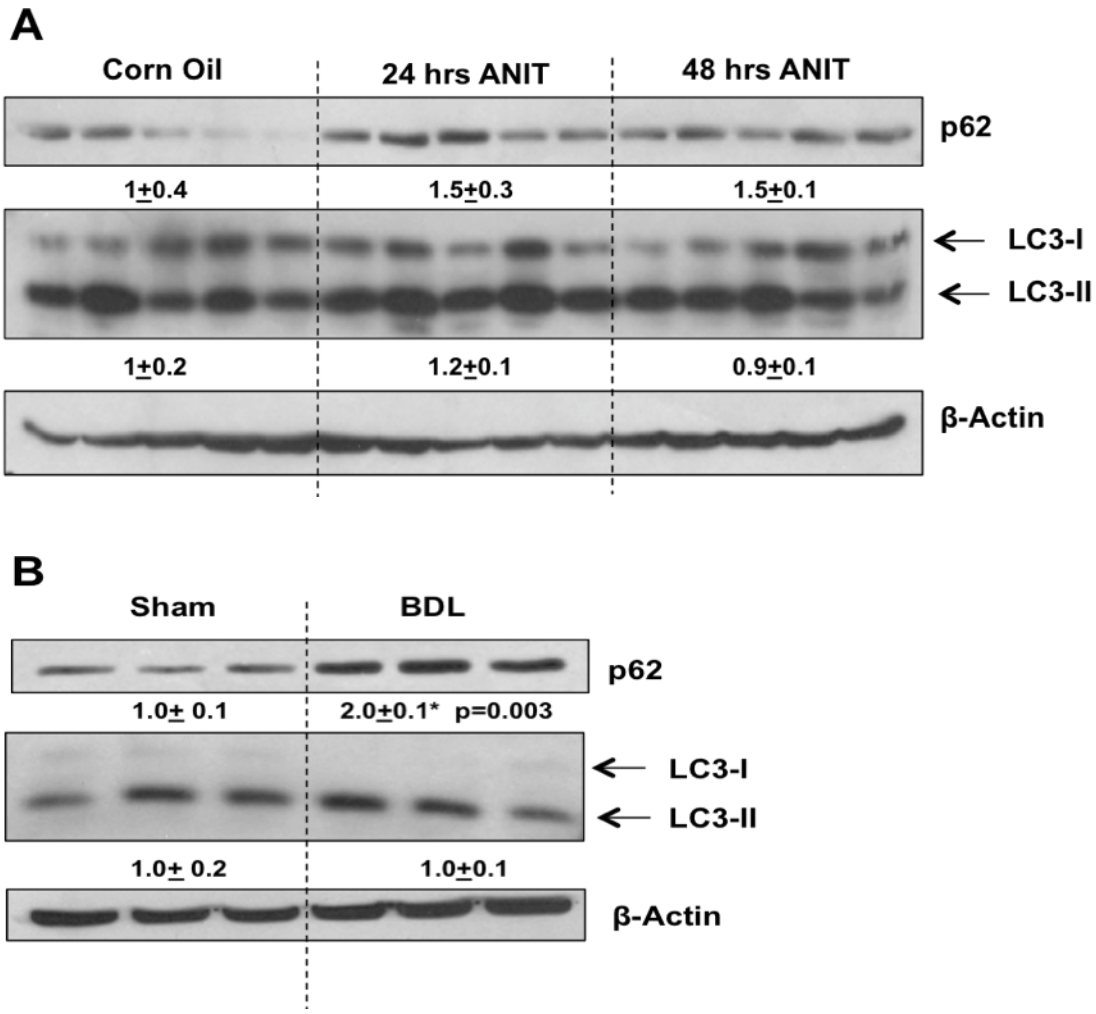
Increased p62 and LC3-II protein expression in FXR KO mouse livers.
(A) Total lysates from 3 and 6 month old FXR+/+ and FXR-/- mouse livers were subjected to immunoblot analysis. Digital data are densitometry analysis and presented as a ratio of wild type mice (n=4-5). **(B)** mRNA was isolated from 3 months old FXR+/+ and FXR-/- KO mouse livers and qRT-PCR was performed. The gene expression levels were normalized to β-actin and shown as fold increase over wild type mice (n=6-7). **(C)** Total liver lysates from 3 months old $FXR^{fl/fl}$:Alb-Cre^{-} and $FXR^{fl/fl}$:Alb Cre^{+} mouse livers were subjected to immunoblot and densitometry analysis .

Accumulation of Hepatic p62 Protein in Intrahepatic and Extrahepatic Cholestasis Mouse Models

We demonstrated that whole body FXR KO, but not hepatic specific FXR KO mice have p62 and LC3-II protein accumulation, which suggests bile acids impair autophagy. To confirm that bile acids, not FXR deficiency, inhibit autophagy, we examined the p62 and LC3-II protein levels in other cholestasic mouse models. α-naphthylisothiocyanate (ANIT) is a widely used chemical to induce intrahepatic cholestasis in rodents (Becker and Plaa 1965; Capizzo and Roberts 1970). ANIT is detoxified in the liver by glutathione (GSH) conjugation, and ANIT-GSH compound is transported into the bile, where ANIT is dissociated from GSH. The free ANIT then selectively damages bile-duct epithelial cells resulting in cholangitis, and subsequent intrahepatic cholestasis (Carpenter-Deyo, Marchand et al. 1991; Dietrich, Ottenhoff et al. 2001). In the ANIT model, we found that ANIT induces p62, but not LC3-II, protein expression by 1.5 fold at both 24 and 48 hours after ANIT administration **(Figure 3.2A)**. Bile duct ligation (BDL) is the most common used model for extrahepatic cholestasis since it closely mimics the pathogenesis of obstructive cholestasis (Schaffner, Bacchin et al. 1971). BDL induces hepatic and serum bile acids accumulation at 6 hours to 14 days (Kinugasa, Uchida et al. 1981; Zhang, Hong et al. 2012). We found similar pattern with BDL after three days as ANIT, in which p62 protein expression was increased two fold, but the LC3-II protein expression was unchanged **(Figure 3.2B)**. The reasons for the lack of increase in LC3-II protein level in ANIT-treated and bile duct ligated mice are not clear, but could be due to the differences in the models in comparison to FXR KO mice. However, it is also possible that the transcriptional level of p62 and LC3 could be altered in these models.

Nevertheless, the p62 accumulation suggests that the autophagic clearance of p62 and its cargo might be impaired.

Figure 3.2



Increased p62 protein expression in ANIT-treated and bile duct ligated mouse livers.
(A) Total lysates from 0, 24 and 48 hours ANIT-treated mouse livers were subjected to immunoblot analysis. Digital data are densitometry analysis and presented as a ratio of wild type mice (n=5). **(B)** Mice underwent sham or bile duct ligation surgery, and total liver lysates were collected three days after the surgery. Total lysates were subjected to immunoblot analysis. Digital data are densitometry analysis and presented as a ratio of wild type mice (n=3).

Hydrophobic BAs Decreased Viability in Primary Cultured Mouse Hepatocytes

Three major primary BAs were chosen for the *in vitro* studies, TCA, CA and CDCA. TCA was chosen because serum and hepatic levels of TCA increased to around 1 mM in BDL mice (Zhang, Hong et al. 2012). CA was chosen because serum levels of CA increased to 200-250 μM in mice subjected to BDL for 7 days (Marschall, Wagner et al. 2006), and CA has been widely used in feeding experiments in mice that show significant toxicity in mouse liver (Sinal, Tohkin et al. 2000; Kim, Morimura et al. 2007). We also chose CDCA because serum levels of CDCA increased in BDL mice, and CDCA increases the expression of inflammatory genes in cultured mouse hepatocytes (Allen, Jaeschke et al. 2011). Ursodeoxycholic acid (UDCA) is chosen because it is a therapeutic bile acid used in treating cholestasis, and alleviates cholestasic liver disease by protecting cholangiocytes against cytotoxic hydrophobic bile acids, stimulating hepatobiliary secretion, and protection against bile acid-induced apoptosis (Paumgartner and Beuers 2002). Finally, deoxycholic acid (DCA), a secondary bile acid, is chosen because DCA has been reported to induce GFP-LC3 puncta formation in rodent hepatocytes (Zhang, Park et al. 2008) and human esophageal cells (Roesly, Khan et al. 2012) and LC3-II protein expression in colon epithelial cells (Payne, Crowley-Skillicorn et al. 2009). The hydrophobicity index of BAs is UDCA<TCA<CA<CDCA<DCA, in which UDCA is considered as hydrophilic BA and TCA is considered as neutral BA (Heuman 1989; Thomas, Pellicciari et al. 2008).

CDCA and DCA are well-known hepatotoxins and induce hepatocyte death *in vitro* (Scholmerich, Becher et al. 1984). Indeed, viability assay analysis showed that CDCA and DCA, but not UDCA, CA or TCA are toxic in a dose-dependent manner at 6

hours **(Figure 3.3A)**. Moreover, TCA and CDCA also showed toxicity in a time-dependent manner, but the rest of the BAs didn't display time-dependent toxicity. More importantly, CQ is toxic in a time dependent manner, in which 80% of cells were viable at 6 hours, but only 12% of cells were viable at 24 hours **(Figure 3.3B)**. Caspase-3 is a death protease that is activated when cleaved; therefore, cleaved caspase-3 is the hallmark of apoptosis (Porter and Janicke 1999). Indeed, CDCA induced hepatocyte apoptosis via caspase-3 cleavage at 100 and 200 µM, but CA did not initiate caspase-3 cleavage. Altogether, the data show that more hydrophobic BAs induce hepatocyte apoptosis via cleaved caspase-3 mediated apoptosis, whereas, more hydrophilic BAs have minimal effects on hepatocyte viability under the culture conditions.

Figure 3.3

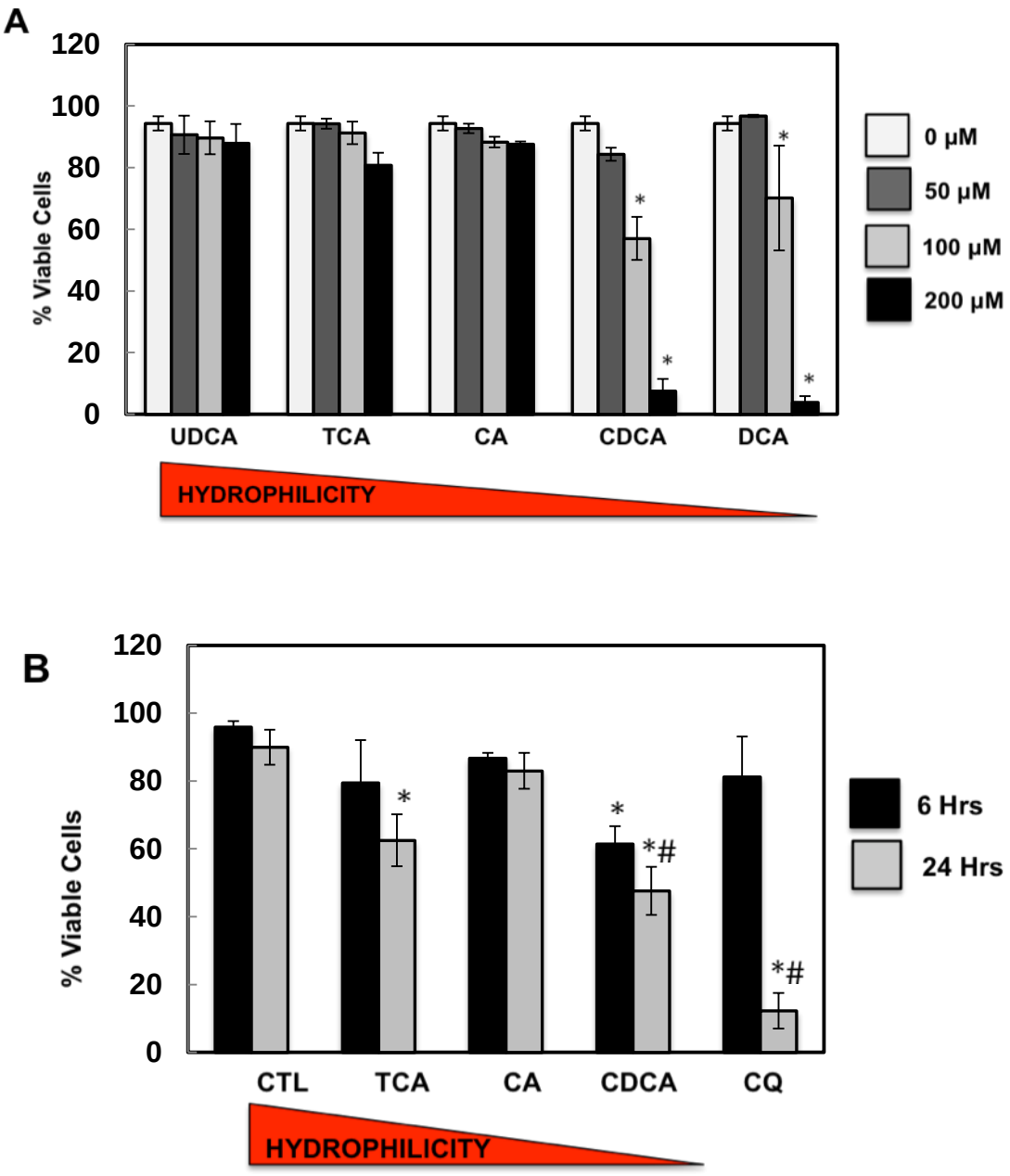
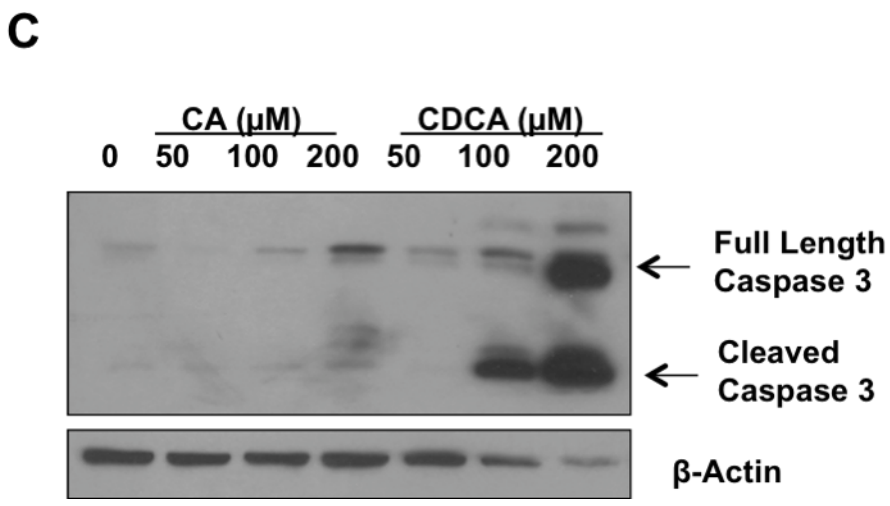

A
120
100
80
60
40
20
0
% Viable Cells
UDCA
TCA
CA
CDCA
DCA
HYDROPHILICITY
0 µM
50 µM
100 µM
200 µM
B
120
100
80
60
40
20
0
% Viable Cells
CTL
TCA
CA
CDCA
CQ
HYDROPHILICITY
6 Hrs
24 Hrs

Figure 3.3 Cont'd



Hydrophobic BAs Decreased Hepatocyte Viability via Cleaved-Caspase-3 Mediated Apoptosis.
(A&B) Cells were stained with propidium iodide (1 μg/mL) and Hoechst 33342 (1 μg/mL) followed by fluorescence microscopy. Both apoptotic (condensed and fragmented nuclei) and PI positive cells were considered dead cells, and the percentage of viable cells was calculated. More than 300 cells were counted from each experiment and data are means± SEM from 3 independent experiments. **A** is dose-response viability assay, and **B** is time-dependent viability assay. **(C)** Primary hepatocytes were treated with different concentrations of CA and CDCA for 6 h. Whole cell lysates were subjected to immunoblot analysis for caspase-3. ***** $p<0.05$ vs control. # $p<0.05$ vs 6 hours treatment.

BAs Increased p62 and LC3-II Proteins in Primary Cultured Mouse Hepatocytes

Since hepatic BA levels were elevated in FXR KO mice (Sinal, Tohkin et al. 2000; Kim, Morimura et al. 2007), we determined whether BAs would directly modulate p62 and LC3-II levels. Exposure of primary hepatocytes to TCA, CA and CDCA increased p62 and LC3-II protein levels in a time-dependent manner (**Figure 3.4A**). Moreover, TCA, CA, CDCA and DCA, but not UDCA exposure increased p62 and LC3-II protein expressions in a dose-dependent manner **(Figure 3.4B)**. We have previously found that unlike the liver tissue, it was difficult to detect the LC3-I form in the primary cultured mouse hepatocytes. This either could be due to the high basal level of autophagy in primary cultured or the antibody that we used preferentially binding to LC3-II form (Ding, Ni et al. 2009; Ding, Li et al. 2010). Exposure of primary hepatocytes to CQ, which suppresses autophagy by increasing lysosomal pH, also increased p62 and LC3-II protein levels in a time-dependent manner (**Figure 3.4C**). The increased cell death at 24 hours treatment might play a role in the changes of the levels of the proteins that we assessed. Indeed, BAs treatment increased the protein levels of p62, which is consistent with the 6 hours treatment. In contrast to the results after 6 hours treatment, CDCA actually decreased LC3-II levels after 24 hours treatment likely due to the increased cell death **(Figure 3.4D).** Consistent with the immunoblotting analysis, BA- and CQ-treated hepatocytes had increased number and size of p62 dots (**Figure 3.4E**). BA treatment did not increase mRNA levels of *Map1lc3b* or *Sqstm1/p62,* but both CA and CDCA increased the mRNA level of *Shp,* a well-known FXR target gene that is induced by BAs (**Figure 3.4F**). CQ treatment slightly increased the expression of *Map1lc3b and Sqstm1/p62* but decreased the expression *of Shp* **(Figure 3.4F)***.* These

results indicate that BA-induced p62 and LC3-II protein accumulations are due to post-translational regulation but not due to regulation of gene transcription in primary mouse hepatocytes.

No Increased Autophagic Flux in BA-treated Primary Mouse Hepatocytes

We next performed autophagic flux assays in BA-treated hepatocytes. Primary hepatocytes were exposed to BAs in the presence or absence of CQ for 6 h. As can be seen, LC3-II levels were increased in BA-treated cells except for UDCA and were further increased in the presence of CQ, but the co-treatment of hydrophobic BAs with CQ had almost the same LC3-II levels as treatment with CQ alone (**Figure 3.5A**). Moreover, the co-treatment of UDCA with CQ had more LC3-II level than treatment with CQ alone, which suggests that UDCA induced autophagic flux. Furthermore, the number of GFP-LC3 puncta increased in BA-treated cells, but the number of GFP-LC3 puncta was not further increased in cells co-treated with BAs and CQ compared to cells that were treated with CQ alone (**Figure 3.5B and 3.5C**). To further determine whether BAs-induced impaired autophagic flux would require FXR, we treated primary cultured FXR-/- hepatocytes with BAs in the presence of absence of CQ for 6 h. Similar to the results that we observed in WT hepatocytes, both CA and CDCA increased p62 protein level and did not further increase the LC3-II level in the presence of CQ compared to the CQ alone treatment **(Figure 3.5D)**. These results indicate that more hydrophobic BAs did not increase autophagic flux in both WT and FXR-/- hepatocytes. In contrast, hydrophilic BA, UDCA appeared to induce autophagy flux, which suggests that UDCA may be protective due to the induction of autophagy. The lack of increased autophagic flux, together with increased p62 levels by BAs, suggests that hydrophobic BAs may

impair autophagic degradation in hepatocytes independent of FXR.

Figure 3.4

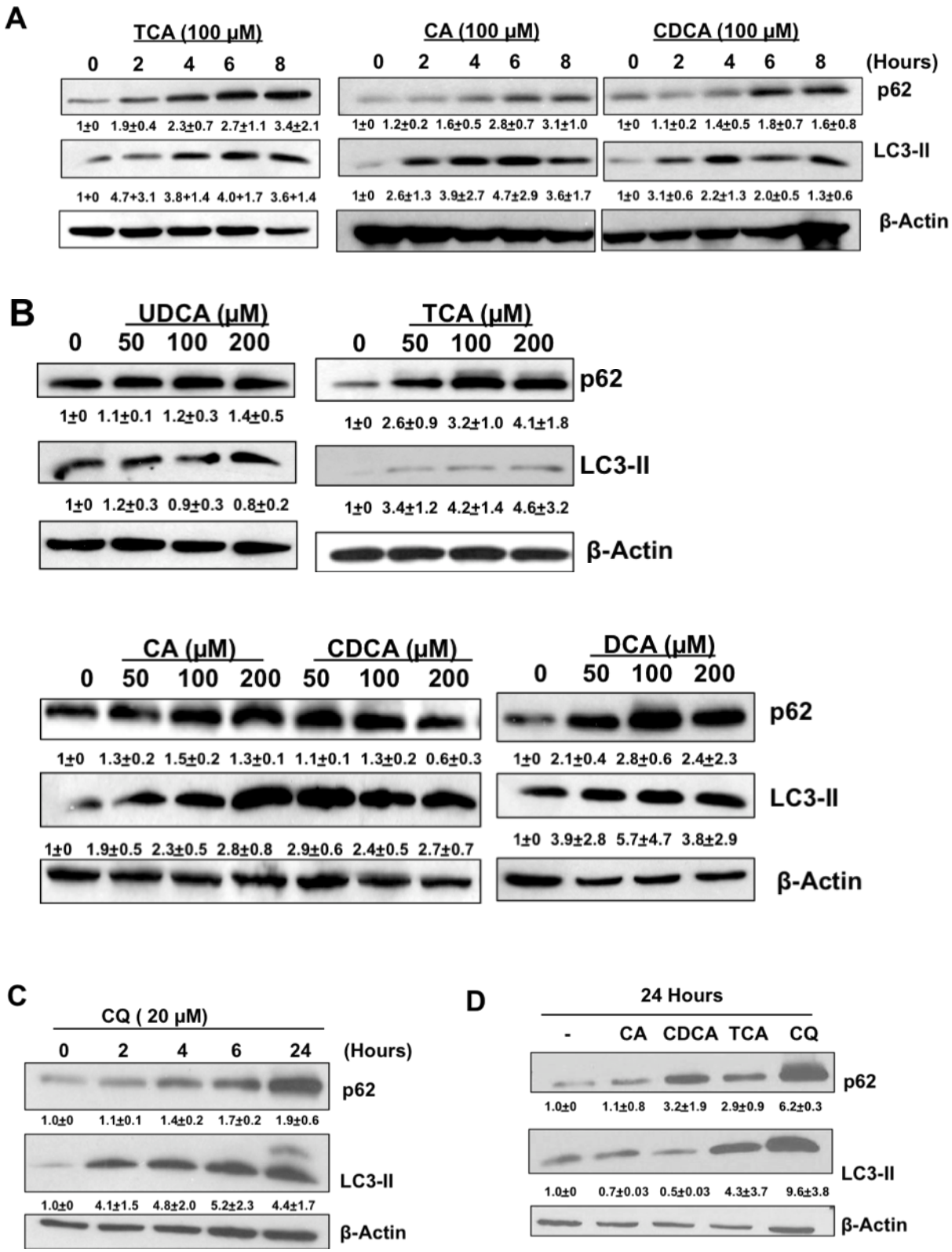
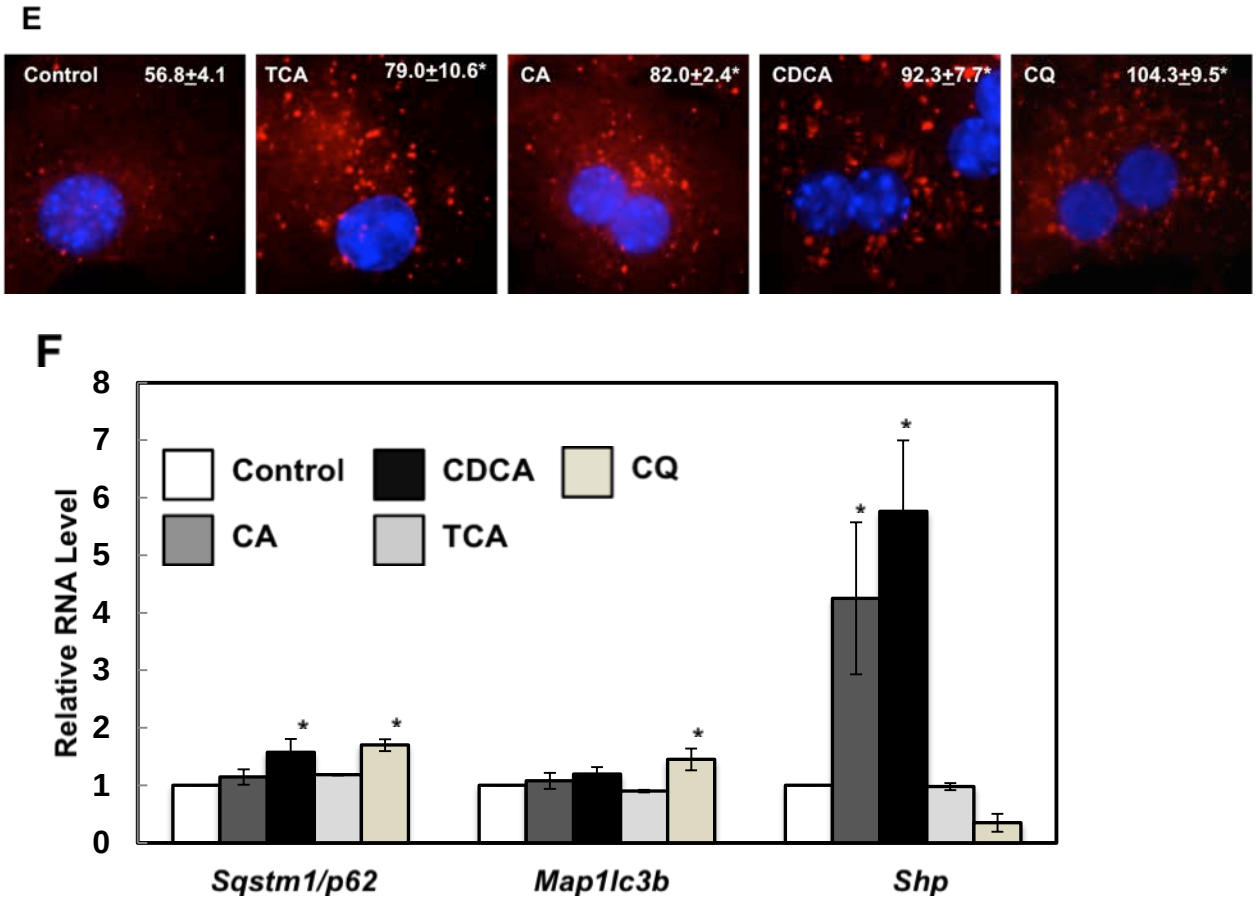
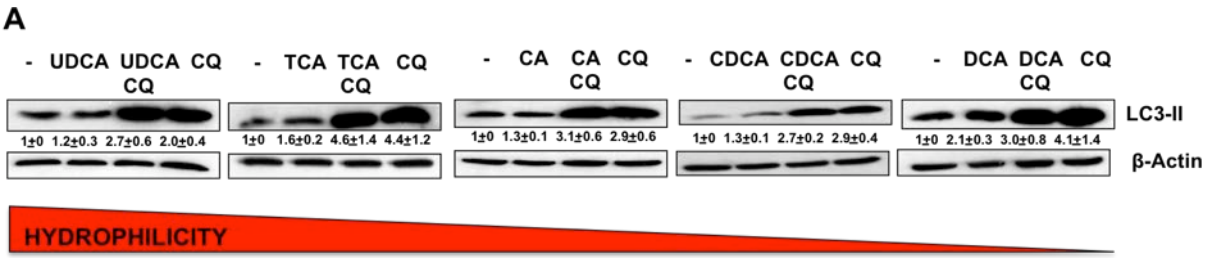

A
TCA (100 μM)
CA (100 μM)
CDCA (100 μM)
0 2 4 6 8 (Hours)
p62
1±0 1.9±0.4 2.3±0.7 2.7±1.1 3.4±2.1
1±0 1.2±0.2 1.6±0.5 2.8±0.7 3.1±1.0
1±0 1.1±0.2 1.4±0.5 1.8±0.7 1.6±0.8
LC3-II
1+0 4.7+3.1 3.8+1.4 4.0+1.7 3.6+1.4
1±0 2.6±1.3 3.9±2.7 4.7±2.9 3.6±1.7
1±0 3.1±0.6 2.2±1.3 2.0±0.5 1.3±0.6
β-Actin
B
UDCA (μM)
TCA (μM)
0 50 100 200
p62
1±0 1.1±0.1 1.2±0.3 1.4±0.5
1±0 2.6±0.9 3.2±1.0 4.1±1.8
LC3-II
1±0 1.2±0.3 0.9±0.3 0.8±0.2
1±0 3.4±1.2 4.2±1.4 4.6±3.2
β-Actin
CA (μM)
CDCA (μM)
DCA (μM)
0 50 100 200 50 100 200 0 50 100 200
p62
1±0 1.3±0.2 1.5±0.2 1.3±0.1 1.1±0.1 1.3±0.2 0.6±0.3
1±0 2.1±0.4 2.8±0.6 2.4±2.3
LC3-II
1±0 1.9±0.5 2.3±0.5 2.8±0.8 2.9±0.6 2.4±0.5 2.7±0.7
1±0 3.9±2.8 5.7±4.7 3.8±2.9
β-Actin
C
CQ (20 μM)
0 2 4 6 24 (Hours)
p62
1.0±0 1.1±0.1 1.4±0.2 1.7±0.2 1.9±0.6
LC3-II
1.0±0 4.1±1.5 4.8±2.0 5.2±2.3 4.4±1.7
β-Actin
D
24 Hours
- CA CDCA TCA CQ
p62
1.0±0 1.1±0.8 3.2±1.9 2.9±0.9 6.2±0.3
LC3-II
1.0±0 0.7±0.03 0.5±0.03 4.3±3.7 9.6±3.8
β-Actin

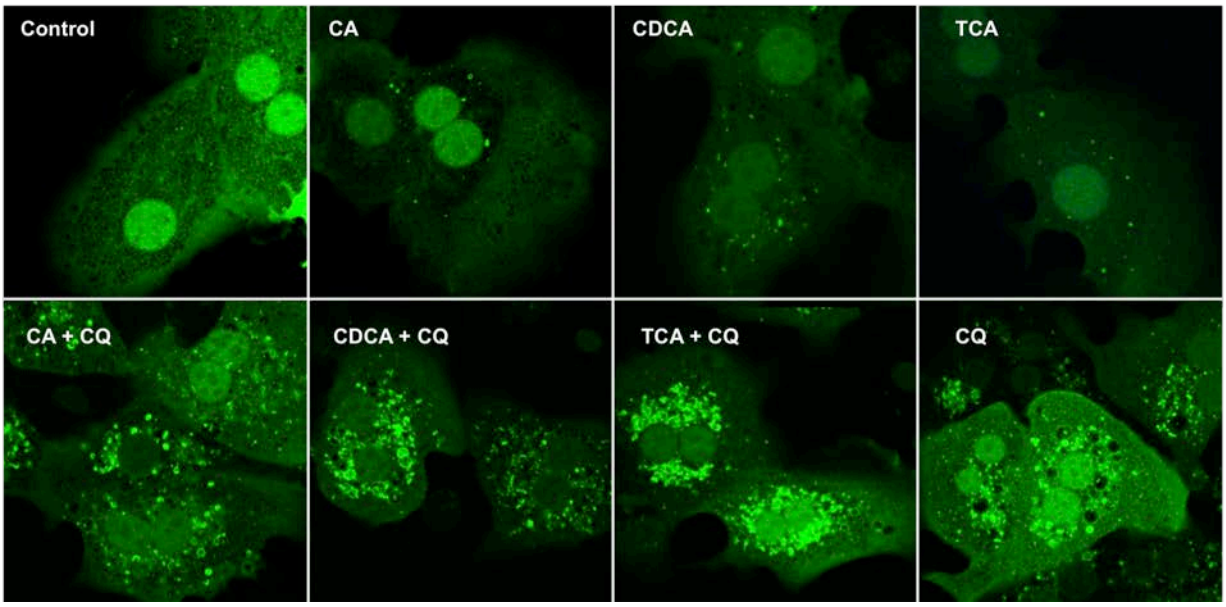
Figure 3.4 Cont'd



BAs increased p62 and LC3-II protein levels in hepatocytes in a time- and dose-dependent manner.
Primary hepatocytes were treated with CA, CDCA, or TCA for 0, 2, 4, 6 and 8 h **(A)** or **(B)** with different concentrations of BAs for 6 h. Whole cell lysates were subjected to immunoblot analysis. Densitometry analysis data are presented as a ratio of control (n=3-4). **(C)** Primary mouse hepatocytes were treated with 100 μM of CA, CDCA, TCA and CQ (20 μM) for 24 hrs. Total cell lysate were subjected to immunoblot analysis, and densitometry analysis was performed as described in **(A)** (n=3). **(D)** Primary hepatocytes were treated with CQ for various time points and total cell lysates were subjected to immunoblot analysis, and densitometry analysis was performed as described in **(A)** (n=3). **(E)** Hepatocytes were treated with 100 μM of TCA, CA or CDCA for 6 h and immunostained for p62 followed by fluorescence microscopy. Representative images are shown. The number of p62 puncta per cell was quantified (>20 cells were counted in each experiment from at least 3 independent experiments). **(F)** Primary hepatocytes were treated as in **(E)** and qRT-PCR was performed. The gene expression levels were normalized to β-actin and shown as fold increase over control (n=3).

Figure 3.5


A
- UDCA UDCA CQ CQ
- TCA TCA CQ CQ
- CA CA CQ CQ
- CDCA CDCA CQ CQ
- DCA DCA CQ CQ
1±0 1.2±0.3 2.7±0.6 2.0±0.4
1±0 1.6±0.2 4.6±1.4 4.4±1.2
1±0 1.3±0.1 3.1±0.6 2.9±0.6
1±0 1.3±0.1 2.7±0.2 2.9±0.4
1±0 2.1±0.3 3.0±0.8 4.1±1.4
LC3-II
β-Actin
HYDROPHILICITY


B
Control
CA
CDCA
TCA
CA + CQ
CDCA + CQ
TCA + CQ
CQ

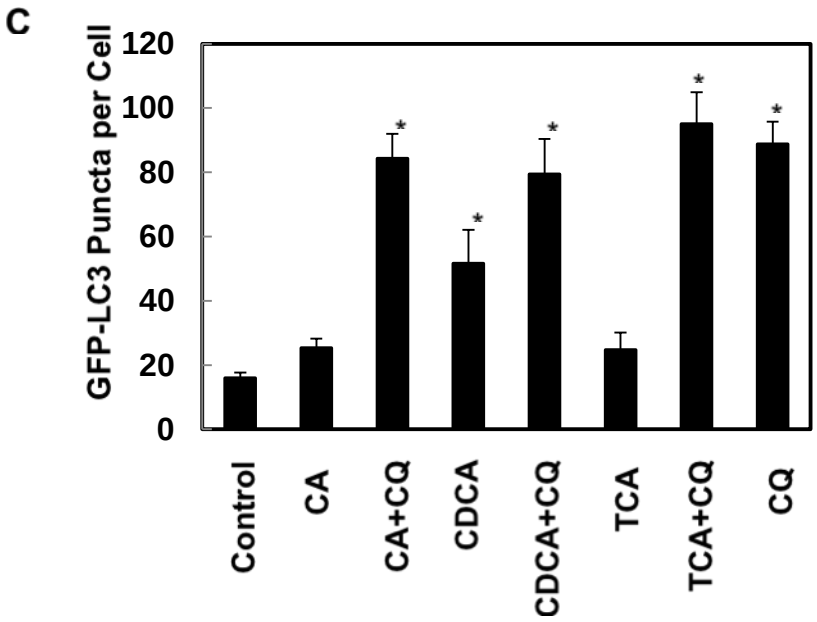
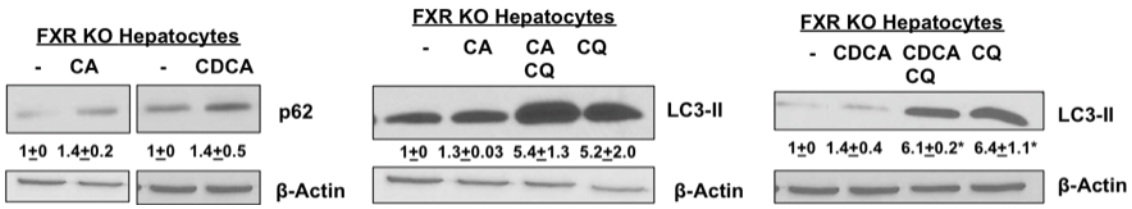

C
GFP-LC3 Puncta per Cell
120
100
80
60
40
20
0
Control
CA
CA+CQ
CDCA
CDCA+CQ
TCA
TCA+CQ
CQ

Figure 3.5 Cont'd

D



UDCA but not more hydrophobic BAs increased autophagic flux in hepatocytes.
(A) Primary hepatocytes were treated with 100 μM of UDCA, TCA, CA, CDCA, or DCA with or without CQ (20 μM) for 6 h. Total cell lysates were subjected to immunoblot analysis followed by densitometry analysis. Digital data were presented as a ratio of control (n=4-8). **(B)** Primary hepatocytes were infected with adenoviral GFP-LC3 (100 viral particles per cell) overnight and treated with as in **(A)** followed by fluorescence microscopy. Representative GFP-LC3 images are shown. (**C)** The number of GFP-LC3 dots per cell was determined (>20 cells were counted in each experiment from at least 3 independent experiments). **(D)** Primary FXR−/− hepatocytes were treated with 100 μM of CA or CDCA, with or without CQ (20 μM) for 6 h. Total cell lysates were subjected to immunoblot analysis followed by densitometry analysis. Data were presented as a ratio of control (n = 3).

BAs did not Affect Lysosome or Proteasome Function in Hepatocytes

Since BAs increased LC3-II and p62 levels in hepatocytes, a phenotype typically induced by the lysosomal inhibitor CQ, although the effects induced by BAs were less potent compared to CQ. We next determined the effects of BAs on lysosomal function. DQ-BSA is BSA that is heavily labeled with a BODIPY dye that it self-quenches. Once it enters lysosomal compartments, DQ-BSA is cleaved by lysosomal proteases, which causes release of a single, dye-labeled fluorescent peptide. Thus, the fluorescence intensity of DQ-BSA can be used to reflect lysosomal function (Klionsky, Abdalla et al. 2012). We found BAs did not alter the fluorescence intensity released from DQ-BSA compared to control cells, but CQ treatment significantly decreased the fluorescence intensity (**Figure 3.6A**). Furthermore, cathepsin B activity was similar among cellular lysates prepared from BA-treated hepatocytes compared to lysates from control cells. As a positive control, lysates from E64D (a cysteine protease inhibitor)-treated cells had significantly decreased cathepsin B activity (**Figure 3.6B**). Because p62 protein levels may also be regulated by the proteasome, we next determined proteasome activity in BA-treated hepatocytes. Treatment with BAs did not alter the proteasome activity in hepatocytes but the addition of MG132 (a proteasome inhibitor) in the lysates decreased proteasome activity (**Figure 3.6C**). Collectively, these results suggest that unlike CQ, BAs do not directly disrupt lysosomal integrity or function. The accumulated p62 and LC3-II in BA-treated hepatocytes could be due to an impaired autophagic process upstream of lysosomal degradation, but it is not due to impaired proteasome function.

Figure 3.6

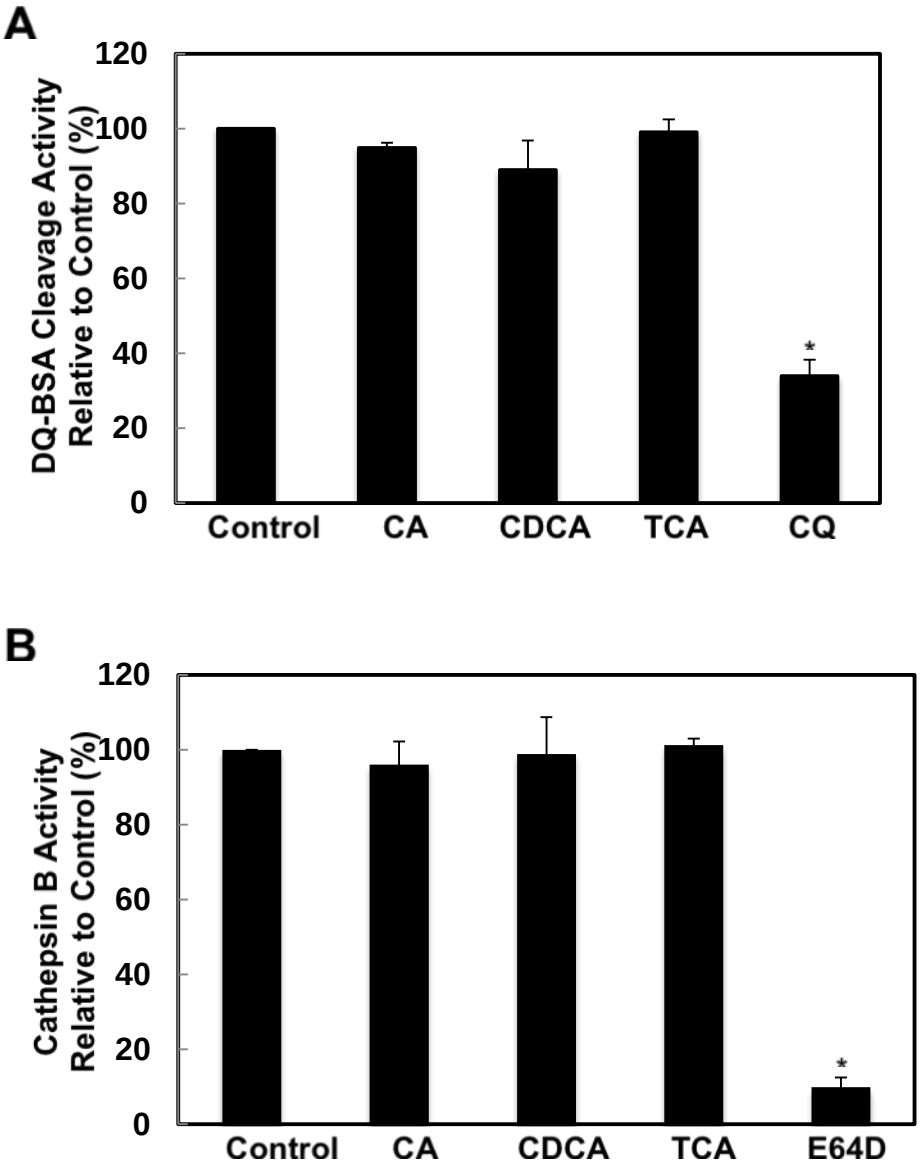
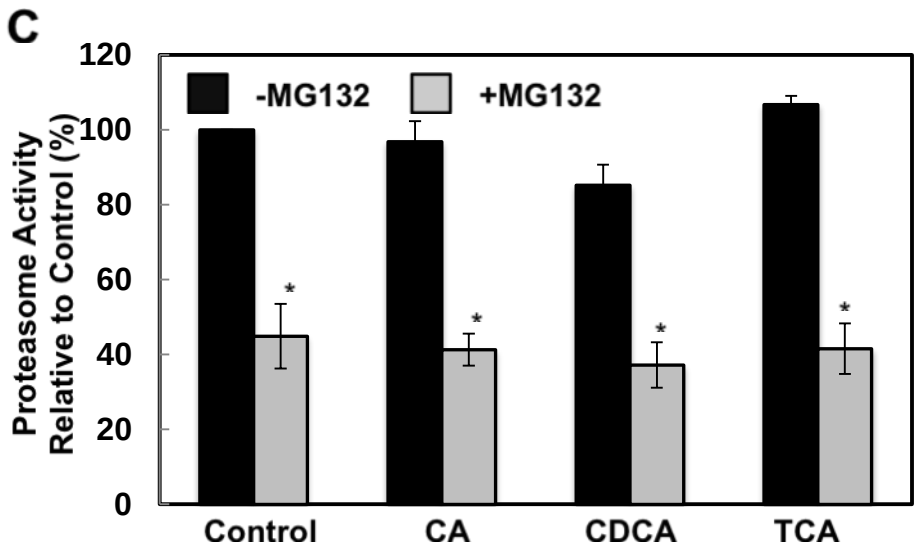

A
DQ-BSA Cleavage Activity
Relative to Control (%)
120
100
80
60
40
20
0
*
Control
CA
CDCA
TCA
CQ
B
Cathepsin B Activity
Relative to Control (%)
120
100
80
60
40
20
0
*
Control
CA
CDCA
TCA
E64D

Figure 3.6 Cont'd



BAs did not directly affect lysosomal or proteasome functions.
(A) Primary hepatocytes were pre-loaded with DQ-BSA (5 µg/ml) for 1 hr and then treated with BAs for 6 h. Fluorescence intensity from cleaved DQ-BSA was measured using total cell lysates. Hepatocytes were treated with BAs for 6 h, and total cell lysates were either incubated with z-RR-AMC for cathepsin B activity (**B**) or Suc-LLVY-AMC with or without MG132 (2 µm) for proteasome activity measurement **(C)** (n=3).

Figure 3.7

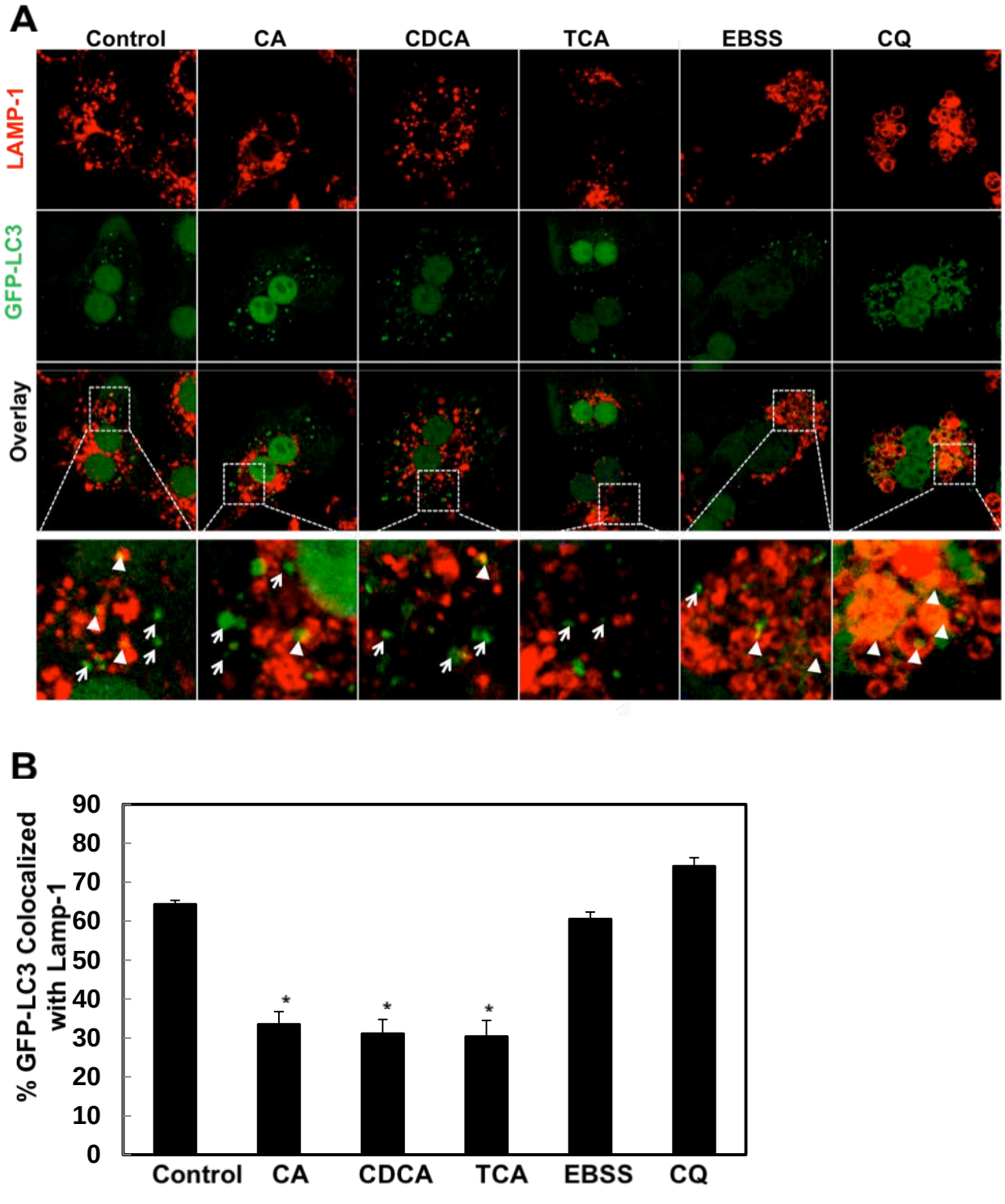
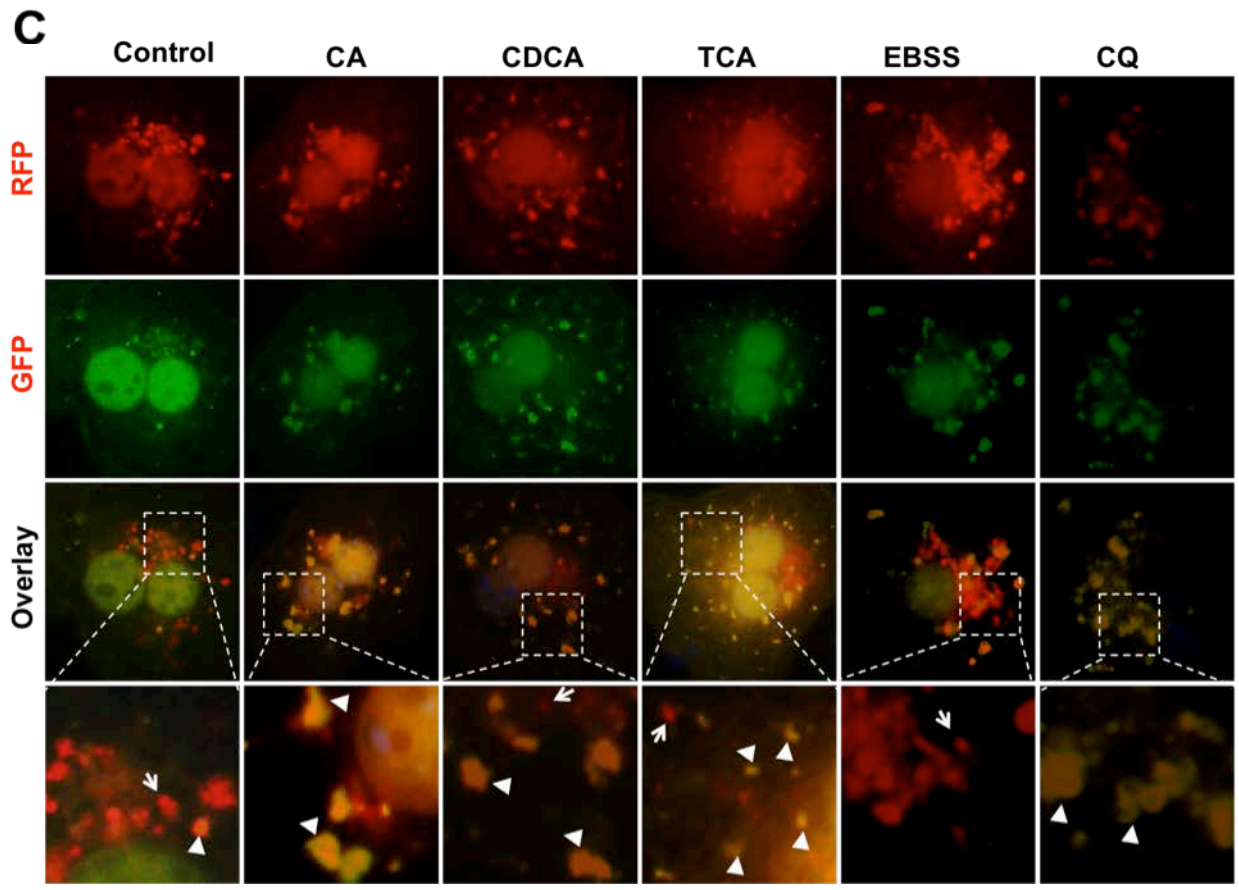

A
Control
CA
CDCA
TCA
EBSS
CQ
LAMP-1
GFP-LC3
Overlay
B
% GFP-LC3 Colocalized with Lamp-1
90
80
70
60
50
40
30
20
10
0
Control
CA
CDCA
TCA
EBSS
CQ
*
*
*

Figure 3.7 Cont'd



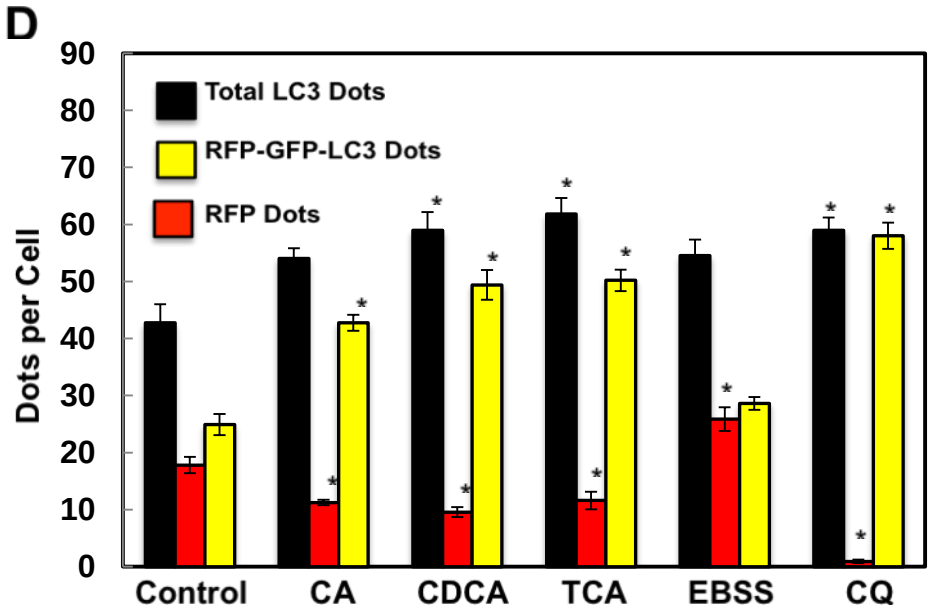
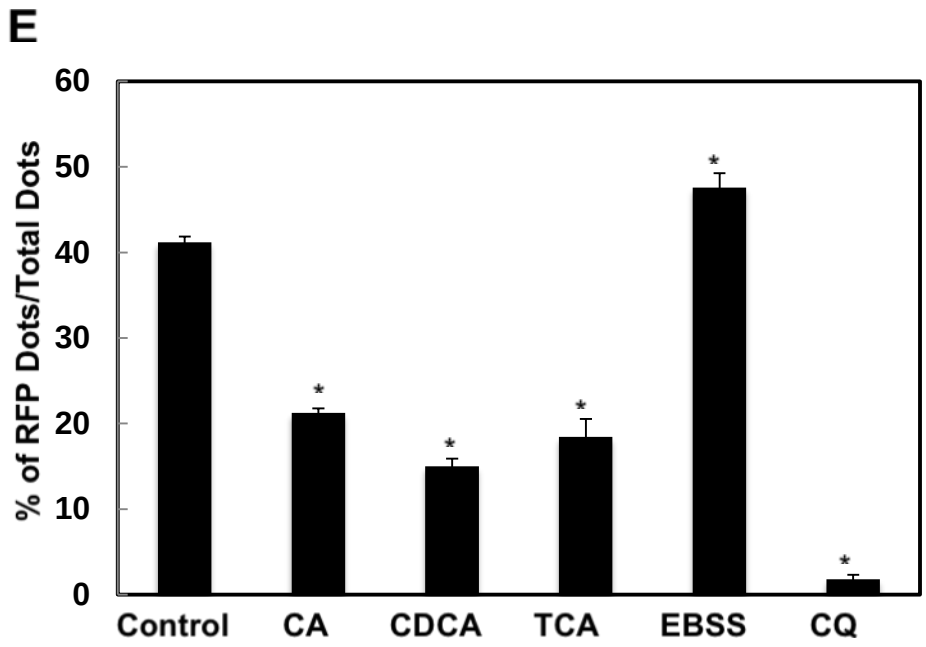


Figure 3.7 Cont'd



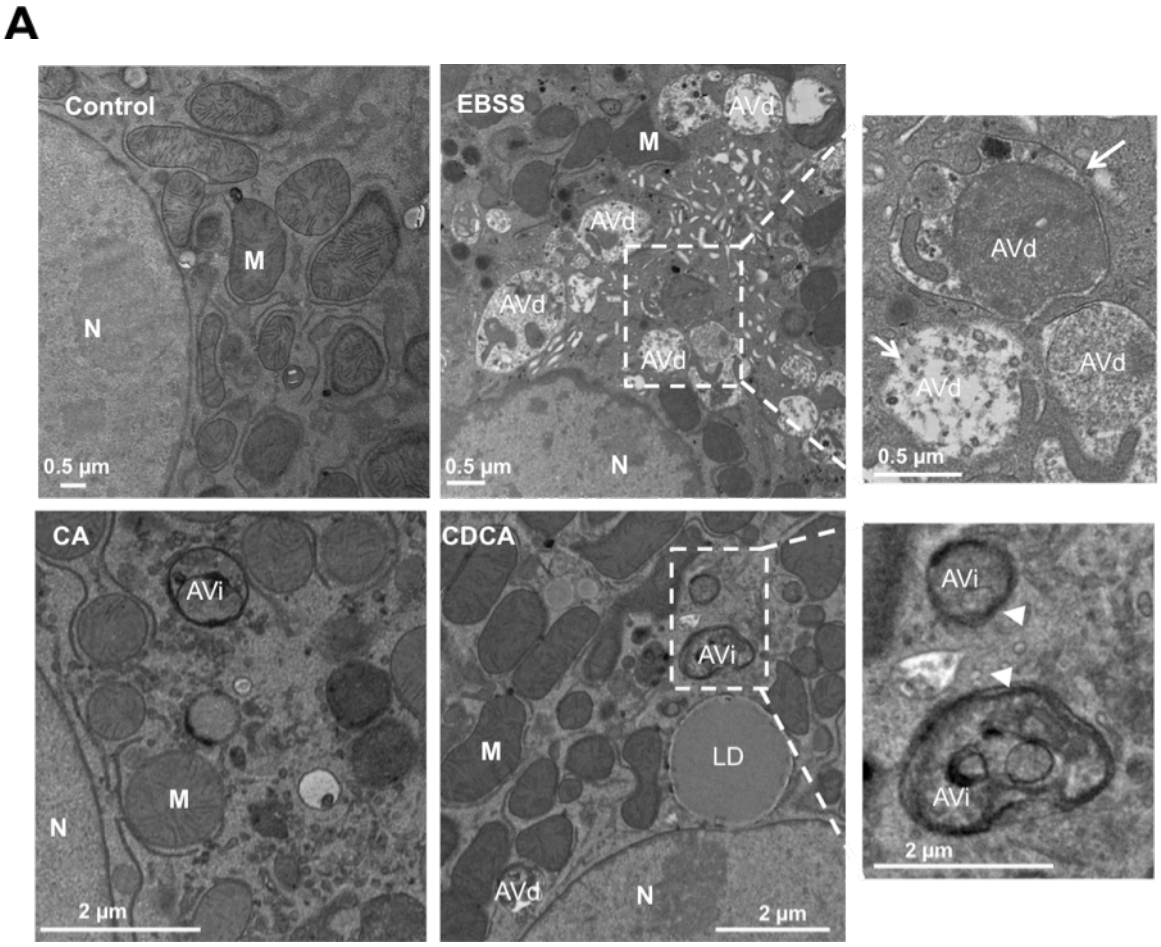
BAs impaired autophagosomal-lysosomal fusion.
(A) Primary hepatocytes were infected with adenoviral GFP-LC3 (100 viral particles per cell) overnight and then treated with 100 μM of BAs, or 20 μM of CQ or EBSS for 6 h. Cells were immunostained for LAMP-1 followed by confocal microscopy. Representative images are shown, and the lower panels are enlarged images from the boxed areas. Arrow heads: yellow dots (colocalization of GFP-LC3 puncta with LAMP-1) and arrows: GFP-LC3 puncta only. **(B)** Percentage of GFP-LC3 puncta colocalized with LAMP-1 (> 20 cells were counted in each experiment from at least 3 independent experiments). **(C)** Primary hepatocytes were transfected with RFP-GFP-LC3 plasmid for 24 h and then treated as in (**A**). Representative images are shown, and the lower panels are enlarged images from the boxed areas. Arrow heads: yellow dots (RFP-GFP-LC3 puncta) and arrows: RFP-LC3 puncta only. (**D**) Total RFP-LC3 and RFP-GFP-LC3 puncta were quantified and percentage of RFP-LC3 only puncta was calculated **(E)** (> 20 cells were counted in each experiment from at least 3 independent experiments).

BAs Inhibited Autophagosomal-Lysosomal Fusion

To determine whether BAs would affect the fusion of autophagosomes with lysosomes, we performed immunostaining for the lysosomal associated membrane protein 1 (LAMP-1), a lysosomal outer membrane protein, and quantified the colocalization of LAMP-1 with GFP-LC3 puncta after BA treatment. We found that cells treated with BAs decreased the colocalization of GFP-LC3 puncta with LAMP-1 compared to control cells or cells cultured in Earle's Balanced Salt Solution (EBSS), which represent basal autophagy or starvation-induced autophagy (**Figure 3.7A and 3.7B**), respectively. We also found that CQ treatment slightly increased the colocalization of GFP-LC3 puncta with LAMP-1 compared to untreated cells, suggesting that CQ did not affect the fusion of autophagosomes with lysosomes. To further confirm that BAs might impair the maturation of autophagosomes, we transfected hepatocytes with tandem RFP-GFP-LC3. In this assay, RFP fluorescence is more stable in acidic compartments, and GFP fluorescence is rapidly quenched, so mature autolysosomes will have red puncta. Blocking the fusion of an autophagosome with a lysosome or suppressing lysosomal degradation (i.e., increase lysosomal pH by CQ) increases the number of yellow puncta (Kimura, Noda et al. 2007; Ni, Bockus et al. 2011). Consistent with the GFP-LC3 and LAMP-1 colocalization results, the number of RFP only positive dots was significantly higher in control cells or amino acid-starved cells than in cells treated with BAs or CQ (**Figure 3.7C-E**). EM analysis also revealed that amino acid-starved cells had more single membrane autolysosomes (Avd) with degraded contents (**Figure 3.8A, arrows, and Figure 3.8B-C**), whereas cells treated with BAs had more double membrane early autophagosomes (Avi) enclosing undegraded contents (**Figure**

3.8A, arrow heads, and Figure 3.8B-C). Taken together, these data collectively suggest that BAs may impair the fusion of autophagosomes with lysosomes in hepatocytes.

Figure 3.8


A
Control
M
N
0.5 μm
EBSS
AVd
M
AVd
AVd
AVd
0.5 μm
N
AVd
AVd
AVd
0.5 μm
CA
AVi
M
N
2 μm
CDCA
AVi
M
LD
AVd
N
2 μm
AVi
AVi
2 μm

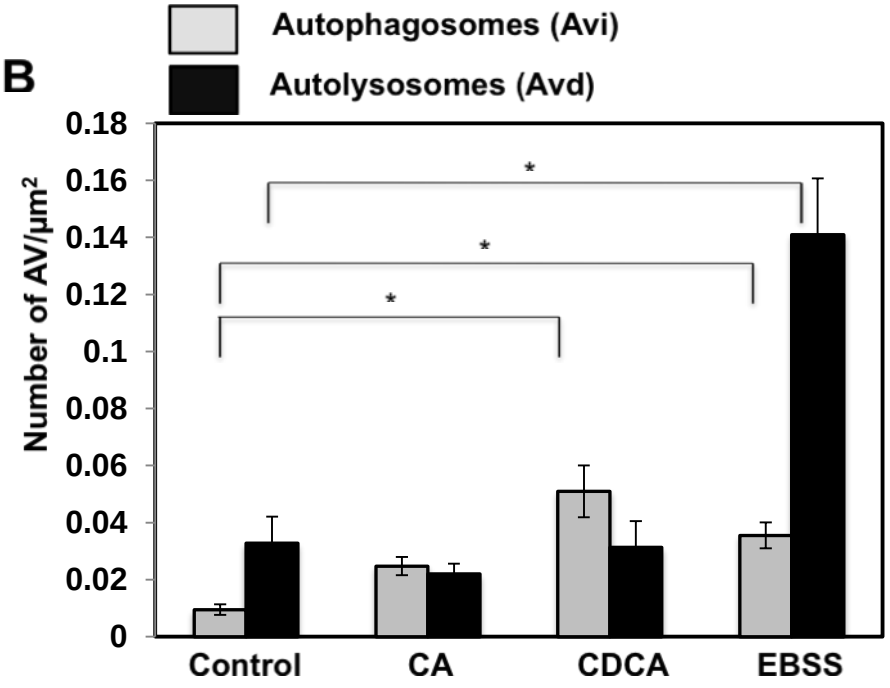
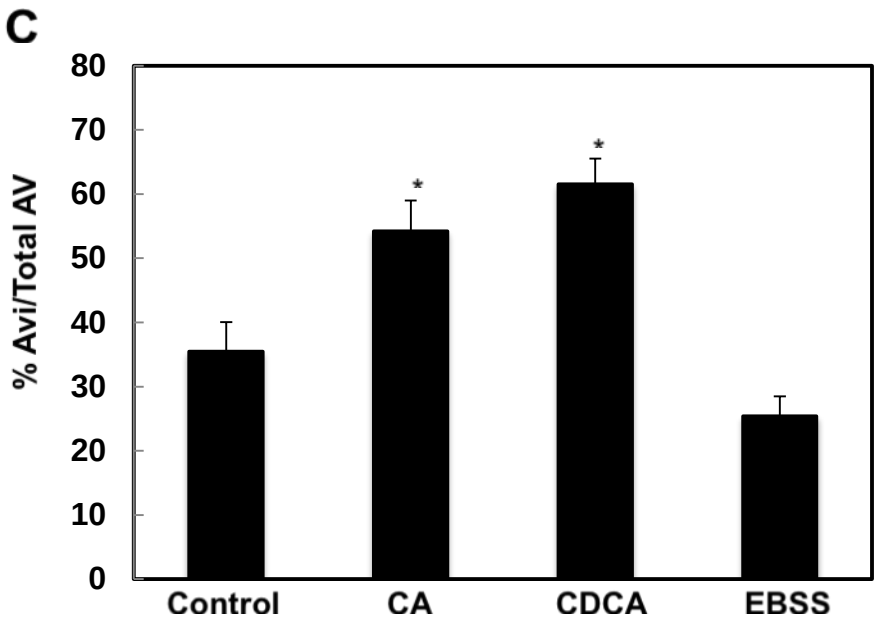

B
Autophagosomes (Avi)
Autolysosomes (Avd)
Number of AV/μm²
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
*
*
Control
CA
CDCA
EBSS

Figure 3.8 Cont'd

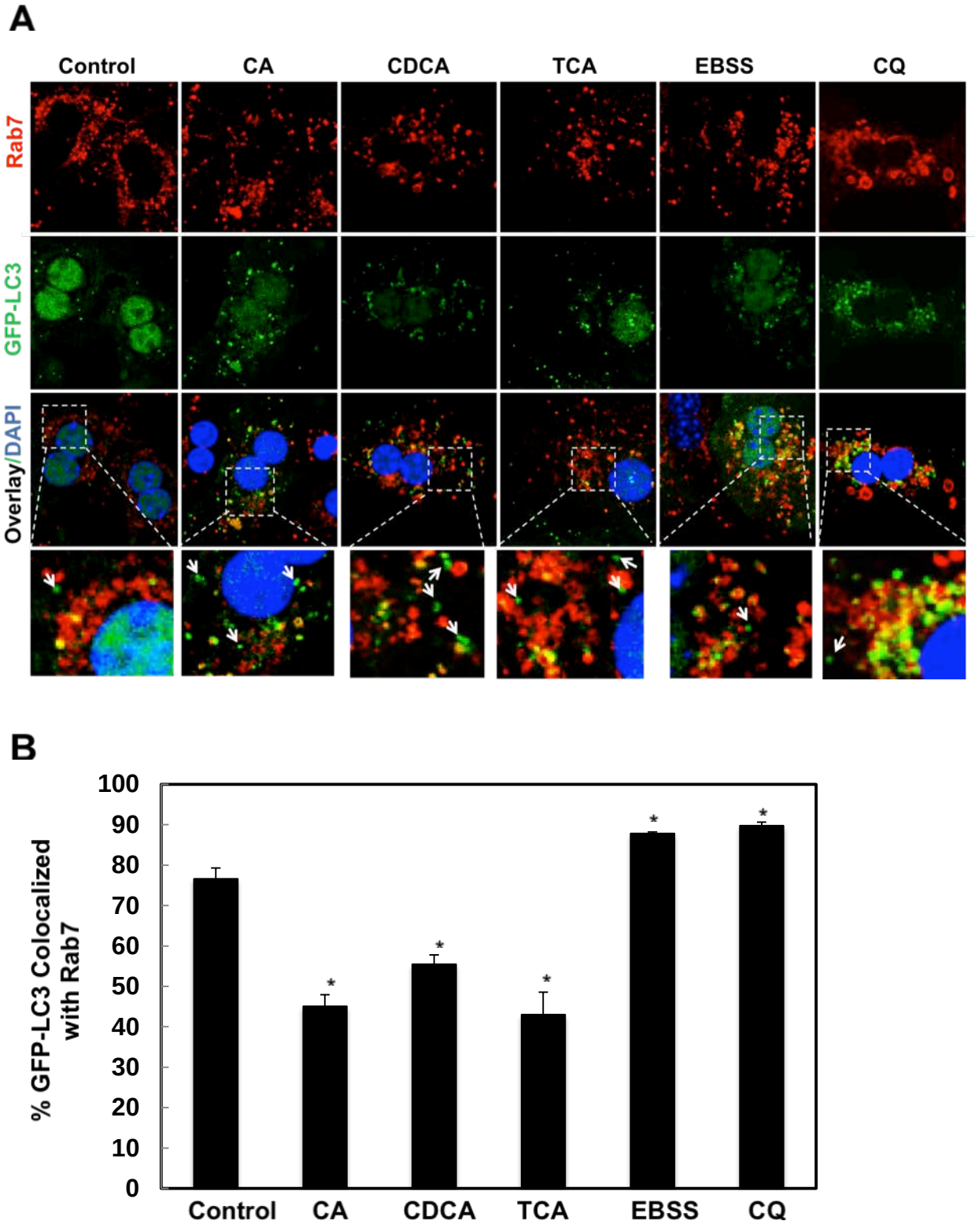


BAs induced accumulation of early autophagosomes.
(A) Hepatocytes were treated with 100 µM of CA or CDCA or EBSS for 6 h. Representative EM images are shown. Right panels are enlarged images from the boxed areas. Arrow heads: early autophagosome (Avi) and arrows: autolysosome (Avd). M: mitochondria, N: nuclei. **(B)** Early autophagosomes (AVi) and late autophagosomes (AVd) were quantified and **(C)** AVi/AVd ratio is presented (>20 different cell sections).

BAs Decreased Levels of Rab7 and Its Targeting to Autophagosomes in Hepatocytes

Rab7, a small GTPase protein, is important for regulating the fusion of autophagosomes with lysosomes (Jager, Bucci et al. 2004) (Chua, Gan et al. 2011). Therefore, we determined if BAs affected Rab7-mediated fusion of autophagosomes with lysosomes. Indeed, the Rab7 staining displayed a peri-nuclear pattern in non-treated hepatocytes that is quite similar to the LAMP-1 staining, which is consistent with the notion that most Rab7 were localized on the late endosomal/lysosomal compartments. More importantly, we found that around 78% of GFP-LC3 puncta were colocalized with Rab7 positive compartments, which was decreased to approximately 40-55% in hepatocytes treated with BAs **(Figure 3.9A-B)**. In contrast, almost 90% of GFP-LC3 puncta were colocalized with Rab7 positive compartments in amino acid-starved or CQ-treated hepatocytes. Furthermore, treatment with BAs also decreased the protein levels of Rab7, but not LAMP-1 or LAMP-2 (**Figure 3.9C**). These results suggest that BAs impair autophagosomal-lysosomal fusion likely due to decreased Rab7-mediated fusion events. The possible cellular events induced by BAs that might cause the inhibition of autophagic flux in hepatocytes were proposed in **Fig 3.10**.

Figure 3.9


A
Control
CA
CDCA
TCA
EBSS
CQ
Rab7
GFP-LC3
Overlay/DAPI
B
% GFP-LC3 Colocalized with Rab7
100
90
80
70
60
50
40
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20
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0
Control
CA
CDCA
TCA
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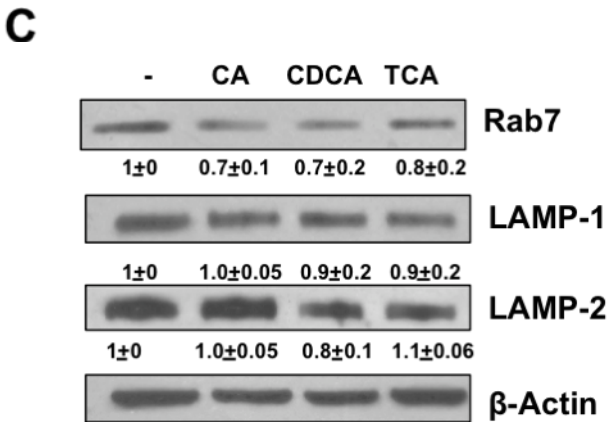
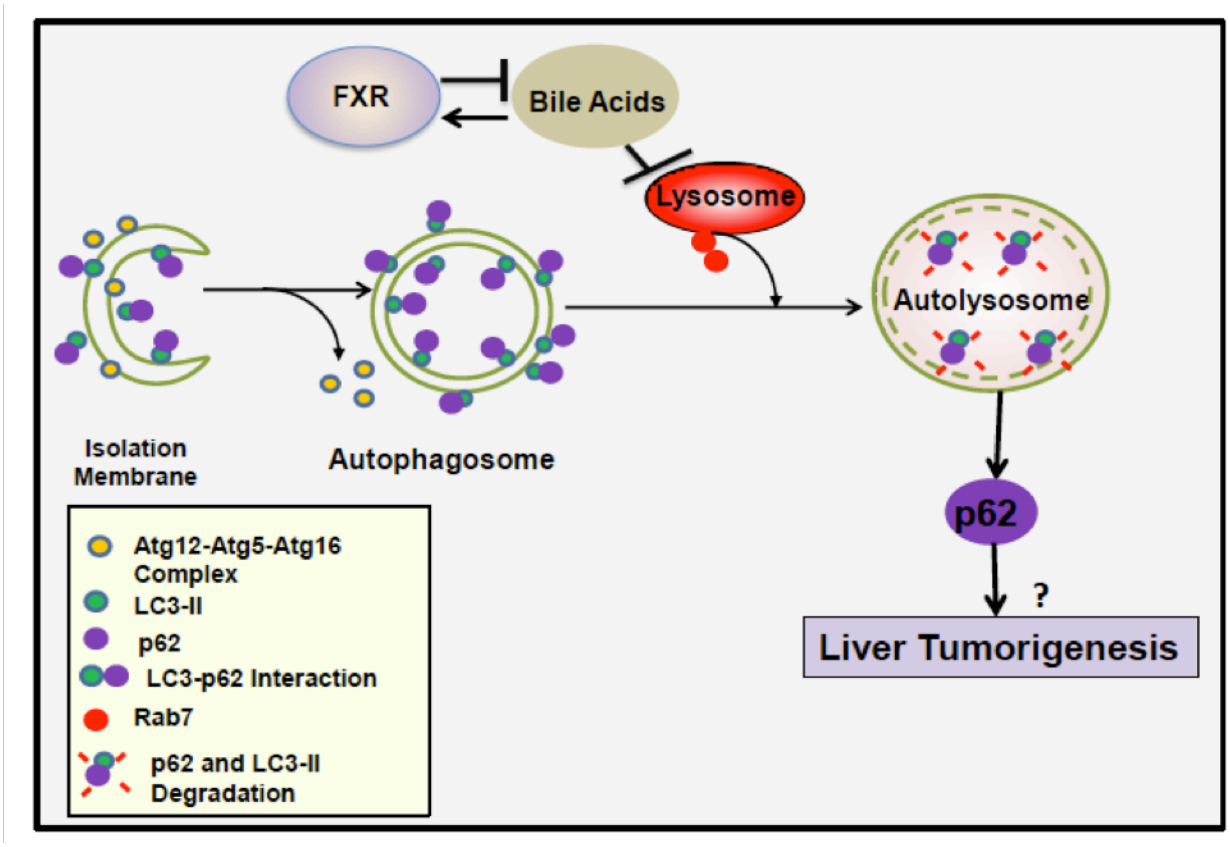
Figure 3.9 Cont'd



BAs decreased the colocalization of GFP-LC3 with Rab7.

(A) Primary hepatocytes were infected with adenovirus GFP-LC3 (100 viral particles per cell) overnight and then treated with BAs, CQ or EBSS for 6 h. Cells were then immunostained with Rab7 followed by confocal microscopy. Representative images are shown, and the lower panels are enlarged images from the boxed areas. Arrows: GFP-LC3 puncta only. **(B)** Percentage of GFP-LC3 colocalized with Rab7 was quantified (> 20 cells were counted in each experiment from at least three independent experiments). (**C**) Hepatocytes were treated with BAs for 6 h and total cell lysates were subjected to immunoblot analysis followed by densitometry analysis as described in Figure 1A (n=3).

Figure 3.10



BAs inhibit autophagic flux by suppressing Rab7-mediated autophagsomal-lysosomal fusion in hepatocytes.
A proposed model that BAs inhibit autophagic flux by suppressing Rab7-mediated autophagsomal-lysosomal fusion in hepatocytes. During autophagy induction, small pieces of isolation membranes grow to form double-membrane autophagosomes, a process that is regulated by Atg5-Atg12-Atg16 complex and LC3-PE conjugation (LC3-II). LC3 also directly interacts with autophagy substrate protein p62 and recruit p62 into autophagosomes. Autophagosomes then fuse with lysosomes to form autolysosomes, where LC3-II and p62 are degraded. This process is mediated by Rab7 and other fusion proteins on either autophagosomes or lysosomes. BAs activate FXR whereas activated FXR negatively regulates synthesis of hepatic BAs. BAs inhibit the fusion of autophagosomes with lysosomes in hepatocytes likely due to decreased expression of Rab7 and its targeting to autophagosomes, resulting in impaired autophagic flux. Impaired autophagic flux leads to the accumulation of LC3-II and p62. Accumulated p62 may contribute to liver tumorigenesis.

3.4 DISCUSSION

BAs are known to exert hepatotoxicity and induce apoptosis by activating either the death receptor or extrinsic apoptosis pathways or necrosis (Jaeschke, Gores et al. 2002; Woolbright and Jaeschke 2012; Woolbright, Antoine et al. 2013). However, the role of BAs in hepatic autophagy is not clear. A previous study showed that deoxycholic acid (DCA) treatment increased the number of GFP-LC3 puncta in primary rat hepatocytes (Zhang, Park et al. 2008). While the authors concluded that DCA induced autophagy in primary rat hepatocytes, their conclusion was questionable because no autophagic flux assay was conducted in their study. We also found that BAs increased LC3-II protein levels as well as the number of GFP-LC3 puncta, which are in agreement with this previous report. However, results from further autophagic flux assays clearly indicated that BAs inhibit autophagic flux in hepatocytes.

How do BAs inhibit autophagic flux? Several mechanisms may lead to decreased autophagic flux, which include: (1) inhibition of the upstream induction of autophagy and autophagosome biogenesis; (2) direct inhibition of lysosomal function; or (3) inhibition of autophagosomal-lysosomal fusion. We found that BA-treatment alone caused increased LC3-II levels and GFP-LC3 puncta; therefore, it is less likely that BAs inhibit upstream autophagosome biogenesis. Furthermore, our results also demonstrated that BAs did not affect cathepsin B or lysosomal proteolytic activity, suggesting that BAs may not directly impair lysosomal function. This is in agreement with a previous study reporting that TCA did not inhibit cathepsin activity or alter lysosomal pH in rat liver or in isolated rat hepatocytes (Larocca, Pellegrino et al. 1999). Therefore it is likely that BAs might decrease autophagic flux by impairing autophagosomal-lysosomal fusion. Indeed, results from multiple different approaches including colocalization of GFP-LC3 with

LAMP-1, tandem RFP-GFP-LC3 assay and EM studies strongly support that BAs inhibit autophagosomal-lysosomal fusion. We also found that BA treatment not only decreased Rab7 protein levels, but also decreased its targeting to GFP-LC3 positive compartments, which could eventually lead to decreased autophagosomal-lysosomal fusion. Increased intracellular levels of Ca^{2+} by thapsigargin has been shown to block the recruitment of Rab7 to autophagosomes resulting in defects for autophagosomal-lysosomal fusion (Ganley, Wong et al. 2011). Interestingly, BAs have also been shown to induce endoplasmic reticulum stress and elevated intracellular Ca^{2+} in rat hepatocytes (Tsuchiya, Tsuji et al. 2006). It would be interesting to determine the possible role of intracellular Ca^{2+} in BA-mediated inhibition of autophagosomal-lysosomal fusion.

What is the significance of the inhibition of hepatic autophagy by BAs? A large body of evidence suggests that FXR may act as a tumor suppressor against liver tumorigenesis. FXR may act on multiple levels to suppress liver tumorigenesis via control of BA homeostasis, prevention of hepatocyte apoptosis, reduction of reactive oxygen species production, inhibition of hepatic inflammation, as well as activation of the expression of *Shp* (Wang, Fu et al. 2013). In contrast, an aberrant high level of BAs has been shown to act as a liver tumor promoter. Firstly, clinical studies reveal a close association between cholestatic liver diseases and liver cancer (Jansen 2007). Secondly, a CA-enriched diet strongly promotes diethylnitrosamine initiated liver tumorigenesis in mice (Yang, Huang et al. 2007). Thirdly, FXR KO mice, which have elevated levels of hepatic BAs, develop spontaneous liver tumors that are significantly reduced by feeding the mice with cholestyramine, a BA-sequestering resin (Yang,

Huang et al. 2007). However, it is not clear how BAs promote liver tumorigenesis, although it has been suggested that high levels of BAs might induce DNA damage to inactivate tumor suppressor genes, cell death, and the inflammatory response in the liver (Wang, Fu et al. 2013). Autophagy has been known to serve as a tumor suppressor. *Beclin-1* heterozygous mice that have decreased expression of *Beclin-1*, a gene involved in the autophagy process, have increased spontaneous tumors in multiple tissues including liver (Qu, Yu et al. 2003). Furthermore, liver-specific Atg5 or Atg7 KO mice also have increased liver tumors but the tumor progression was significantly blunted in the Atg7/p62 double KO mice (Inami, Waguri et al. 2011; Takamura, Komatsu et al. 2011), supporting the notion that autophagy is a bona fide tumor suppressor and increased p62 levels could be one of the important factors responsible for the tumorigenesis associated with impaired-autophagy. We recently demonstrate that further deletion of Nrf2 completely abolished tumorigenesis in liver-specific Atg5 KO mice, suggesting that Nrf2 is the major player downstream of p62 to promote liver tumorigenesis (Ni, Woolbright et al. 2014). It will be interesting to determine whether Nrf2 is activated by BAs or increased in FXR KO mouse livers in the future. We previously found that FXR can directly bind to the *Sqstm1/p62* gene as determined by chromatin immunoprecipitation (Williams, Thomas et al. 2012). However, when mice were treated with a synthetic FXR agonist, we found that the mRNA and protein expression levels of *Sqstm1/p62* only increased in mouse ileum but not in liver. How FXR differentially regulate *Sqstm1/p62* expression in different mouse tissues is still not clear. However, we found that p62 protein accumulated in FXR KO mouse liver, suggesting that other transcriptional factor (s) would be important in the regulating the

expression of *Sqstm1/p62.* Nevertheless, the findings that BAs inhibited autophagic flux in primary cultured mouse hepatocytes and in mouse liver in the present study suggest that decreased autophagy may be a novel mechanism that accounts for the liver tumorigenesis induced by BAs, and for the tumorigenesis observed in FXR KO mice.

In conclusion, we demonstrated that BAs inhibit autophagic degradation *in vitro* and may also play a role in the impaired hepatic autophagy in FXR KO mice *in vivo*. We further demonstrated that BAs decreased the expression of Rab7 and targeting to the autophagosome resulting in decreased autophagosomal-lysosomal fusion. Autophagy deficiency has been shown to cause liver injury and promote liver tumorigenesis, and increased concentrations of BAs have been linked to spontaneous liver tumor development in FXR KO mice. Therefore, our results suggest a possible link between BAs and impaired autophagy in BA-induced hepatotoxicity and liver tumorigenesis. Modulating autophagy function could be a promising therapeutic approach for preventing cholestasis and related liver tumorigenesis.

CHAPTER 4:

SPECIFIC AIM 2

4.1 Specific Aim 2

The role of FXR, a master bile acid homeostasis regulator, in alcohol-induced liver injury is unknown. Increased bile acids have been shown to inhibit autophagy, and FXR KO mice have impaired hepatic autophagy. We previously showed that autophagy protects against ethanol-induced steatosis and liver injury. We also showed that activation of hepatic FoxO3a increased autophagy and protects against ethanol-induced steatosis and liver injury in mice. Therefore, **I hypothesized that FXR may be required for ethanol-induced FoxO3a-mediated autophagy in mouse livers and deletion of FXR may exacerbate liver injury due to impaired autophagy after ethanol treatment.**

Figure 4.1

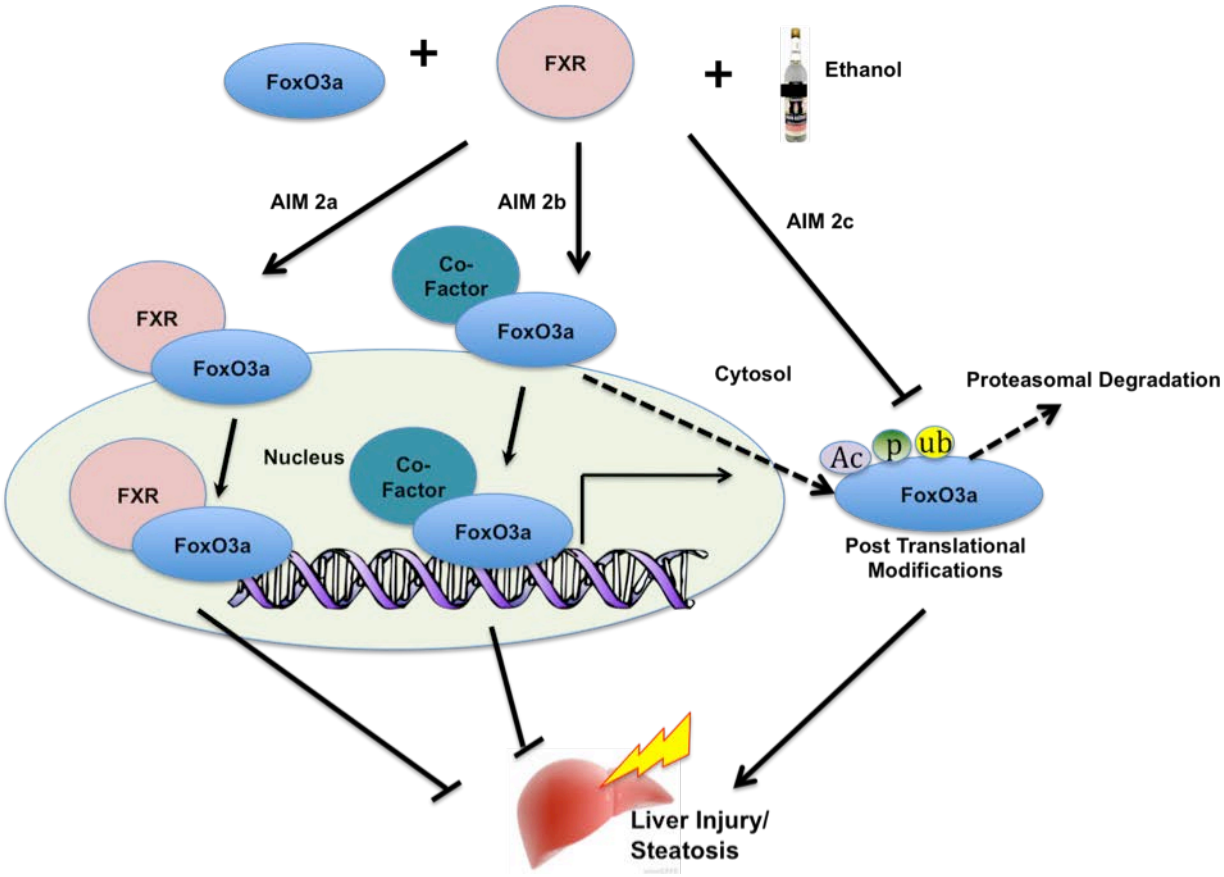


Diagram of the Specific Aim 2.

Aim 2a is to determine if FXR interacts with and promotes FoxO3a stabilization and translocation to the nucleus. Aim 2b is to examine if FXR regulates interaction of FoxO3a with its cofactors. Aim 2c is to investigate if FXR is involved with modulation of post-translational modifications of FoxO3a. FXR regulation of alcohol-induced FoxO3a activation is expected to be a protective mechanism against alcohol-induced liver injury and steatosis.

4.2 Aim 2a: To determine the interaction of FXR with FoxO3a after ethanol treatment.

The objective of this aim is to determine if FXR is required for FoxO3a stabilization and translocation to the nucleus allowing FoxO3a to initiate cell protective gene expressions in response to ethanol exposure. **I hypothesized that FXR is required for FoxO3a activation and translocation to nucleus to initiate transcription of FoxO3a target genes.** This hypothesis is formed based on the preliminary data that showed the difference in the gene expression profiles between ethanol treated WT and FXR KO mouse livers. The data revealed that in ethanol treated FXR KO mouse livers that ethanol-induced increases in mRNA of FoxO3a target genes were abolished. The data suggest that FXR may be required for FoxO3a stabilization and translocation in response to acute ethanol exposure. However, the mechanism of how FXR regulates FoxO3a is not known.

4.3 Aim 2b: To determine that FXR is required for the interaction of co-factors with FoxO3a. My long-term goal is to examine to what degree FXR is involved with modulating the interactions of known co-factors with FoxO3a and subsequently modifying the activation of FoxO3a. The objective of this aim is to investigate to what degree FXR regulates the interactions of the co-factors with FoxO3a using both *in vivo* and *in vitro* models. **I hypothesized that FXR may be necessary for activation of FoxO3a by co-factors.** My hypothesis was based on previous published data showing that FoxO3a interacts with several co-factors including PGC-1α and various nuclear receptors to either upregulate or downregulate gene transcription (van der Vos and Coffer 2008). FXR also shares a couple of co-factors, PGC-1α and Sirt1, with FoxO3a

(Kemper 2011). Currently, it is not known if FXR regulates the interaction of FoxO3a with the activating co-factors.

4.4 Aim 2c: To determine that FXR affects the post-translational modifications of FoxO3a and the nuclear stabilization of FoxO3a. My long-term goal is to identify what post-translational modifications of FoxO3a is regulated by FXR in response to ethanol exposure and how it regulates the localization of FoxO3a. The objective is to identify the post-translational modifications that are modified in response to ethanol in WT and FXR KO mice. **I hypothesized that FXR KO mice livers have altered post-translational modifications and cellular localization of FoxO3a in response to ethanol exposure compared to WT mouse livers.** My hypothesis was based on previously published data showing that FoxO3a activation and localization is regulated by a number of post-translational modifications including phosphorylation, acetylation, and ubiquitination. FXR is an emerging key protein in regulation of many signaling pathways particularly in glucose and lipid metabolism in liver. Similiarly, FoxO3a has been also shown to be involved in glucose and lipid homeostasis. Sirt1 is a known co-factor regulating both FoxO3a and FXR by acetylating both molecules. Moreover, the FXR- SHP pathway inhibits miRNA-34a to positively regulate hepatic Sirt1 levels (Lee, Padhye et al. 2010). Sirt1 deacetylates FoxO3a, which targets FoxO3a to Skp2-mediated ubiquitination and degradation (Wang, Chan et al. 2012). Intriguingly, acetylation of FoxO3a also results in impaired DNA-binding function (Daitoku, Sakamaki et al. 2011). It has been reported that chronic ethanol administration down regulates

Sirt1 in rat and mouse livers (Lieber, Leo et al. 2008; You, Liang et al. 2008). However the role of Sirt1 in cross-talking between FoxO3a and FXR currently is not known.

CHAPTER 5:

FARNESOID X RECEPTOR REGULATES FORKHEAD BOX O3A ACTIVATION IN ETHANOL-INDUCED AUTOPHAGY AND HEPATOTOXICITY.

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1. Manley S, Ni HM, Williams JA, Kong B, DiTacchio L, Guo GL, and Ding WX. Farnesoid X Receptor Regulates Forkhead Box O3a Activaiton in Ethanol-Induced Autophagy and Hepatotoxicity. Redox Biology. In Press.

5.1 INTRODUCTION

Alcohol is widely consumed in the United States and worldwide, and can be beneficial in moderation. Excessive alcohol consumption and abuse may result in alcoholic liver disease (ALD), a major contributor of liver diseases and deaths (Ding, Manley et al. 2011; Gao and Bataller 2011). Pathogenesis of ALD initiates with simple steatosis in the majority of patients and progresses to more severe pathologies including fibrosis, alcoholic hepatitis, and cirrhosis in a fraction of the patients. In exceptional cases, ALD may progress to hepatocellular carcinoma (Gao and Bataller 2011). Binge drinking is a form of alcohol abuse defined by consuming more than 5 drinks (males) or 4 drinks (females) in a two-hour period (Wechsler and Austin 1998). About one out of three adults displays high-risk drinking patterns including binge drinking, but less attention has been given to acute alcohol liver injury despite the fact that binge drinking is more common than chronic alcohol abuse (Ding, Manley et al. 2011). At the physiological level, binge drinking induces glycogen depletion, acidosis, and hypoglycemia (Zakhari and Li 2007). At the cellular level, binge drinking results in mitochondria damage, ablated insulin signaling, steatosis, and free radical generation (Bailey and Cunningham 2002; Carmiel-Haggai, Cederbaum et al. 2003; Lieber 2004; He, de la Monte et al. 2007; Lu and Cederbaum 2008). Paradoxically, binge drinking induces autophagy as a cellular protective mechanism to selectively degrade damaged mitochondria (mitophagy) and lipid droplets (lipophagy), whereas suppression of autophagy by pharmacological inhibitors or small interfering RNAs exacerbates alcohol-induced hepatotoxicity and steatosis (Ding, Li et al. 2010; Ding, Li et al. 2011).

Autophagy is a cellular lysosomal degradation pathway responsible for degradation of cellular protein and damaged organelles to promote cell survival (Mizushima, Yoshimori et al. 2010). The autophagy process is characterized by formation of circular double membrane structures containing cargo known as autophagosomes. Autophagosomes then fuse with lysosomes to form autolysosomes to complete the degradation of the autophagic cargo (Mizushima, Yoshimori et al. 2010). More than 30 *Atg* genes have been identified as participants in autophagy in yeast, and most of them have mammalian homologues (Klionsky and Emr 2000). Among them, two ubiquitin conjugation systems include Atg7 (E1-like protein), Atg3 (E2-like) and Atg5-Atg12-Atg16 complex (E3 ligase) play an essential role to mediate the conjugation of phosphatidylethanolamine (PE) to microtubule associated protein light chain 3 (LC3) (Kabeya, Mizushima et al. 2000; Ohsumi 2001). This conjugated form of LC3 is known as LC3-II, and the unconjugated form of LC3 is referred as LC3-I (Kamada, Sekito et al. 2004). LC3-II is targeted to the autophagosomal membrane, where the inner membrane LC3-II is degraded and the outer membrane LC3-II is de-conjugated and recycled (Kirisako, Ichimura et al. 2000). Induction of autophagy or inhibition of autophagy degradation causes LC3-II accumulation; therefore, LC3-II is used as a marker to monitor autophagic flux (Klionsky, Abdalla et al. 2012). Sequestome-1 (Sqstm1)/p62 is another specific autophagy substrate that also can be used to monitor autophagic flux, and is accumulated in autophagy deficient cells or mouse liver (Komatsu, Waguri et al. 2007; Kirkin, Lamark et al. 2009; Ni, Boggess et al. 2012).

FoxO3a is a member of the FoxO transcription factor family and regulates the transcription of genes involved with apoptosis, oxidative stress, cell-cycle transition and

DNA repair (Huang and Tindall 2007; Tzivion, Dobson et al. 2011). FoxO3a also regulates the autophagy related gene expression in skeletal muscles (Mammucari, Milan et al. 2007; Zhao, Brault et al. 2007), cardiomyocytes (Sengupta, Molkentin et al. 2011), and liver (Ni, Du et al. 2013). Multiple post-translational modifications including phosphorylation, ubiquitination, acetylation, and methylation regulate the FoxO3a cellular localization and DNA-binding affinity (Huang and Tindall 2007; Tzivion, Dobson et al. 2011). Akt is the canonical regulator of FoxO3a by phosphorylating FoxO3a on serine 253 and sequestering FoxO3a in the cytosol, which renders FoxO3a unable to bind DNA and induce gene transcription (Tzivion, Dobson et al. 2011). We have recently demonstrated that acute ethanol treatment inhibits Akt-mediated phosphorylation of FoxO3 resulting in nuclear FoxO3a retention and increased transcription of autophagy genes in mouse livers and primary mouse and human hepatocytes (Ni, Du et al. 2013).

Farnesoid X receptor (FXR), the master regulator of bile acids homeostasis, is a member of the nuclear hormone receptor superfamily and is highly expressed in liver and intestines (Forman, Goode et al. 1995; Sinal, Tohkin et al. 2000; Kim, Morimura et al. 2007). Bile acids are identified as the endogenous ligands for FXR, and bile acid-mediated FXR activation increases the expression of SHP, which serves as a negative regulator of bile acid synthesis. As a result, FXR knockout (KO) mice display elevated hepatic bile acid level due to the lack of SHP-mediated inhibition on bile acid synthesis (Sinal, Tohkin et al. 2000; Kim, Morimura et al. 2007). We recently demonstrated that FXR KO mice display impaired hepatic autophagy due to increased hepatic bile acid levels. Furthermore, bile acids suppress autophagosomal-lysosomal fusion (Manley, Ni

et al. 2014). Recent studies showed that activation of FXR protected against ethanol-induced hepatotoxicity and steatosis (Xie, Zhong et al. 2013; Livero, Stolf et al. 2014). However, whether FXR would play a role in ethanol-induced autophagy is not known.

In the present study, we found that FXR was critical for protecting against acute ethanol-induced hepatotoxicity and steatosis by indirectly promoting ethanol-induced nuclear FoxO3 retention and activation. In response to acute ethanol treatment, there was an increased Akt activation resulting in decreased nuclear FoxO3a retention and FoxO3a-mediated expression of autophagy related genes in FXR KO mice. Acute ethanol treatment also promoted mitochondrial spheroid formation in FXR KO mouse livers.

5.2 MATERIALS AND METHODS

Reagents. The following antibodies were used: Atg5 (PM050) from MBL International (Woburn, MA), p62 (H00008878-M01) and PGC-1α (PAB-12061) from Abnova (Taipei, Taiwan), Beclin-1 (sc11427), FXR (sc13063), CHOP/GADD152 (sc793), HA (sc805), mitofusin-1 (sc50330), and Parkin (sc32282) from Santa Cruz Biotechnology (Santa Cruz, CA), beta-actin (a5541), Bip/GRP78 (G9043), Flag (F3165), and mitofusin-2 (M6444) from Sigma (St. Louis, MO), acetylated lysine (9441S) serine 473 phosphorylated Akt (4058), Akt (9272), serine 9 phosphorylated GSK3β (5558S), GSK3β (5676S), serine 253 phosphorylated FoxO3a, FoxO3a (2497), GAPDH (2118), Lamin A/C (2032), and Sirt1 (2028) from Cell Signaling Technology (Beverly, MA), and CYP2E1 (ab28146) from Abcam (Cambridge, MA). The rabbit polyclonal anti-LC3B antibody was described previously (Ding, Ni et al. 2009). The secondary antibodies used for immunoblotting analysis were HRP-conjugated goat anti-mouse (115-035-062), rabbit (111-035-045), and rat (111-035-143) and a Dylight 549 conjugated goat anti-rabbit (111-505-144) antibody from Jackson ImmunoResearch (West Grove, PA) or an HRP-conjugated goat anti-rabbit antibody (31460) from Thermo Fisher Scientific (Waltham, MA). Ethanol was from Pharmaco (Brookfield, CT). All other chemicals were from Sigma-Aldrich, (St. Louis, MO), Thermo Fisher Scientific (Waltham, MA), Invitrogen (Carlsbad, CA), or EMD Millipore (Billerica, MA).

Animal Experiments. Wild type C57BL/6 and FXR -/- C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). 2-4 months age matched male wild type and FXR -/- mice were used in this study. All mice were provided with humane care

according to the NIH guidelines, and the Institutional Animal Care and Use Committee at the University of Kansas Medical Center approved all procedures.

Mouse Ethanol Binge Treatment. The ethanol binge model was modified from the model Carson and Pruett established, which has been shown to closely mimic human binge ethanol consumption including blood alcohol levels and behavioral effects (Carson and Pruett 1996). The mice were fasted for 6 hours, and then administered with 33% (v/v) ethanol at a cumulative dose of 4.5 g/kg body weight by four equally divided gavage in 15 minutes intervals. Control mice received same volume of double-distilled water. For WAY-363450 treatment, the mice were administered 30 mg/kg body weight WAY-362450 after 6 hours fast and prior to ethanol treatment. Control and ethanol-only treated mice received same volume of PEG400:Tween20 solution (80:20 ratio). Mice were sacrificed 16 hours later after the treatment, and serum and liver samples were harvested. Serum alanine aminotransferase (ALT) levels and H&E staining were used to assess ethanol-induced liver injury. Total liver lysates were obtained by using radio-immunoprecipitation assay buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl (lauryl) sulfate).

Cell Culture and Transfection. Human embryonic kidney 293A cells were cultured in DMEM medium with FBS and L-glutamate and transiently transfected with HA tagged FoxO3, Flag tagged FXR, HA or Flag plasmid constructs using TurboFect Transfection reagent (Thermo Fisher Scientific) for 24 hours. Total cell lysates then were extracted using HA cell lysis buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 2 mM EDTA pH 7.5, 1 mM EGTA pH 7.5, 1% Triton X-100, protease inhibitors).

Hepatic Triglyceride Analysis. Frozen liver tissues (50-100 mg) were grounded to fine powder using mortar and pestle. The powdered tissue was incubated in 1 ml of chloroform/methanol (2:1) mix with vigorous shaking for 1 hour at room temperature. 200 µl of double-distilled water was added, and the mixture was centrifuged for 5 minutes at 3000 x g at 4°C. The aqueous upper phase layer then was collected and air-dried at room temperature. The dried pellet was dissolved in tert-butanol and Triton X-114/methanol (2:1) solution. Hepatic triglyceride analysis was performed with a colormetric assay kit according to manufacturer's instructions (Pointe Scientific, Ann Arbor, MI).

Immunoblot Assay. Equal amount of nuclear fraction (15 µg), cytosol fraction (30 µg), or total liver lysates (50 µg) were separated by SDS-PAGE gel and transferred to PVDF membranes. The membranes were immunoblotted with primary antibodies followed by HRP-conjugated secondary antibodies. The membranes then were developed with either Pierce Supersignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL) or Millipore Immobilon Western chemiluminescent HRP substrate (Billerica, MA). Densitometry was performed using ImageJ software and further normalized using beta-actin or GAPDH, and expressed as means $\pm$ SEM.

qPCR. RNA was isolated from liver tissues using TRIzol (Life Technologies, Waltham, MA) and reverse transcribed into cDNA by RevertAid reverse transcriptase (Thermo Fisher Scientific, Waltham, MA). Real-time PCR was performed on a Bio-Rad CFX384™ real-time PCR detection system using iTaq™ Universal SYBR® Green Supermix (Bio-Rad). The following genes were probed with quantitative PCR using *β-actin* gene as loading control: *Atg5, Becn-1, Map1lc3b, MnSOD, p21, FoxO3a, Sqstm-1,*

and *Shp.* Primer sequences were as follows: β-actin, 5'-TGTTACCAACTGGGACGACA-3' and 5'-GGGGTGTTGAAGGTCTCAAA-3'; Atg5, 5'-GACCACAAGCAGCTCTGGAT-3' and 5'-GGTTTCCAGCATTGGCTCTA-3'; Becn-1 (Atg6), 5'-TGATCCAGGAGCTGGAAGAT-3' and 5'-CAAGCGACCCAGTCTGAAAT-3'; FoxO3a, 5'-AGCCGTGTACTGTGGAGCTT-3' and 5'-TCTTGGCGGTATATGGGAAG-3'; Map1lc3, 5'-CCGAGAAGACCTTCAAGCAG-3' and 5'-ACACTTCGGAGATGGGAGTG-3'; MnSod, 5'-GGCCAAGGGAGATGTTACAA-3' and 5'-AGACACGGCTGTCAGCTTCT-3'; p21, 5'-CGGTGGAACTTTGACTTCGT-3' and 5'-CAGGGCAGAGGAAGTACTGG-3'; Sqstm1/p62, 5'-AGAATGTGGGGGAGAGTGTG-3' and 5'-TCGTCTCCTCCTGAGCAGTT-3'; and Shp, 5'-CTGCAGGTCGTCCGACTATT-3' and 5'-ACCTCGAAGGTCACAGCATC-3'.

Immunoprecipitation. Total cell lysates (200 µg) or total liver lysates (500 µg) were incubated with a FoxO3a antibody (sc11351, Santa Cruz) overnight at 4°C rotating and pulled down with protein A/G agarose beads (sc2003, Santa Cruz) or EZview™ Red ANTI-FLAG® M2 Affinity Gel (F2425, Sigma). Immunoblot analysis was performed with immunoprecipitated and input proteins.

Chromatin Immunoprecipitation. Fresh liver sections were minced or frozen liver sections were homogenized and cross-linked with 1% formaldehyde in PBS. Following incubation, the livers were quenched with 250 mM glycine. Crude nuclear extracts were obtained using hypotonic buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40) and nuclear lysis buffer (1%SDS, 5 mM EDTA, 50 mM Tris-HCl pH 8.0). The chromatin extracts were then sheared using Active Motif Q800R sonicator to 200-500 base pair fragments. Chromatin proteins (600 µg) were immunoprecipitated with a

FoxO3a antibody (sc11351X) from Santa Cruz Biotechnology (Santa Cruz, CA) binded with Dynabead Protein A (10001D) from Invitrogen/Dynal (Oslo, Norway). DNA was then extracted from immunoprecipitated and input chromatin using GeneJet PCR Purification Kit (K0701) from Thermo Scientific (Waltham, MA). qPCR was performed and three FoxO3a binding sites in the *Map1LC3b* promoter site were probed. Primer sequences were as follows: 1379 to 1608 base pairs upstream, 5'-CATGCCTTGGGACACCAGAT-3' and 5'-ACCTTCTTCAAGTGCTGTTTGT-3'; 3397 to 3595 base pairs upstream, 5'-TTTGACCAAACAGGGTTTCC-3' and 5'-CCCTCCAGGTGTTTGTGATAA-3'; and 4673 to 4801 base pairs upstream, 5'-CCTCAGCTGGCTAAGAGCAT-3' and 5'-CCC AAG GAT CTC AAC CAA AC-3'. The primer sequences were obtained from a previous report (Zhao, Brault et al. 2007).

Fluorescence and Electron Microscopy. Liver sections were fixed with 4% paraformaldehyde then incubated in 20% sucrose in PBS and embedded in optimal cutting temperature (OCT) solution at -20°C. Liver cyrosections were immunostained with FoxO3a antibody followed by Dylight 549 conjugated secondary antibody and Hoechst 33342 staining. The images of sections were acquired under a Nikon Eclipse 200 fluorescence microscope with MetaMorph software. For electron microscopy (EM), liver sections were fixed with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4), followed by 1% OsO_4. After dehydration, thin sections were cut and stained with uranyl acetate and lead citrate. Digital images were obtained using a JEM 1016CX electron microscope.

Nuclear Fractionation. Mouse liver cytosol and nuclear proteins were extracted using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific)

according to manufacturer's instructions. In brief, 50-100 mg of liver was homogenized in cytoplasmic extraction reagent I, followed by addition of cytoplasmic extraction reagent II, and centrifuged. The supernatant was collected as cytoplasmic fraction, and the insoluble pellet was suspended in nuclear extraction reagent and centrifuged. The resulting supernatant contains nuclear fraction.

Statistic Analysis. All experimental data were expressed as means ± SE and subjected to Student t-test or one-way analysis of variance with Holm-Sidak post hoc test or one-way analysis of variance on ranks with Dunn post hoc test where appropriate. *$p<0.05$ was considered significant.

5.3 RESULTS

Acute ethanol treatment exacerbated liver injury and steatosis in FXR KO mice compared to WT mice.

Acute ethanol treatment increased serum alanine aminotransferase (ALT) levels and hepatic triglyceride levels in wild type (WT) mice, which is consistent with our previous report (Ding, Li et al. 2010). Compared to WT mice, acute ethanol-treated FXR KO mice had higher serum ALT and hepatic triglyceride levels, suggesting ethanol treatment exacerbated liver injury and steatosis in FXR KO mice (**Figure 5.1A & B**). Notably, control FXR KO mice already had a mild increase of serum ALT, which is consistent with previous reports suggesting the lack of FXR causes mild liver injury (Yang, Huang et al. 2007)(**Figure 5.1A**). However, there was no difference in hepatic triglycerides level between age matched WT and FXR KO control mice **(Figure 5.1B)**. Moreover, there was no difference in the liver weight/body weight ratio between control and ethanol-treated WT and FXR KO mice **(Figure 5.1C)**. Hematoxylin and eosin staining revealed that ethanol induced mild hepatic steatosis in WT mice, which was further exacerbated in FXR KO mice **(Figure 5.2).** In line with these findings**,** oil red o staining for lipids showed that ethanol treatment increased oil red o staining in WT mouse livers compared to control mice. However, oil red o staining was much more severe in ethanol-treated FXR KO mouse livers compared to ethanol-treated WT mouse livers, suggesting that lack of FXR exacerbates ethanol-induced hepatic steatosis **(Figure 5.3)**. Furthermore, EM analysis also revealed that ethanol treatment increased the number of lipid droplets in WT mouse livers. The number of lipid droplets was further increased in ethanol-treated FXR KO mouse livers compared to WT mouse

livers **(Figure 5.4A & B)**. Taken together, these data indicate that FXR deficiency exacerbates acute ethanol-induced liver injury and steatosis.

Figure 5.1

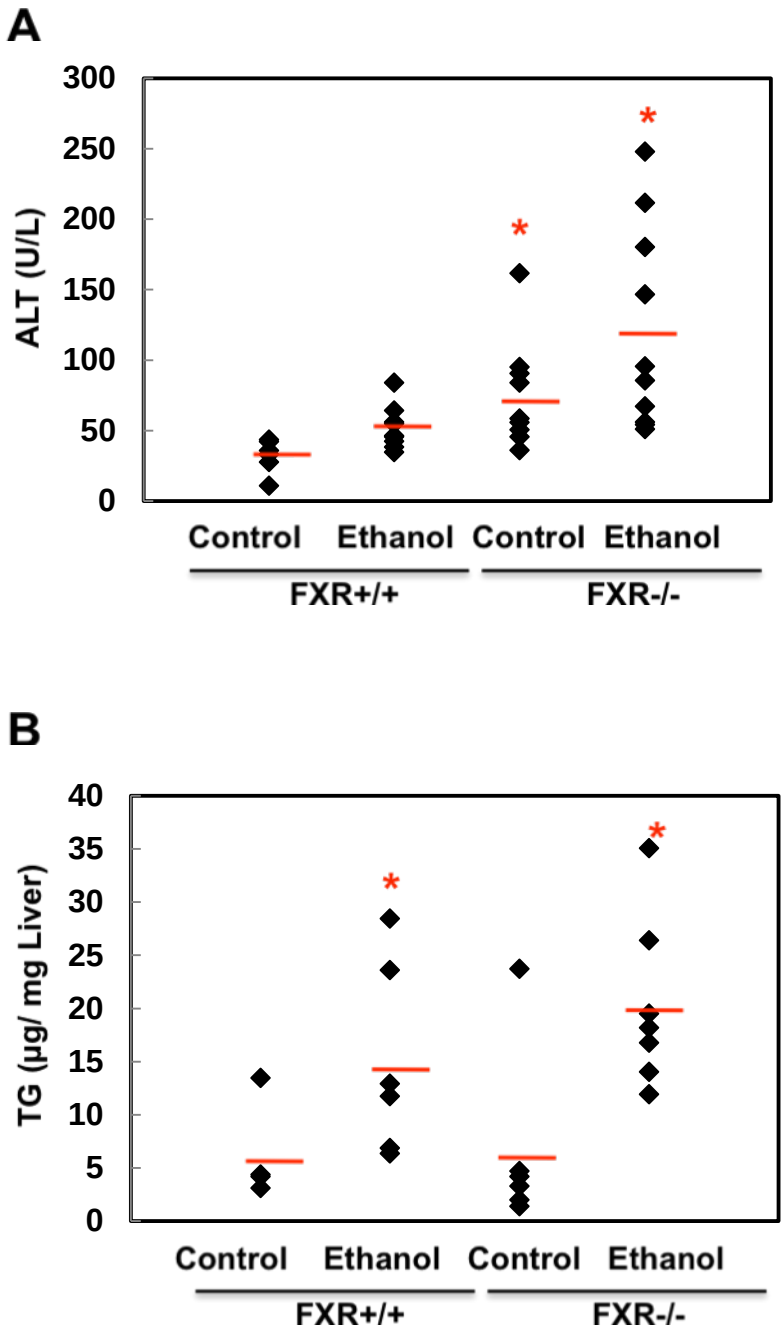
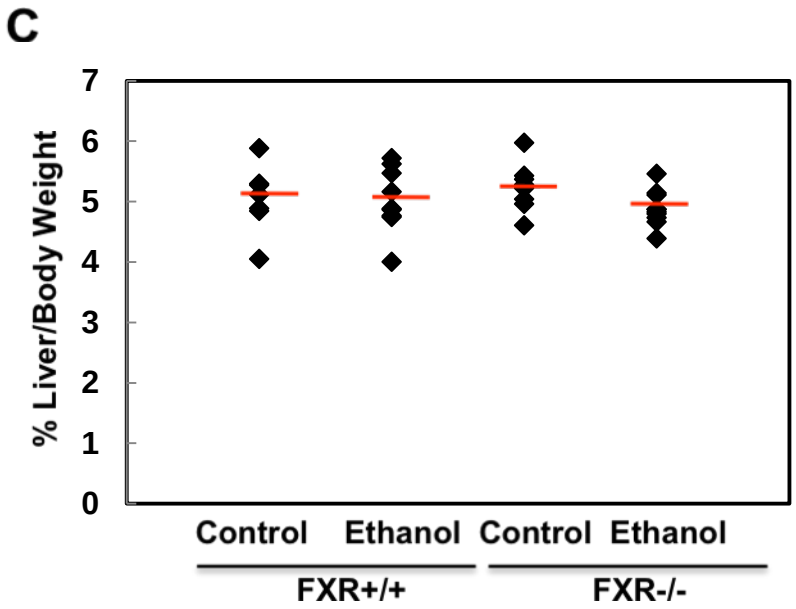

A
300
250
200
150
100
50
0
ALT (U/L)
*
*
Control
Ethanol
Control
Ethanol
FXR+/+
FXR-/-
B
40
35
30
25
20
15
10
5
0
TG (µg/ mg Liver)
*
*
Control
Ethanol
Control
Ethanol
FXR+/+
FXR-/-

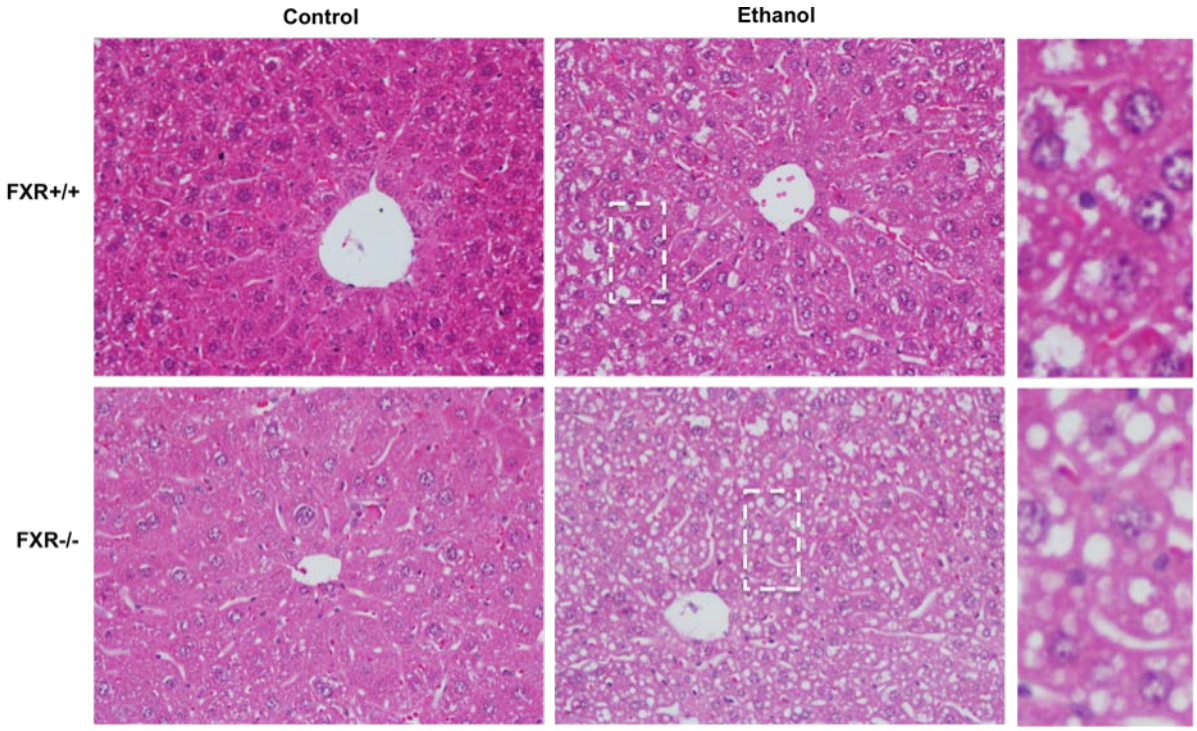
Figure 5.1 Cont'd



FXR KO mice exhibited increased ethanol-induced liver injury and steatosis.

Age matched WT and FXR KO mice were treated with 4.5 g/kg ethanol or water by gavage for 16 hours. Serum alanine aminotransferase (ALT) **(A)** and hepatic triglycerides (TG) **(B)** were measured (n=4-10). Liver/body weight ratio was calculated **(C)** (n=4-10)**.** *: $p<0.05$

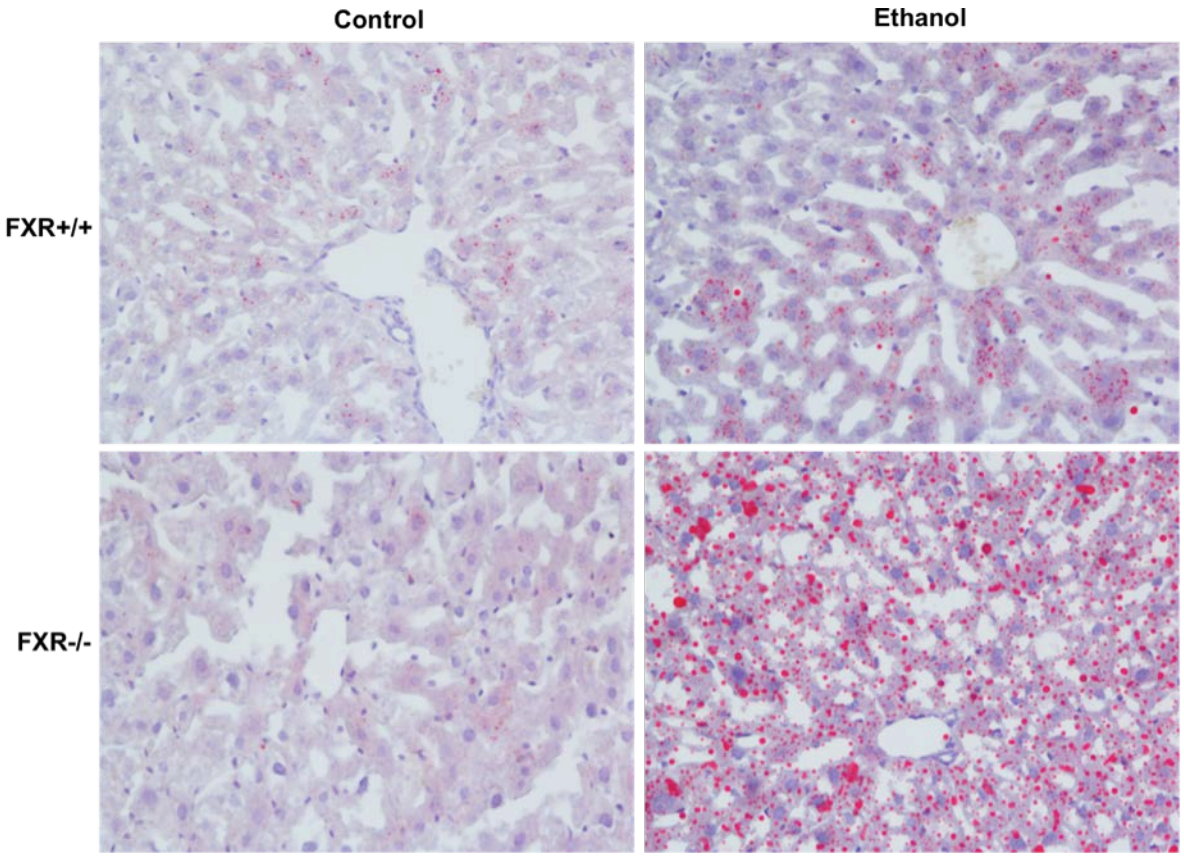
Figure 5.2



H & E staining showed that FXR KO mice exhibited increased ethanol-induced steatosis.

Age matched WT and FXR KO mice were treated with 4.5 g/kg ethanol or water by gavage for 16 hours. Representative H&E stain images are shown. Right images are the enlarged images from the box area.

Figure 5.3



Oil Red O staining revealed FXR KO mice exhibited increased ethanol-induced steatosis.

Age matched WT and FXR KO mice were treated with 4.5 g/kg ethanol or water by gavage for 16 hours. Representative oil red o stain images are shown.

Figure 5.4

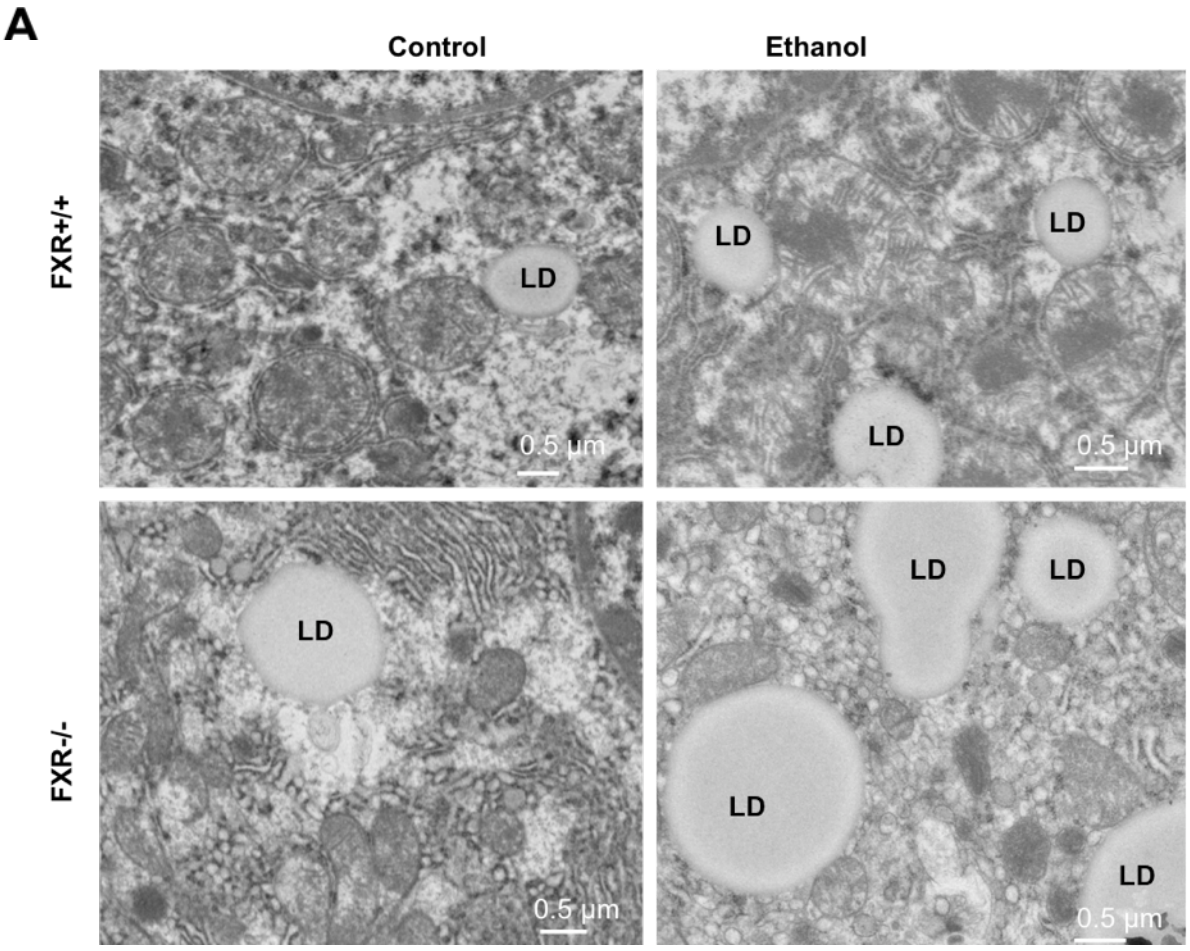

A
Control
Ethanol
FXR+/+
FXR-/-
LD
0.5 μm
B
Number of Lipid Droplet/μm²
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
*
*
*
Control
Ethanol
Control
Ethanol
FXR+/+
FXR-/-

Figure 5.4 Cont'd

FXR KO mice exhibited increased ethanol-induced lipid droplet formation.

Age matched WT and FXR KO mice were treated with 4.5 g/kg ethanol or water by gavage for 16 hours. Representative EM images are shown in **(A)** and lipid droplets (LD) are quantified from at least 24 different liver section **(B)**. *: $p<0.05$

Acute ethanol treatment induced endoplasmic reticulum stress.

Chronic alcohol consumption has been shown to induce endoplasmic reticulum (ER) stress response in mouse livers (Ji 2014). In agreement with previous findings, the EM analysis showed ER dilation, a sign of ER stress in acute ethanol-treated WT mouse livers **(Figure 5.5A)**. The dilated ER seemed to be more severe in ethanol-treated FXR KO mouse livers than that of WT mice. Acute ethanol treatment did not increase binding immunoglobulin protein (Bip), a marker of ER stress. However, CCAAT-enhancer-binding-protein homologous protein (CHOP), another ER stress marker was increased in both WT and FXR KO mouse livers after acute ethanol treatment **(Figure 5.5B)**. These results suggest that ethanol may induce ER stress in the mouse livers, but the whether FXR KO mouse livers had more severe ER stress than that of WT mice need to be further studied. More ER stress markers such as eukaryotic initiation factor 2-alpha (eIF2-α) phosphorylation, Atg4 and activating transcription factor 6 (ATF6) as well as the splicing of x-box binding protein 1 (XBP1) should be examined in the future.

Figure 5.5

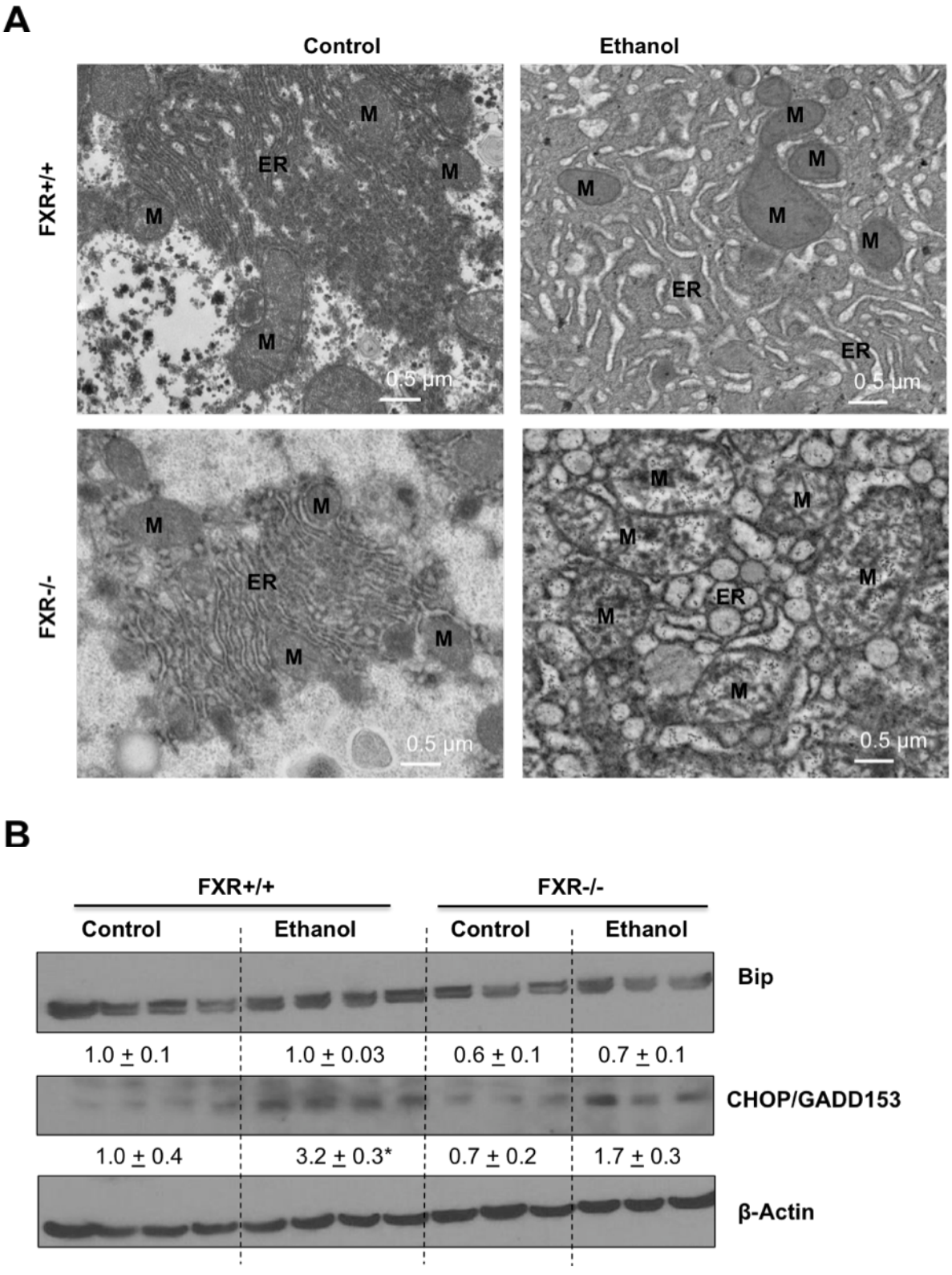

A
Control
Ethanol
FXR+/+
FXR-/-
M
ER
0.5 µm
B
FXR+/+
FXR-/-
Control
Ethanol
Control
Ethanol
Bip
1.0 ± 0.1
1.0 ± 0.03
0.6 ± 0.1
0.7 ± 0.1
CHOP/GADD153
1.0 ± 0.4
3.2 ± 0.3*
0.7 ± 0.2
1.7 ± 0.3
β-Actin

Figure 5.5 Cont'd

Ethanol induced ER stress response.

Age matched WT and FXR KO mice were treated with 4.5 g/kg ethanol or water by gavage for 16 hours. Representative EM images are shown in **(A)**. M- Mitochondria. ER- endoplasmic reticulum. Total liver lysates were subjected to immunoblot analysis for ER stress proteins, Bip and CHOP/GADD153 **(B)**. Densitometry analysis data are presented as a ratio of control (n = 3–4).

Impaired autophagy in acute ethanol-treated FXR KO mouse livers.

We previously demonstrated that FXR KO mice have impaired hepatic autophagy (Manley, Ni et al. 2014). In accordance with previous findings, we showed that control FXR KO mice had higher basal LC3-II and p62 protein levels compared to WT mice. Ethanol treatment slightly increased LC3-II protein levels in WT but not FXR KO mouse livers **(Figure 5.6A)**. However, ethanol treatment increased p62 protein levels in WT mouse livers, which was further increased in FXR KO mouse livers (**Figure 5.6A)**. Ethanol treatment also increased CYP2E1 protein levels in both WT and FXR KO mouse livers **(Figure 5.6B),** consistent with previous reports (Wu and Cederbaum 1993; Caro and Cederbaum 2004). Ethanol treatment increased the mRNA levels of p62 in WT mouse livers but not in FXR KO mouse livers **(Figure 5.6C)**, suggesting that ethanol treatment-increased p62 protein levels could be mediated at the transcriptional level in WT mice but not in FXR KO mice. These data suggest that impaired hepatic autophagy in FXR KO mice results in increased ethanol-induced p62 protein accumulation. EM analysis revealed that ethanol treatment increased the number of autolysosomes in WT mouse livers although did not reach the statistical significance. Consistent with our previous report (Manley, Ni et al. 2014), control FXR KO mice already had increased number of autophagosomes and autolysosomes likely due to impaired fusion of autophagosomes with lysosomes**.** Treatment with acute ethanol further increased the number of autophagosomes and autolysosomes (**Figure 5.7A & B**). Furthermore, FXR KO mouse livers showed increased percentage of autophagosomes in comparison to WT mouse livers, which suggests that fusion of autophagosomes with lysosomes may be impaired **(Figure 5.7C)**. These results

indicate that there was impaired autophagy in FXR KO mouse livers, which might contribute to exacerbated liver injury and steatosis following ethanol treatment.

Figure 5.6

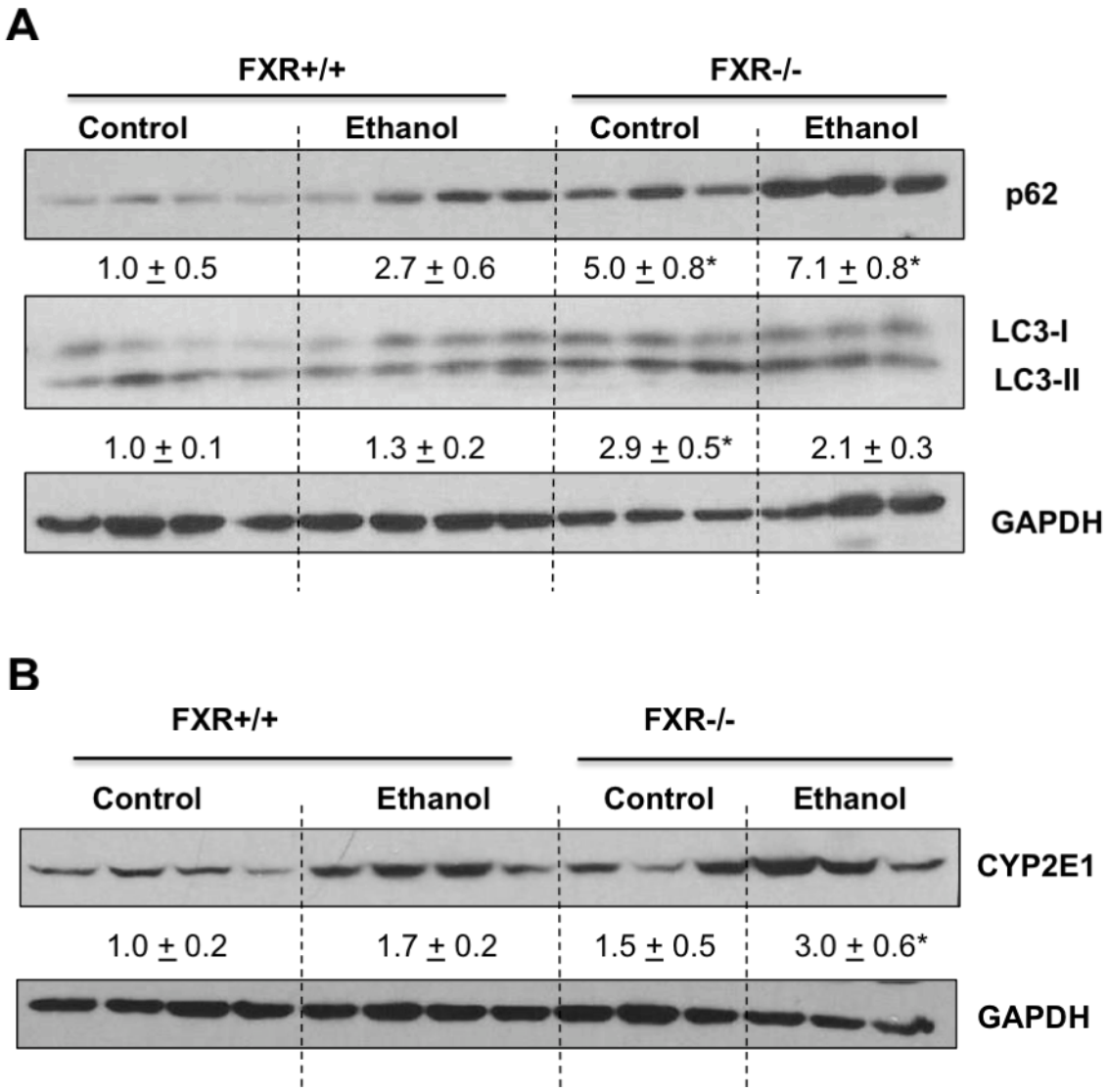
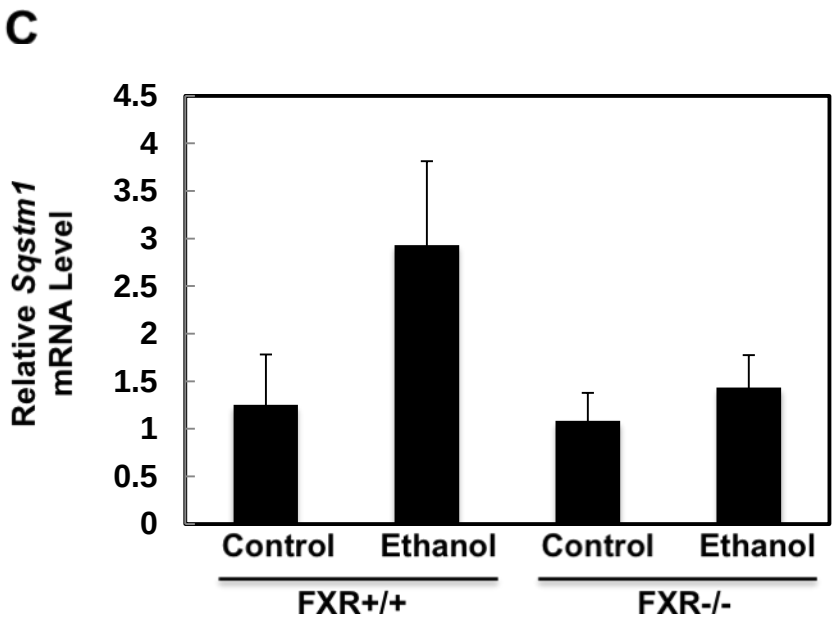

A
FXR+/+
FXR-/-
Control
Ethanol
Control
Ethanol
p62
1.0 ± 0.5
2.7 ± 0.6
5.0 ± 0.8*
7.1 ± 0.8*
LC3-I
LC3-II
1.0 ± 0.1
1.3 ± 0.2
2.9 ± 0.5*
2.1 ± 0.3
GAPDH
B
FXR+/+
FXR-/-
Control
Ethanol
Control
Ethanol
CYP2E1
1.0 ± 0.2
1.7 ± 0.2
1.5 ± 0.5
3.0 ± 0.6*
GAPDH

Figure 5.6 Cont'd



Autophagy was impaired in acute ethanol-treated FXR KO mice.

Age matched WT and FXR KO mice were treated with 4.5 g/kg ethanol or water by gavage for 16 hours. Total liver lysates were subjected to immunoblot analysis for autophagy-related proteins, p62 and LC3 **(A)** and ethanol-metabolizing enzyme, CYP2E1 **(b)**. Densitometry analysis data are presented as a ratio of control (n = 3–4). Hepatic mRNA was isolated and qRT-PCR was performed for *Sqstm1* **(C)**. The gene expression levels were normalized to β-actin and shown as fold increase over wild type mice (n=4-7). *: $p<0.05$

Figure 5.7

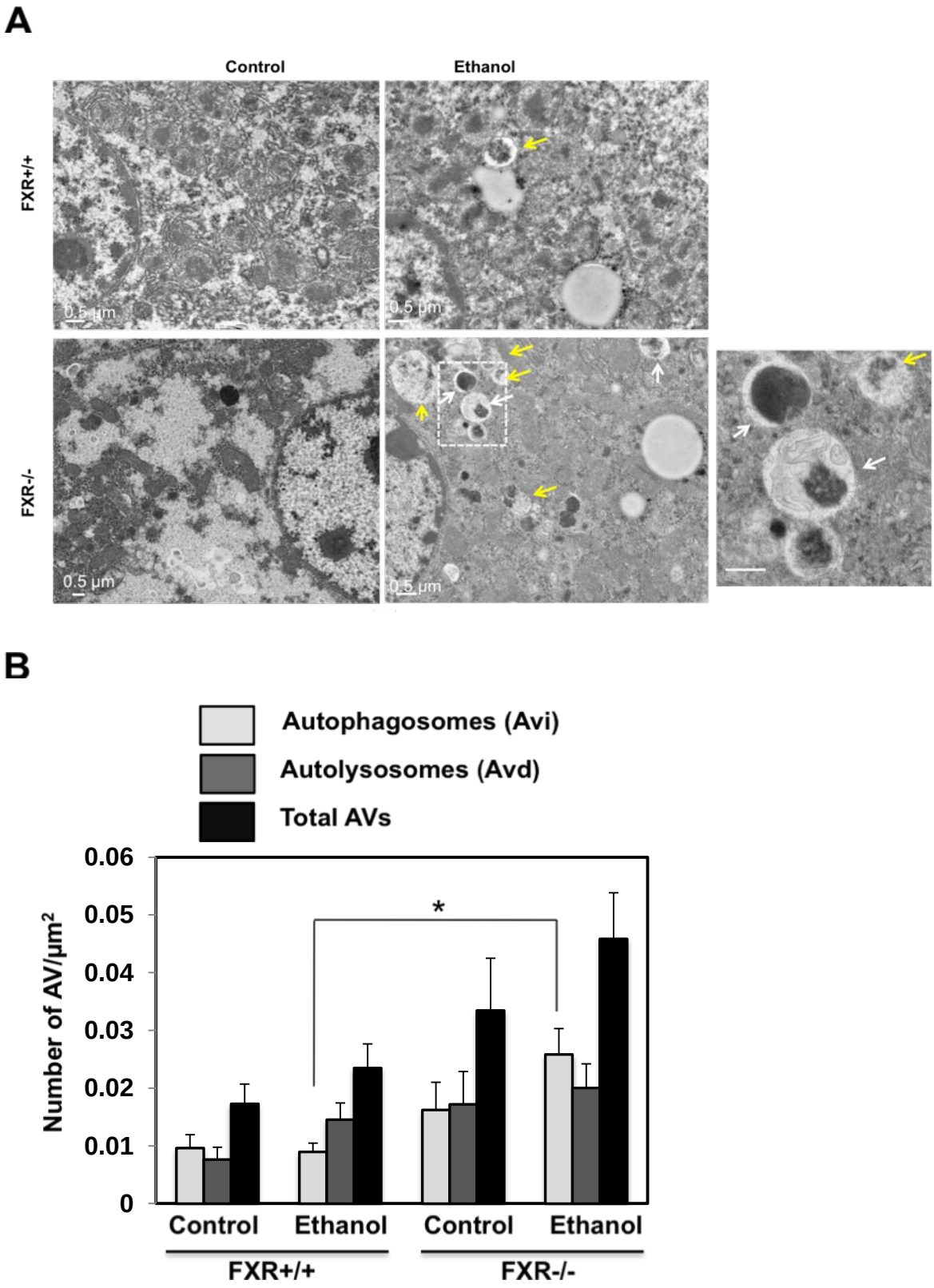
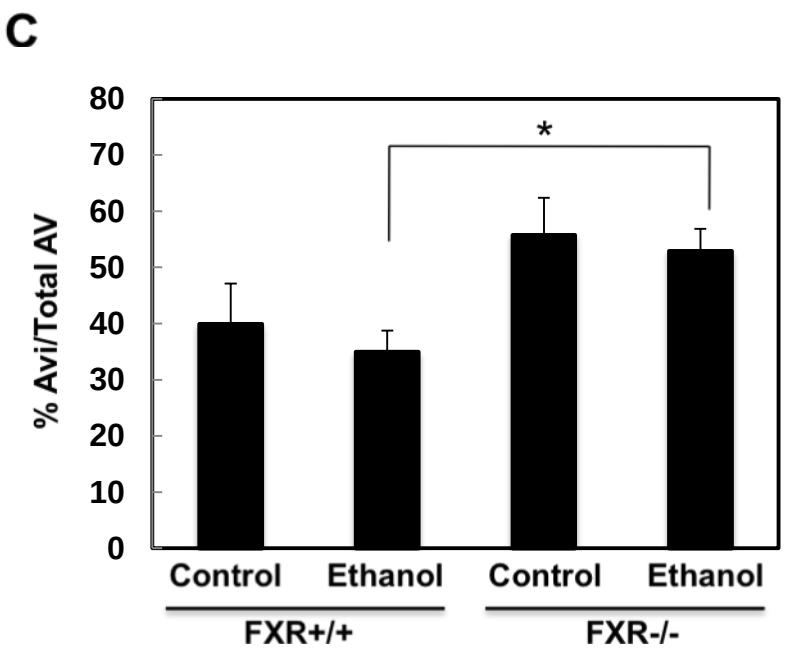

A
Control
Ethanol
FXR+/+
FXR-/-
0.5 μm
0.5 μm
0.5 μm
0.5 μm
B
Autophagosomes (Avi)
Autolysosomes (Avd)
Total AVs
Number of AV/μm²
0.06
0.05
0.04
0.03
0.02
0.01
0
*
Control
Ethanol
Control
Ethanol
FXR+/+
FXR-/-

Figure 5.7 Cont'd



EM Analysis showed that autophagy was impaired in acute ethanol-treated FXR KO mouse livers.

Age matched WT and FXR KO mice were treated with 4.5 g/kg ethanol or water by gavage for 16 hours. Representative EM images are shown in **(A)**. Right image is the enlarged image from the box area. White arrows: early autophagosomes (Avi) and yellow arrows: autolysosomes (Avd) **(A)**. Autophagosomes (Avi) and autolysosomes (Avd) were quantified **(B)** (<24 different liver sections). Ratio of Avi over total AV also was calculated **(C)**. *: $p<0.05$

Lack of FXR impaired acute ethanol-induced FoxO3a activation.

We previously reported that acute ethanol treatment induces FoxO3a-mediated hepatic expression of autophagy related genes (Ni, Du et al. 2013). Consistent with our previous report, ethanol treatment increased the mRNA levels of *Atg5, Becn-1* and *Map1lc3b* in WT mouse livers **(Figure 5.8A)**. In contrast, ethanol treatment did not cause such an increase, and actually showed a slight decrease of hepatic expression of these autophagy related genes in FXR KO mice. In addition to autophagy related genes, ethanol treatment also increased expression of *MnSod*, *p21* and *FoxO3a*, which are known FoxO3a target genes in WT but not in FXR KO mouse livers **(Figure 5.8B)**. *Shp* (*small heterodimer partner*) is a well-known target gene of FXR, and ethanol treatment had no effect on the expression of hepatic *Shp* mRNA level in WT mice, suggesting that acute ethanol treatment may not activate FXR. In contrast, FXR KO mice had dramatically decreased expression of hepatic *Shp*, which was further decreased with ethanol treatment **(Figure 5.8C)**. The immunoblotting analysis showed that ethanol treatment did not increase Atg5 or Beclin-1 protein levels **(Figure 5.9)**. It is possible that these proteins could be used for the induction of autophagosome formation at that particular time point. A detail time-course study for the autophagy-regulating proteins after ethanol treatment may be needed in the future. In conclusion, these results suggest that lack of FXR impairs ethanol-induced FoxO3a activation in mouse livers.

Figure 5.8

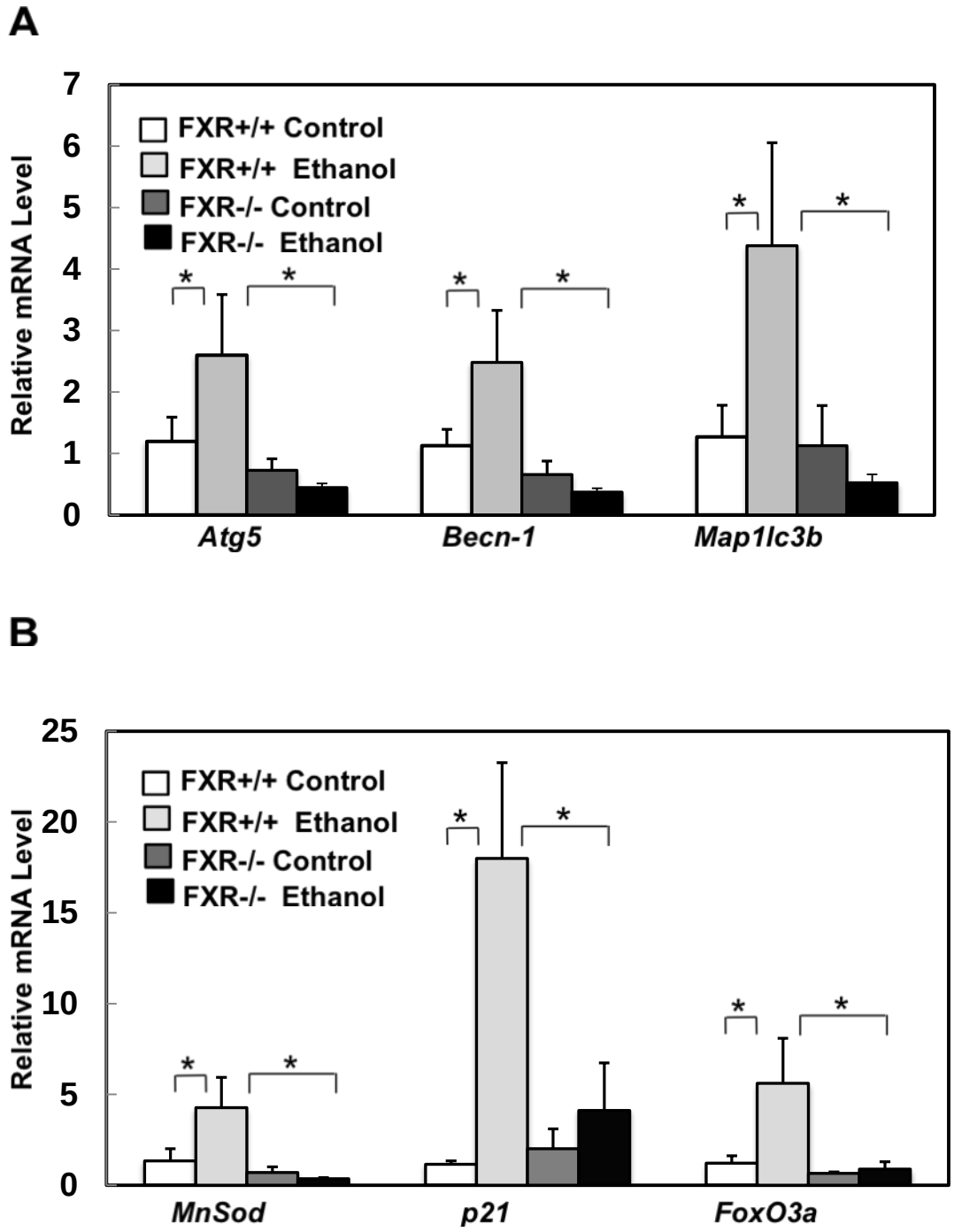
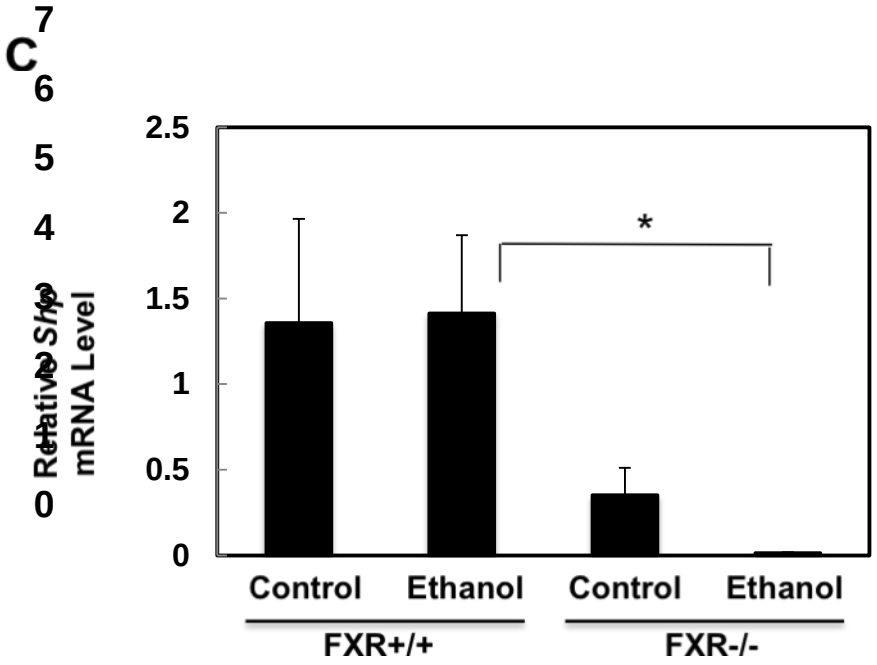

A
Relative mRNA Level
0
1
2
3
4
5
6
7
FXR+/+ Control
FXR+/+ Ethanol
FXR-/- Control
FXR-/- Ethanol
*
*
*
*
*
*
Atg5
Becn-1
Map1lc3b
B
Relative mRNA Level
0
5
10
15
20
25
FXR+/+ Control
FXR+/+ Ethanol
FXR-/- Control
FXR-/- Ethanol
*
*
*
*
*
*
MnSod
p21
FoxO3a

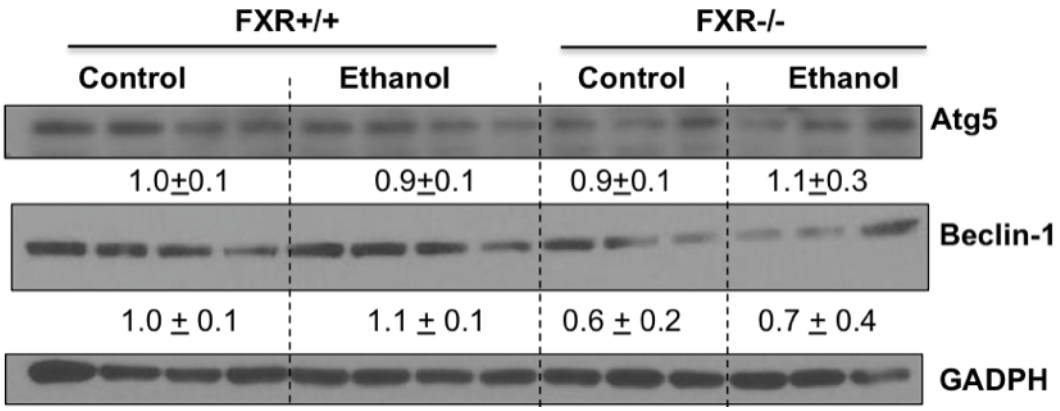
Figure 5.8 Cont'd



Ethanol-induced FoxO3a-mediated transcription of autophagy and FoxO3a target genes was inhibited in FXR KO mouse livers.

Age matched WT and FXR KO mice were treated with 4.5 g/kg ethanol or water by gavage for 16 hours. Hepatic mRNA was isolated and qRT-PCR was performed for autophagy genes, *Atg5, Becn-1, and Map1lc3B* **(A)** and FoxO3a target genes, *MnSOD, p21,* and *FoxO3a* **(B)**. qRT-PCR was also performed for FXR target gene, *Shp* **(c)**. The gene expression levels were normalized to β-actin and shown as fold increase over wild type mice (n=4-7). *: $p<0.05$

Figure 5.9



Ethanol did not increase Atg5 or Beclin-1 protein levels.

Age matched WT and FXR KO mice were treated with 4.5 g/kg ethanol or water by gavage for 16 hours. Total liver lysates were subjected to immunoblot analysis for autophagy-related proteins, Atg5 and Beclin-1. Densitometry analysis data are presented as a ratio of control (n = 3–4).

No direct interaction between FoxO3a and FXR both *in vitro* and *in vivo*.

To determine whether FXR would directly interact with FoxO3a, we transiently transfected with HA-tagged FoxO3a, Flag-tagged FXR, or both in human embryonic kidney (HEK) 293A cells. We found that there was a weak interaction between Flag-FoxO3a and HA-FXR. However, Flag tag only also showed similar weak interaction with HA-FXR, suggesting that the interaction between Flag-FoxO3a and HA-FXR is not specific. Immunoblotting analysis of the total lysate revealed that the transfection was efficient in HEK293 cells **(Figure 5.10A)**. We further found that FoxO3a was present in both nuclear and cytosolic fractions, whereas FXR was dominantly expressed in nuclear fraction, suggesting that the lack of interaction between FXR and FoxO3a was not due to the alterations of cellular localization as a result of the overexpression of these two proteins **(Figure 5.10B)**. To determine whether endogenous FXR in mouse livers would interact with endogenous FoxO3a, and whether this interaction would be altered by ethanol treatment, immunoprecipitation was performed using endogenous liver proteins from mouse livers with or without the ethanol treatment. Similar to the results from the overexpression of FoxO3a and FXR in HEK293 experiment, no interaction between FoxO3a and FXR was found in either control or ethanol-treated mouse livers **(Figure 5.10C)**. These results suggest that FXR may not directly interact with FoxO3a in ethanol-treated mouse livers.

Figure 5.10

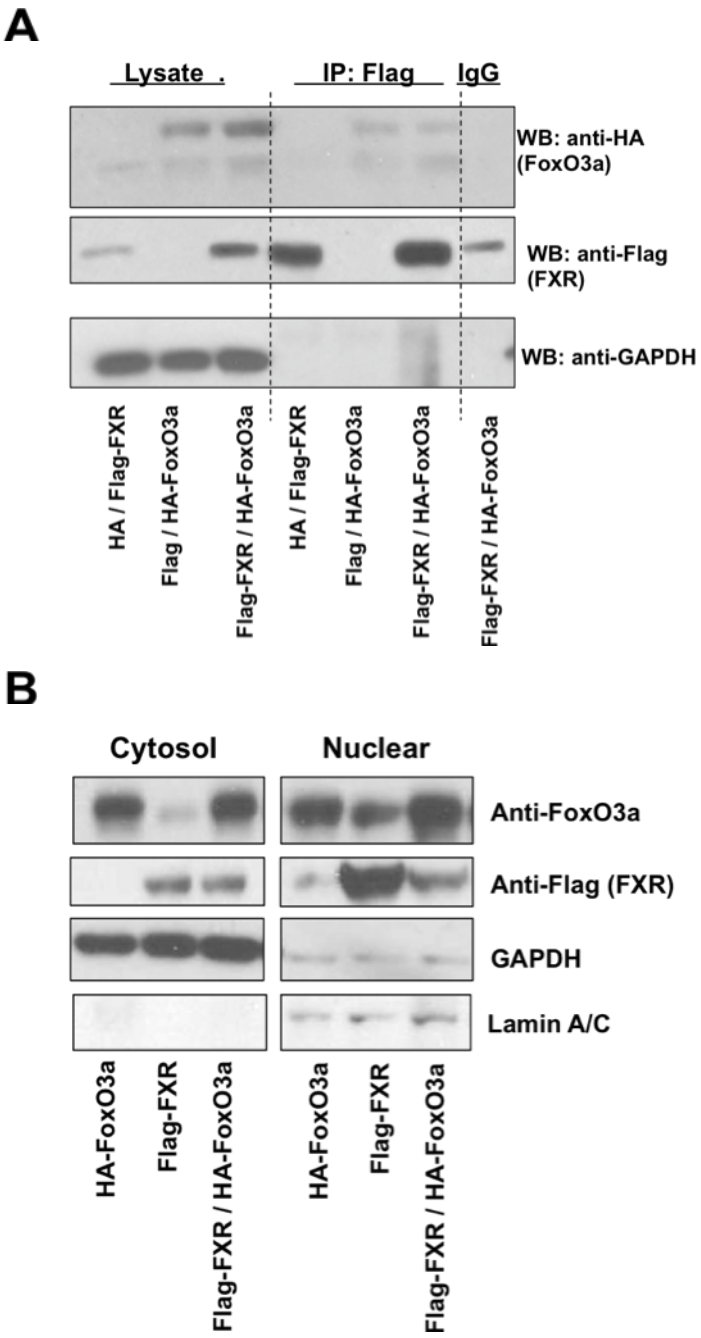
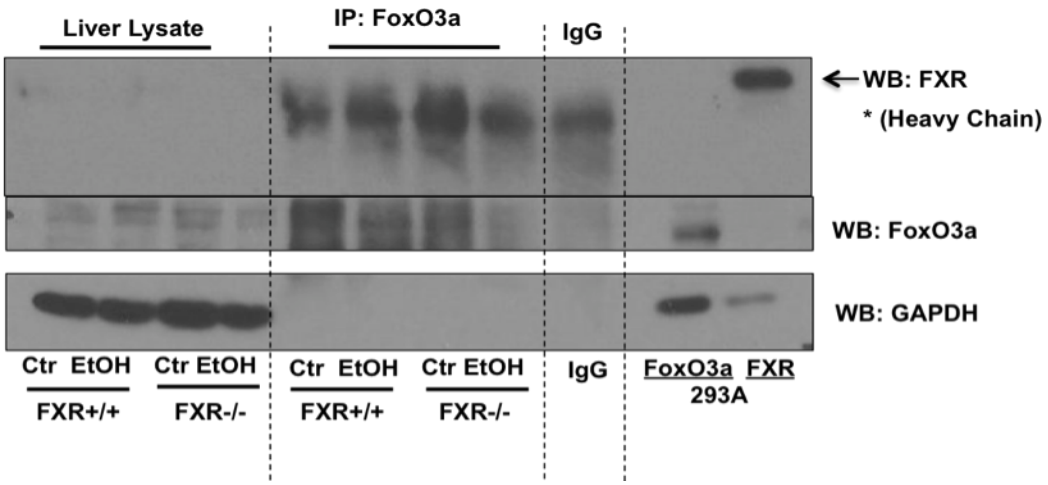

A
Lysate .
IP: Flag
IgG
WB: anti-HA (FoxO3a)
WB: anti-Flag (FXR)
WB: anti-GAPDH
HA / Flag-FXR
Flag / HA-FoxO3a
Flag-FXR / HA-FoxO3a
HA / Flag-FXR
Flag / HA-FoxO3a
Flag-FXR / HA-FoxO3a
Flag-FXR / HA-FoxO3a
B
Cytosol
Nuclear
Anti-FoxO3a
Anti-Flag (FXR)
GAPDH
Lamin A/C
HA-FoxO3a
Flag-FXR
Flag-FXR / HA-FoxO3a
HA-FoxO3a
Flag-FXR
Flag-FXR / HA-FoxO3a

Figure 5.10 Cont'd

C



FXR and FoxO3a did not interact *in vitro* or *in vivo*.

HEK 293A cells were transfected with plasmids containing Flag-FXR, HA-FoxO3a, HA or Flag. Total cell lysates were isolated, and Flag was pulled down by immunoprecipitation and subjected to immunoblot analysis for Flag and HA. Input total lysates from transfected HEK 293A were used as positive controls for FoxO3a and FXR **(A)**. Cytosol and nuclear fractions also were obtained from transfected HEK 293A cells, and immunoblot analysis was performed for Flag and FoxO3a **(B)**. Age matched WT and FXR KO mice were treated with 4.5 g/kg ethanol or water by gavage for 16 hours. FoxO3a in total liver lysates was pulled down by immunopreciptation and subjected to immunoblot analysis for FXR and FoxO3a **(C)**.

Ethanol-induced FoxO3a nuclear translocation was inhibited in FXR KO mouse livers.

We next determined whether lack of FXR would affect the cellular localizations of FoxO3a in ethanol-treated mouse livers. Immunostaining analysis for endogenous FoxO3a revealed that acute ethanol treatment increased the number of cells with nuclear FoxO3a signals in WT but not in FXR KO mouse livers **(Figure 5.11 A&B)**. To confirm the findings from the immunostaining analysis, cytosolic and nuclear fractions were prepared from WT and FXR KO mouse livers followed by immunoblotting analysis. We found that there was a decrease of cytosolic but increase of nuclear FoxO3a in WT mouse livers after ethanol treatment. However, acute ethanol treatment failed to increase nuclear FoxO3a levels in FXR KO mice **(Figure 5.11C)**. These data suggest that acute ethanol-induced nuclear retention of FoxO3a is inhibited in FXR KO mouse livers.

Figure 5.11

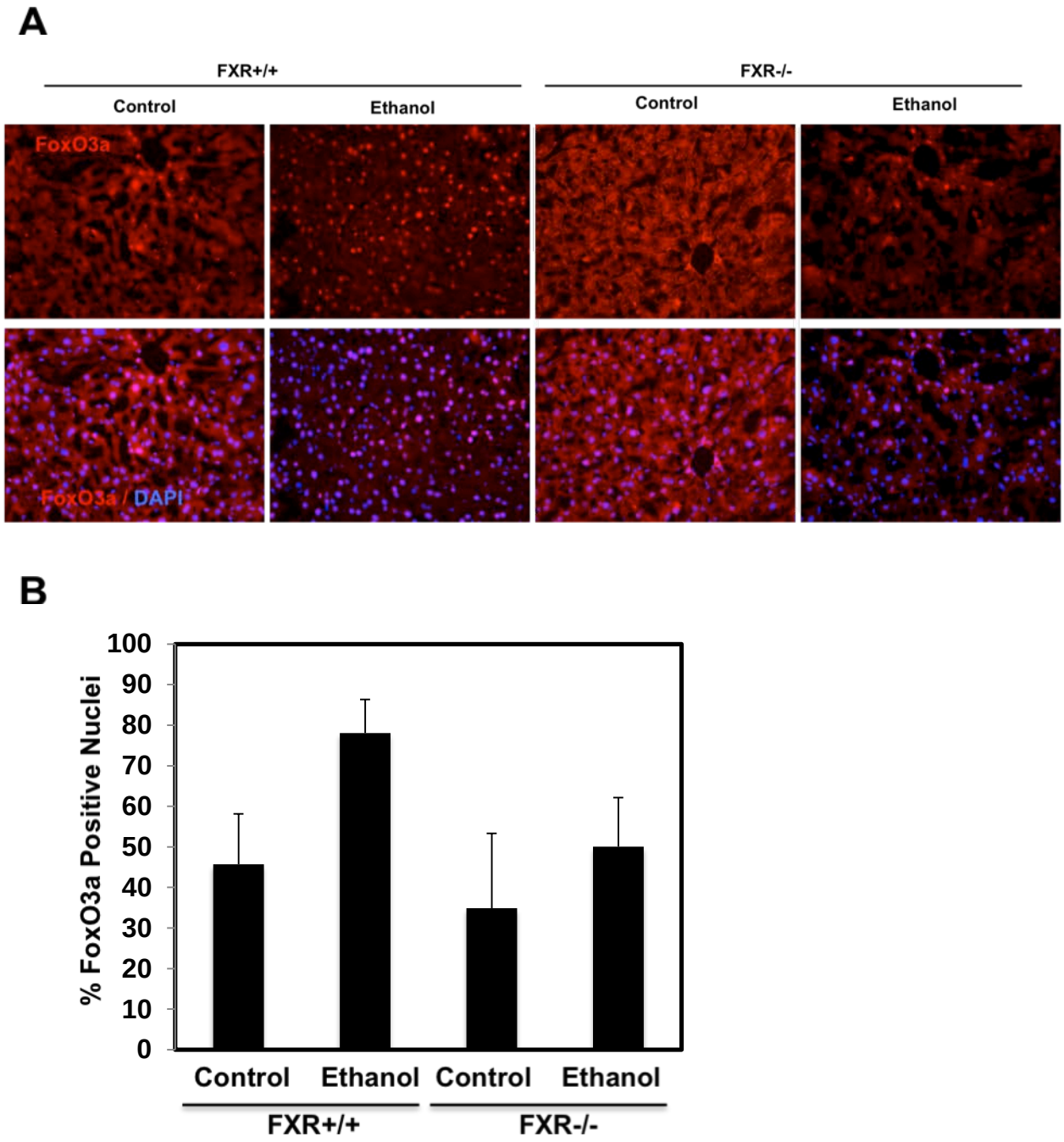
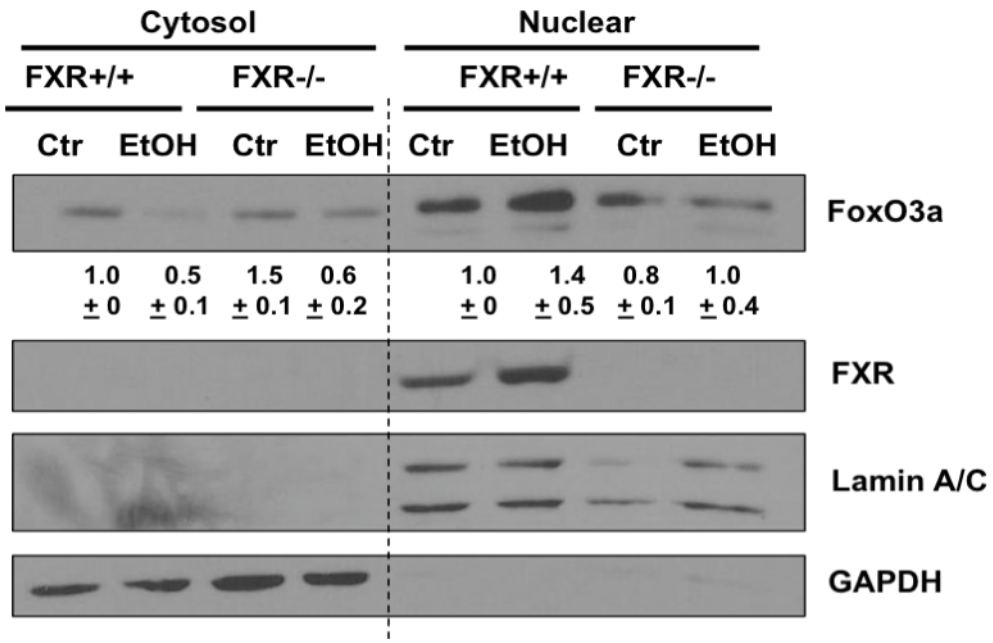

A
FXR+/+
FXR-/-
Control
Ethanol
Control
Ethanol
FoxO3a
FoxO3a / DAPI
B
% FoxO3a Positive Nuclei
100
90
80
70
60
50
40
30
20
10
0
Control
Ethanol
Control
Ethanol
FXR+/+
FXR-/-

Figure 5.11 (cont'd)

C



Ethanol-induced FoxO3a nuclear translocation was diminished in FXR KO mouse livers.

Age matched WT and FXR KO mice were treated with 4.5 g/kg ethanol or water by gavage for 16 hours. Liver cyrosections were immunostained for FoxO3a, and representative images are shown in **(A)**. Nuclei positive for FoxO3a were quantified from at least 3 images **(B)**. Cytosolic and nuclear fractions were isolated from liver and subjected to immunoblot analysis for FoxO3a and FXR **(C)** Densitometry analysis data are presented as a ratio of control (n = 3).

Acute ethanol treatment increased Akt-mediated FoxO3a phosphorylation and inhibited ethanol-induced FoxO3 binding in *Map1lc3b* promoter sites in FXR KO mouse livers

Akt is the canonical regulator of FoxO3a, and sequesters FoxO3 into cytosol and decrease FoxO3a activity by promoting phosphorylation of FoxO3a (Tzivion, Dobson et al. 2011). We found that acute ethanol treatment slightly decreased the phosphorylation of Akt at serine 473 in WT mouse livers, which is consistent with our previous report (Ni, Du et al. 2013). Conversely, we found that acute ethanol treatment decreased phosphorylation of Akt, but dramatically increased the phosphorylation of Akt in FXR KO mouse livers by five-fold **(Figure 5.12A)**. Glycogen synthase kinase beta (GSK3β) can be phosphorylated by Akt and thus serves as an indirect marker for Akt kinase activity. We found that the phosphorylation of GSK3β at serine 9 was reduced in ethanol-treated WT mouse livers. Furthermore, phosphorylation of GSK3β was enhanced by ethanol treatment in FXR KO mouse livers by around 2.5-fold **(Figure 5.12A)**. These data suggest that acute ethanol treatment inhibits Akt activity in WT mouse livers but increases Akt activity in FXR KO mouse livers. As a result, we found that ethanol treatment attenuated the phosphorylation of FoxO3a at serine 253 in WT mouse livers but increased the phosphorylation of FoxO3a in FXR KO mouse livers **(Figure 5.12B)**. Ethanol treatment increased total hepatic FoxO3a protein expression in WT mouse livers by two-fold but decreased the hepatic FoxO3a protein level in FXR KO mice. Since FoxO3a itself is a target gene of FoxO3a, these data thus may also imply that there is a decrease of FoxO3a transcriptional activity in FXR KO mouse livers after ethanol treatment compared to WT mice. We next performed ChIP analysis to

determine whether there would be any changes for FoxO3a binding on its target genes between WT and FXR KO mouse livers after ethanol treatment. It has previously been reported that FoxO3a binds to mouse *Map1lc3b* transcription promoter region in three different sites at approximately 1.5, 3.5 and 4.5 kilo base pairs upstream of the *Map1lc3b* transcription start site (Zhao, Brault et al. 2007). Indeed, ethanol increased FoxO3a binding to all three binding sites in WT mouse livers, but these bindings were significantly diminished in FXR KO mouse livers **(Figure 5.12C)**. Collectively, these data suggest that ethanol treatment activates Akt and increased phosphorylation of FoxO3a resulting in decreased FoxO3a transcriptional activity in FXR KO mouse livers.

Figure 5.12

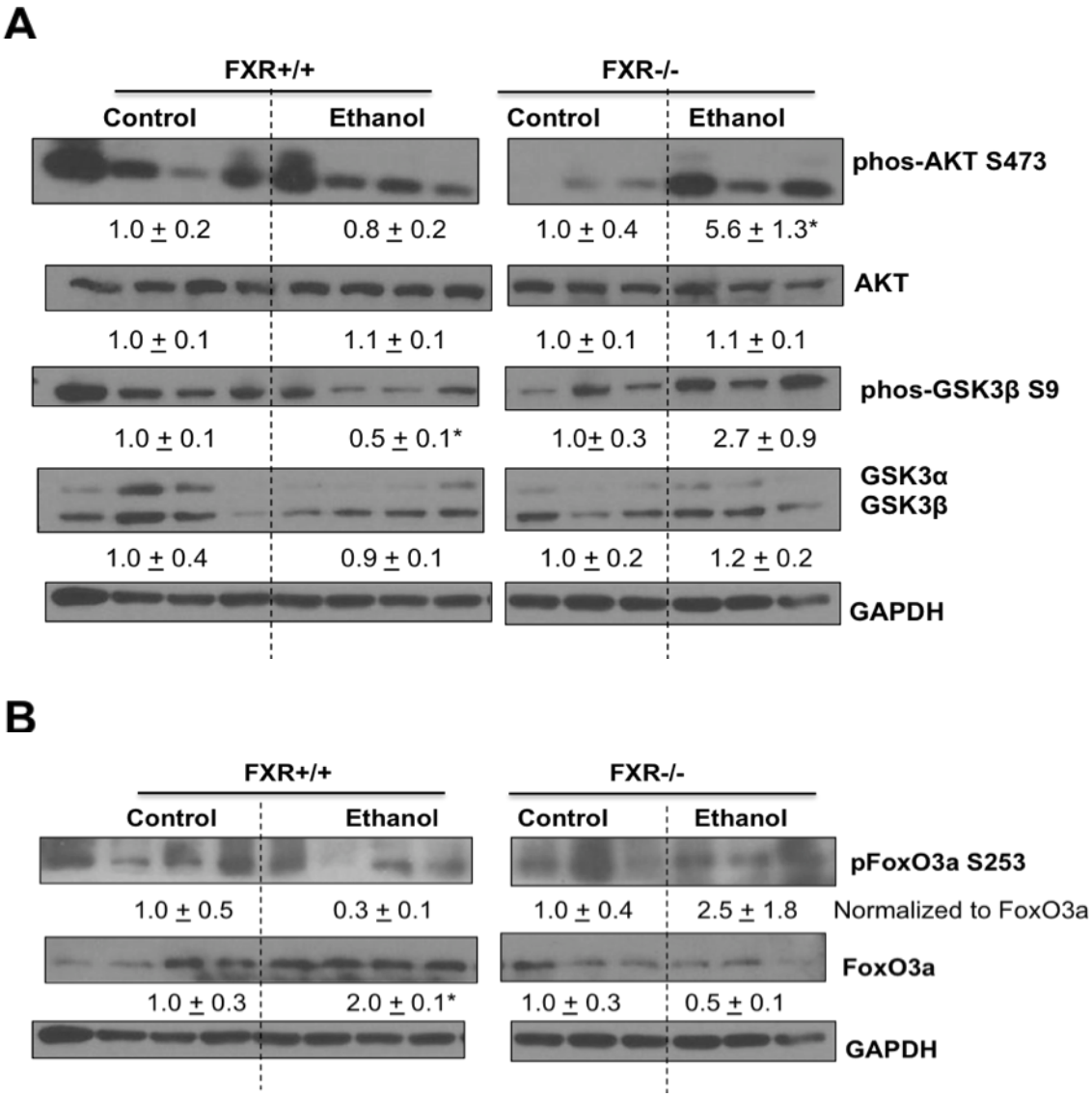
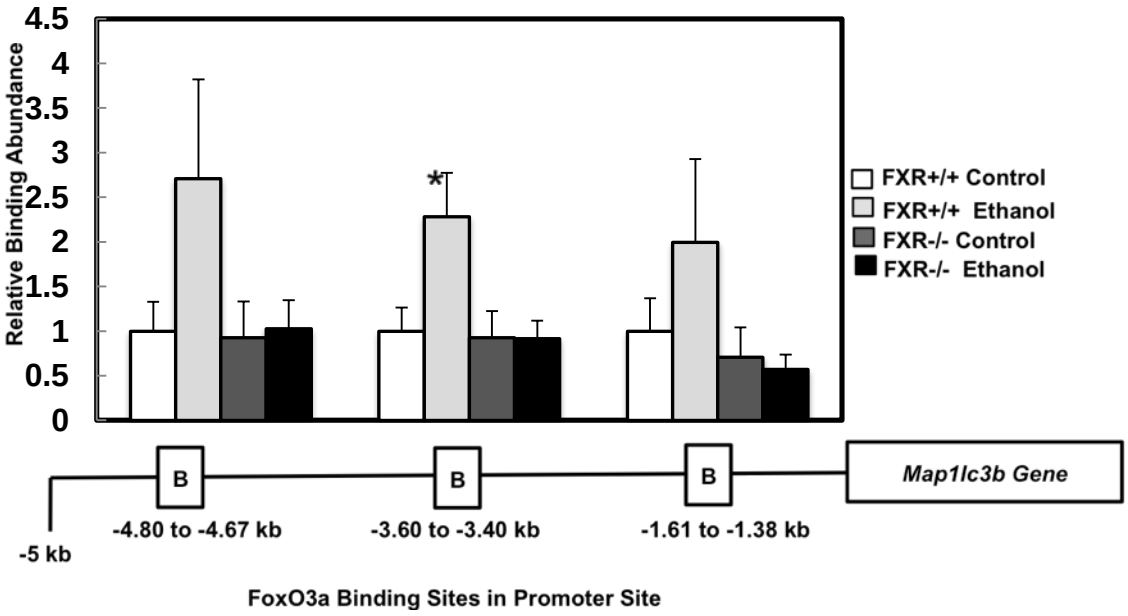

A
FXR+/+
FXR-/-
Control
Ethanol
Control
Ethanol
phos-AKT S473
1.0 ± 0.2
0.8 ± 0.2
1.0 ± 0.4
5.6 ± 1.3*
AKT
1.0 ± 0.1
1.1 ± 0.1
1.0 ± 0.1
1.1 ± 0.1
phos-GSK3β S9
1.0 ± 0.1
0.5 ± 0.1*
1.0± 0.3
2.7 ± 0.9
GSK3α
GSK3β
1.0 ± 0.4
0.9 ± 0.1
1.0 ± 0.2
1.2 ± 0.2
GAPDH
B
FXR+/+
FXR-/-
Control
Ethanol
Control
Ethanol
pFoxO3a S253
1.0 ± 0.5
0.3 ± 0.1
1.0 ± 0.4
2.5 ± 1.8
Normalized to FoxO3a
FoxO3a
1.0 ± 0.3
2.0 ± 0.1*
1.0 ± 0.3
0.5 ± 0.1
GAPDH

Figure 5.12 Cont'd

C



Increased Akt activity resulted in inhibition of ethanol-induced FoxO3a binding in *Map1lc3b* promoter site in FXR KO mouse livers.

Age matched WT and FXR KO mice were treated with 4.5 g/kg ethanol or water by gavage for 16 hours. Total liver lysates were subjected to immunoblot analysis for serine 473 phosphorylated and total AKT, serine 9 phosphorylated and total GSK3β **(A)**, and serine 253 phosphorylated and total FoxO3a **(B)**. Densitometry analysis data are presented as a ratio of control (n = 3–4). Chromatin immunoprecipitation using FoxO3a antibody was performed to probe the three putative FoxO3a binding sites in *Map1lc3b* promoter site. DNA was isolated from chromatin pull-down and qPCR analysis was performed, and data was normalized to input chromatin. Relative binding abundance in comparison to untreated WT control was presented in **(C)**. *: $p<0.05$

Acute ethanol treatment had differential effect on sirtuin-1 protein expression and decreased FoxO3 acetylation in FXR KO mouse livers.

FoxO3a is a promiscuous binding partner, and Sirt1, a NAD-dependent deacetylase, and PGC-1α, a regulator of mitochondria biogenesis, are two reported co-factors of FoxO3a (Brunet, Sweeney et al. 2004; Sandri, Lin et al. 2006; Olmos, Valle et al. 2009; Borniquel, Garcia-Quintans et al. 2010). We found that ethanol treatment did not increase PGC-1α protein levels in WT and FXR KO mouse livers. Consistent with our previous findings, ethanol treatment decreased hepatic Sirt1 protein levels in WT mouse liver. However ethanol treatment increased Sirt1 protein levels in FXR KO mouse livers **(Figure 5.13A)**. Furthermore, ethanol didn't affect acetylation of FoxO3a in WT mouse livers, but decreased acetylation of FoxO3a in FXR KO mouse livers **(Figure 5.13B)**. Collectively, the data suggest that Sirt1 may mediate the decreased acetylation of FoxO3a in FXR KO mouse livers although future studies are needed to further elucidate these possibilities.

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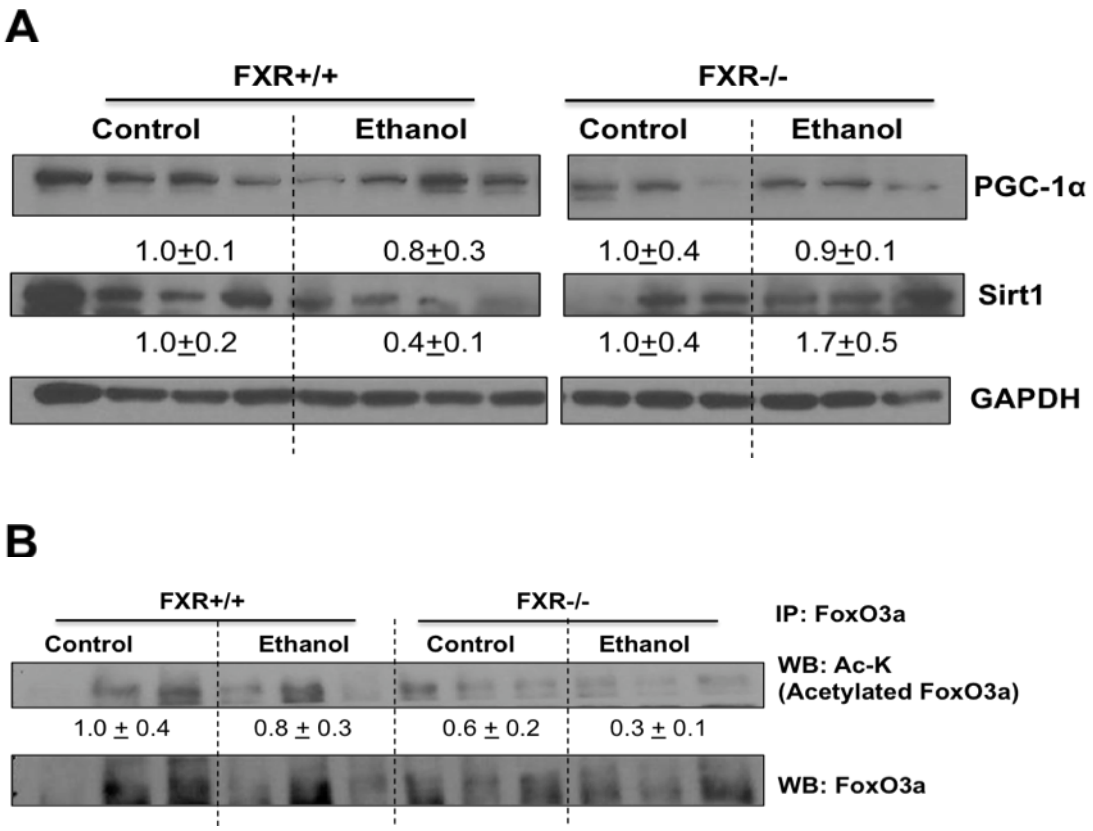
Figure 5.13



Acute ethanol treatment increased Sirt1 protein levels and decreased acetylation of FoxO3a in FXR KO mouse livers.

Age matched WT and FXR KO mice were treated with 4.5 g/kg ethanol or water by gavage for 16 hours. Total liver lysates were subjected to immunoblot analysis for PGC-1α and Sirt1 **(A)**. Densitometry analysis data are presented as a ratio of control (n = 3–4). FoxO3a in total liver lysates was pulled down by immunopreciptation and subjected to immunoblot analysis for acetylated lysine and FoxO3a **(B)**.

Acute ethanol treatment increased the number of mitochondrial spheroid in ethanol-treated FXR KO mouse livers

We previously reported that mitochondrial spheroids are induced and may serve as an alternative mitochondrial quality control pathway in response to stresses that induce mitochondrial damage as a result of impaired conventional mitophagy (Ding, Guo et al. 2012). In ethanol-treated FXR KO mouse livers, we found that some mitochondria underwent a dramatic structural remodeling that form a vesicular-like structure with enveloped contents. Some part of the mitochondria formed a four-membrane structure with squeezed matrix (**Figure 5.14A, arrows**), which is consistent with the formation of mitochondrial spheroids that we previously reported (Ding, Guo et al. 2012). We did not detect any mitochondrial spheroids in either control or ethanol-treated WT mouse livers but quantitative EM analysis showed increased number of mitochondrial spheroids in control FXR KO mouse livers, which was further increased after ethanol treatment (**Figure 5.14B**). These results suggest that acute ethanol treatment may induce the formation of mitochondrial spheroids as an alternative mitochondrial quality control to compensate for the impaired autophagy in FXR KO mouse livers. We previously demonstrated that Parkin negatively regulates the formation of mitochondrial spheroids by promoting the degradation of mitofusin 1 (Mfn1) and Mfn2, two important proteins that regulate mitochondrial fusion (Ding, Guo et al. 2012; Yin and Ding 2013). Interestingly, we found that acute ethanol treatment caused almost 50% loss of hepatic Parkin. While the mechanisms of how ethanol decreased Parkin protein levels in the mouse livers were not clear, we have found that chronic plus binge alcohol treatment (Gao-binge) led to the Parkin mitochondrial translocation

(Williams JA et al, unpublished observations). Thus it is possible that the decreased Parkin in acute ethanol-treated mouse livers was due to the increased autophagic degradation of Parkin positive mitochondria (mitophagy). Interestingly, we also found increased degradation of Mfn1 and Mfn2 in ethanol-treated wild type mouse livers but the levels of Mfn1 and Mfn2 were less affected in FXR KO mouse livers (**Figure 5.14C**). The relatively higher levels of Mfn1 and Mfn2 in ethanol-treated FXO KO mouse livers than that of wild type mice may account for the increased mitochondrial spheroids formation in FXO KO mouse livers. We also found that acute ethanol treatment led to approximately 30% decrease on hepatic Lamp-1 and Lamp-2 proteins in both wild type and FXR KO mice (**Figure 5.14C**). Alcohol consumption has been implicated in impaired lysosomal functions but whether the decreased Lamp-1 and Lamp-2 would contribute to the impaired lysosomal functions need to be further studied. These results suggest that acute ethanol treatment may induce the formation of mitochondrial spheroids as an alternative mitochondrial quality control to compensate for the impaired autophagy in FXR KO mouse livers.

Figure 5.14

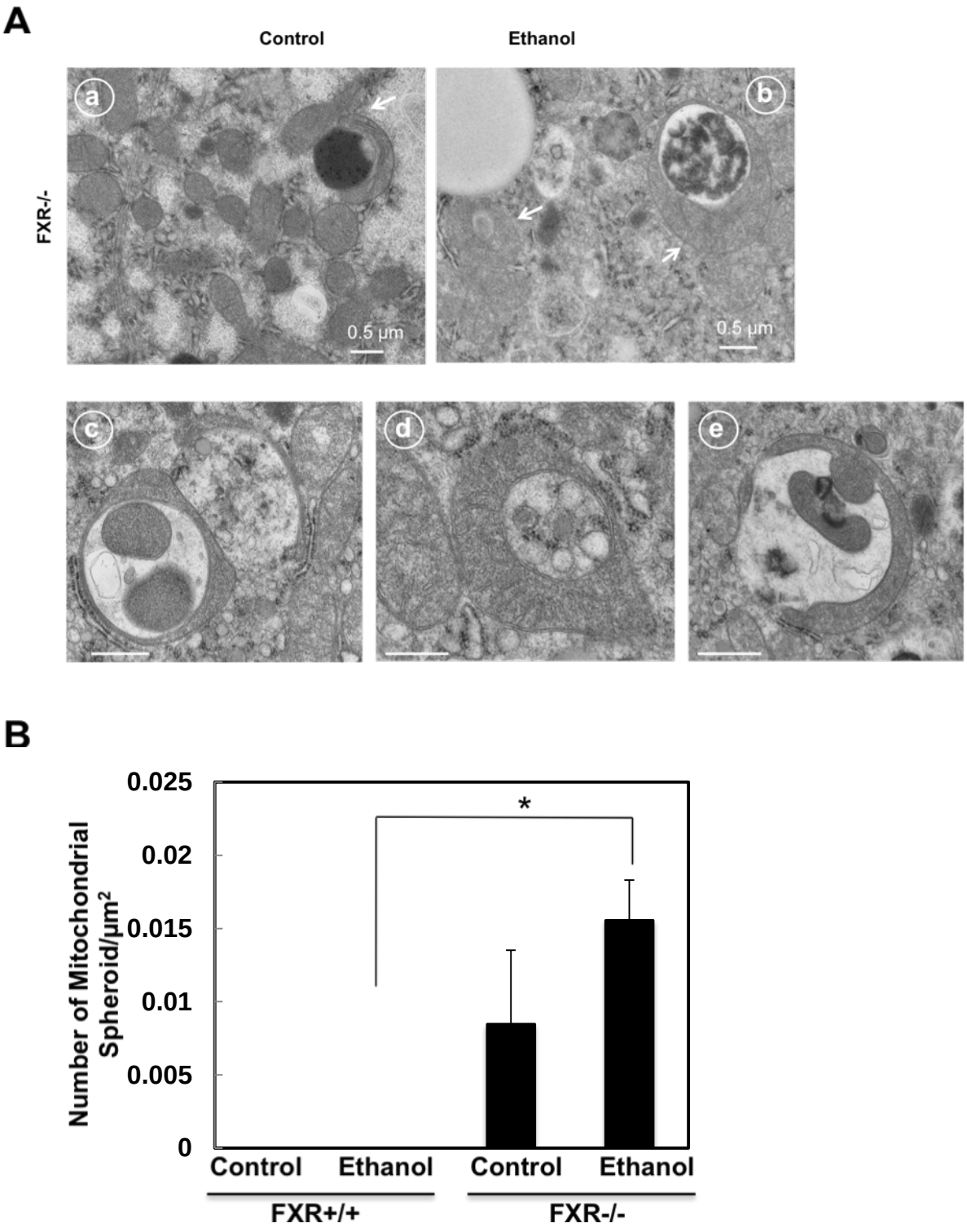
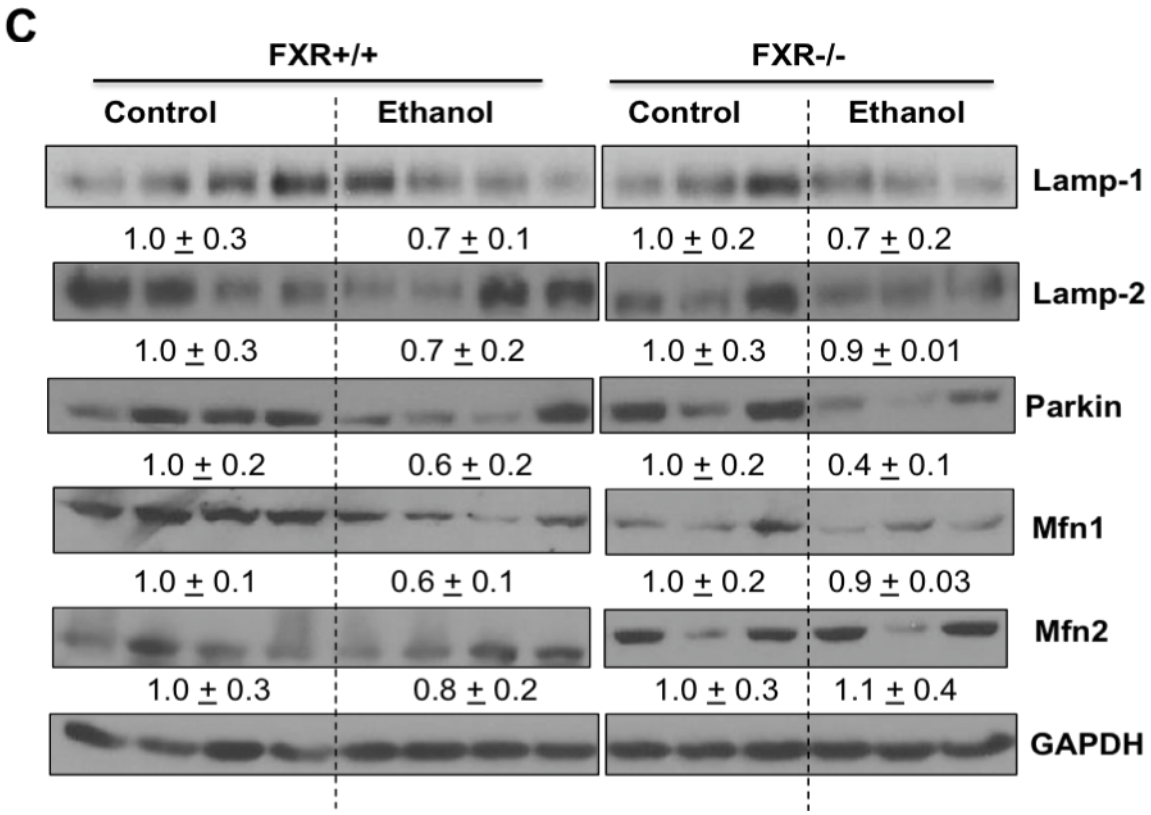

A
Control
Ethanol
FXR-/-
a
b
c
d
e
0.5 μm
0.5 μm
B
Number of Mitochondrial Spheroid/μm²
0.025
0.02
0.015
0.01
0.005
0
*
Control
Ethanol
Control
Ethanol
FXR+/+
FXR-/-

Figure 5.14 Cont'd



Ethanol induced mitochondrial spheroid formation in FXR KO mouse livers.

Age matched WT and FXR KO mice were treated with 4.5 g/kg ethanol or water by gavage for 16 hours. Representative EM images of mitochondrial spheroids from FXR KO control (a) and ethanol-treated (b-e) liver sections were shown **(A)**. Arrows denote mitochondrial spheroids. Mitochondrial spheroid formation was quantified (>24 liver sections) **(B)**. *: $p<0.05$. One-way ANOVA analysis. WT Ethanol vs FXR KO Ethanol. (**C**) Mice were treated as in (**A**), total liver lysates were subjected to western blot analysis followed by densitometry analysis. Densitometry analysis data are presented as a ratio of control (n = 3-4).

FXR activation attenuated ethanol-induced steatosis.

We demonstrated that FXR KO mice have exacerbated ethanol-induced hepatotoxicity and steatosis. Previous reports showed that FXR activation is protective against ethanol-induced hepatotoxicity and steatosis in mice that were fed with Lieber-DeCarli ethanol diet (Livero, Stolf et al. 2014; Wu, Zhu et al. 2014). We treated WT mice with WAY-362450 (WAY), a potent FXR agonist, along with ethanol treatment. Serum ALT was increased in ethanol-treated mice, and was not further decreased with WAY co-treatment. Interestingly, we found that WAY also increased serum ALT levels, but it was not significant **(Figure 5.15A)**. As expected, ethanol, but not WAY treatment increased hepatic triglycerides levels. Ethanol and WAY co-treatment attenuated ethanol-induced increase in hepatic triglycerides level, suggesting that activation of FXR by WAY may protect against ethanol-induced steatosis **(Figure 5.15B)**. Furthermore, ethanol and WAY treatments did not affect liver weight/body weight ratio **(Figure 5.15C)**. These data suggest that FXR activation may protect against ethanol-induced steatosis, but not hepatotoxicity. Furthermore, WAY treatment increased total hepatic FoxO3a protein levels in untreated and ethanol-treated mice, which suggests that FXR activation may promote FoxO3a activation. However, we found that ethanol and WAY treatments and co-treatment did not change total hepatic Beclin-1, Atg5 and LC3-II protein levels. In line with our previous findings, ethanol treatment increased CYP2E1 protein levels with and without WAY. Moreover, WAY treatment itself did not change CYP2E1 protein level **(Figure 5.15D)**. In the future, we need to perform autophagy flux assay to determine whether WAY has any effects on autophagy. Furthermore, the

effects of vehicle, the PEG400:Tween-20 mixture, on autophagy is not known and need to be examined.

The proposed cellular mechanism of how FXR regulates FoxO3a and mitochondria homeostasis is shown in **Figure 5.16**.

Figure 5.15

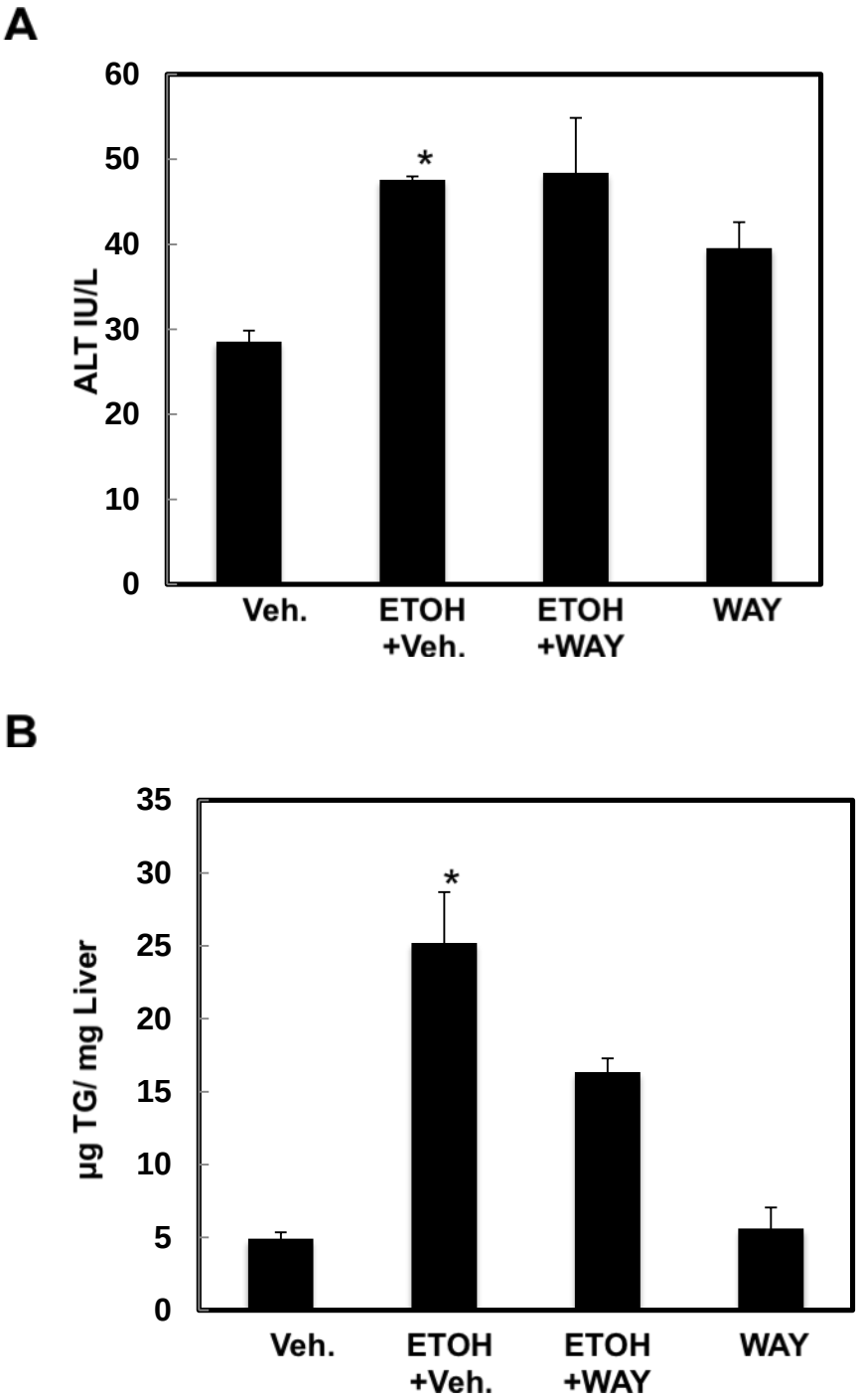

A
60
50
40
30
20
10
0
ALT IU/L
*
Veh.
ETOH +Veh.
ETOH +WAY
WAY
B
35
30
25
20
15
10
5
0
µg TG/ mg Liver
*
Veh.
ETOH +Veh.
ETOH +WAY
WAY

Figure 5.15 Cont'd

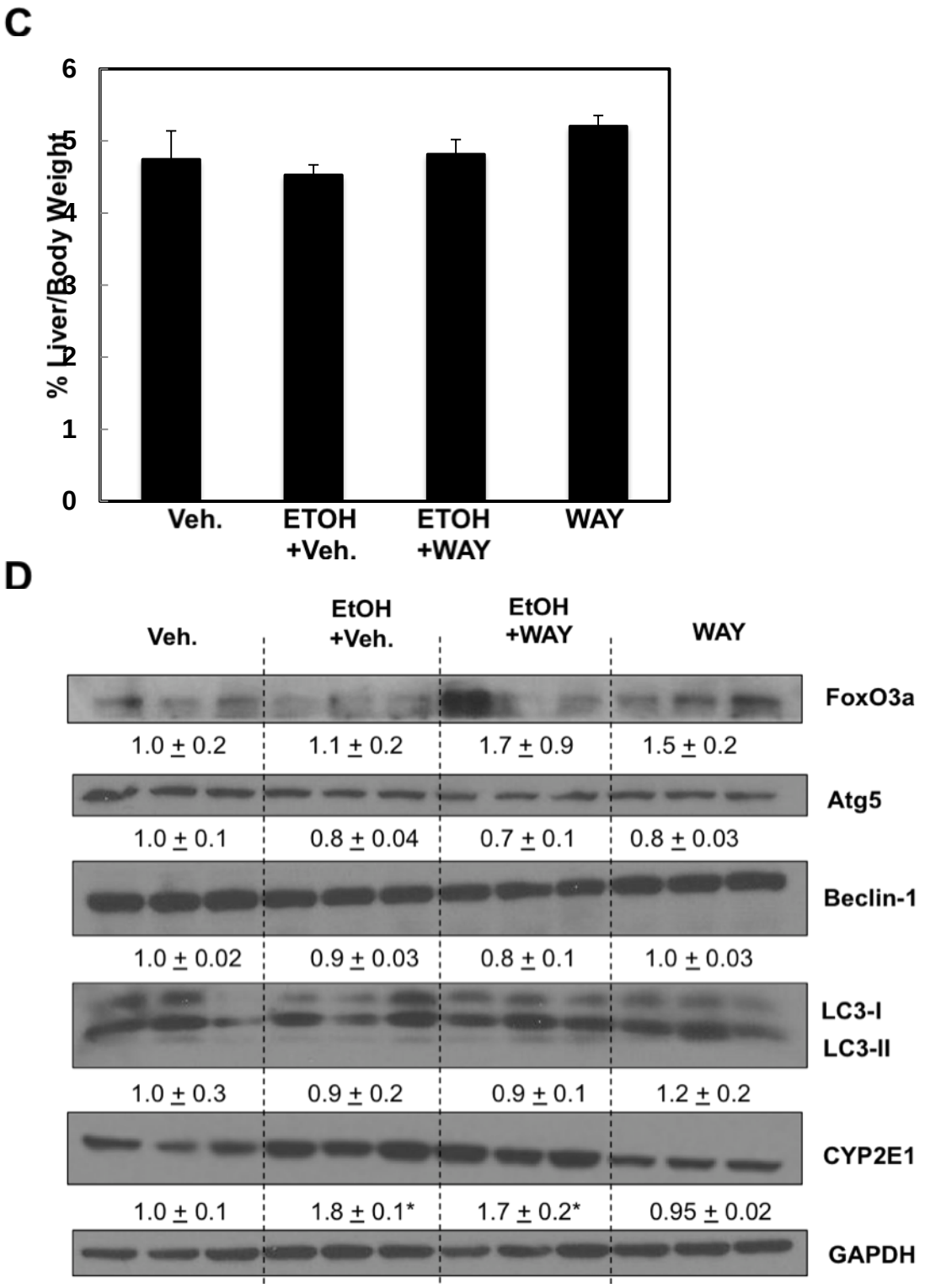
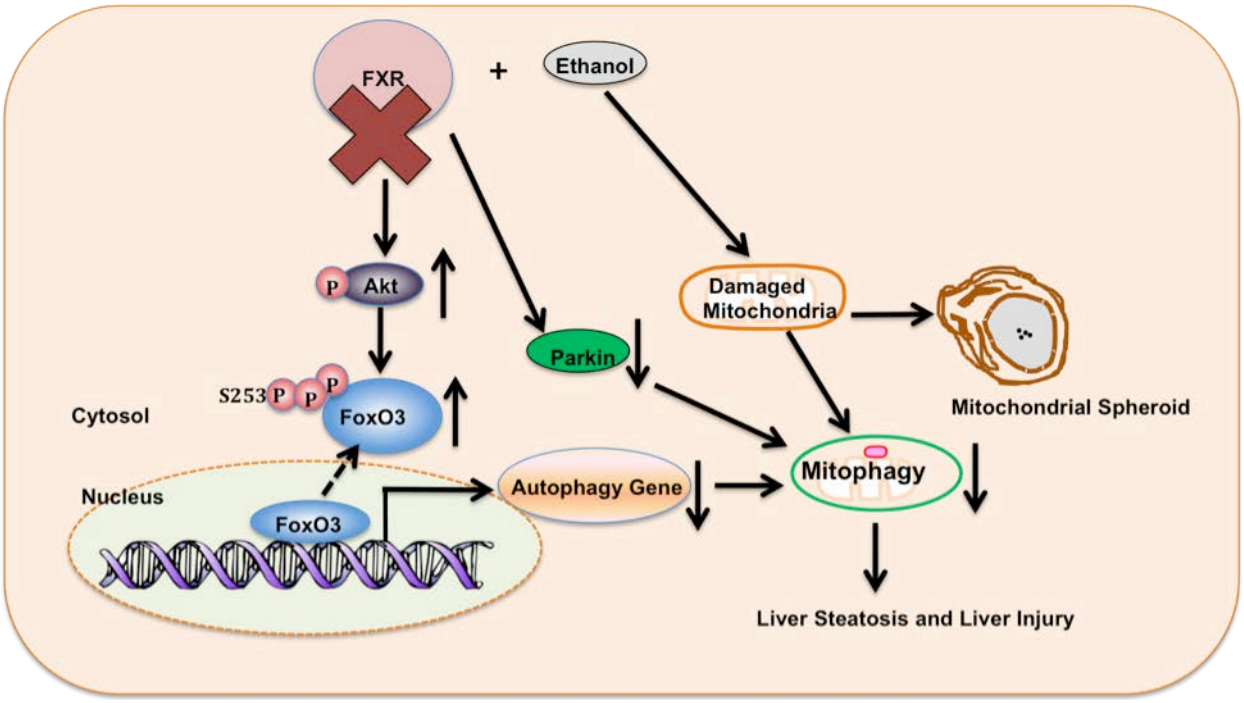


Figure 5.15 Cont'd

FXR activation protected against ethanol-induced steatosis.

WT mice were treated with 4.5 g/kg ethanol and/or 30 mg/kg WAY-362450 by gavage for 16 hours. Serum alanine aminotransferase (ALT) **(A)** and hepatic triglycerides (TG) **(B)** were measured (n=4). Liver/body weight ratio was calculated **(C)** (n=4)**.** Total liver lysates were subjected to immunoblot analysis for FoxO3a, Atg5, Beclin-1, LC3, and CYP2E1 **(D)**. Densitometry analysis data are presented as a ratio of control (n = 3). *: $p<0.05$

Figure 5.16



FXR deficiency results in impaired FoxO3-mediated autophagy induction, decreased mitophagy, and mitochondrial spheroid formation in response to acute ethanol exposure.

Acute ethanol exposure induces FoxO3 activation and mitophagy as a protective mechanism. FXR deficiency promotes Akt activation and subsequent serine 253 phosphorylation of FoxO3, resulting in decreased nuclear FoxO3 retention and abolished transcription of autophagy genes in response to ethanol; therefore, FXR KO mice displays defective hepatic autophagy, and results in impaired mitophagy in FXR KO mice. Impaired mitophagy results in accumulation of damaged mitochondria and oxidative stress; thus, autophagy-independent mitochondrial spheroid formation is initiated. Altogether, impaired FoxO3- mediated autophagy and mitophagy lead to increased liver injury and steatosis.

5.4 DISCUSSION

In the present study, using an acute ethanol binge model, we showed that FXR KO mice had exacerbated steatosis and liver injury in comparison with WT mice. Mechanistically, following acute ethanol treatment, FXR KO mice had impaired hepatic autophagy due to the failure of activating FoxO3a-mediated up-regulation of autophagy related genes and likely reduced fusion of autophagosomes with lysosomes.

It is well known that the nuclear receptor FXR is the master regulator of bile acid homeostasis. FXR deficiency caused an increased concentration of bile acid in the liver and serum due to activation of Cyp7A1 (Sinal, Tohkin et al. 2000; Kim, Morimura et al. 2007). We previously reported that FXR KO mice had reduced hepatic autophagy likely due to impaired fusion of autophagosomes with lysosomes as a result of increased hepatic bile acid levels (Manley, Ni et al. 2014). In addition to removing cytosolic proteins and damaged/excess organelles, autophagy has been known to remove excess lipid droplets, a process named lipophagy (Singh, Kaushik et al. 2009). Impaired autophagy has been reported in many experimental non-alcoholic fatty liver disease and non-alcoholic steatohepatitis models, which was associated with endoplasmic reticulum (ER) stress and post-translational modifications of several autophagy proteins (Yang, Li et al. 2010; Ding, Manley et al. 2011). We also showed that fatty acids differentially regulated autophagy in which saturated fatty acids suppressed whereas unsaturated fatty acids induced autophagy in cultured hepatoma cells (Mei, Ni et al. 2011). In the context of alcoholic liver diseases, we also showed that pharmacological inhibition of autophagy exacerbated acute ethanol-induced steatosis (Ding, Li et al. 2010). Therefore, the impaired autophagy in FXR KO mice is likely one of the major

contributors for the exacerbated hepatic steatosis in acute ethanol-treated FXR KO mice.

Interestingly, it has been reported that chronic ethanol exposure inhibited FXR activation, resulting in up-regulation of bile acid synthesis enzymes and down-regulation of bile acid transporter and in turn elevated hepatic bile acid pool (Xie, Zhong et al. 2013; Wu, Zhu et al. 2014). Moreover, pharmacological activation of FXR using WAY-362450 and 6α-ethyl-chenodeoxycholic acid (6ECDCA) attenuated chronic ethanol exposure-induced liver injury and steatosis (Livero, Stolf et al. 2014; Wu, Zhu et al. 2014). While it is still controversial regarding the autophagy status in the mouse livers after chronic ethanol exposure, it has been suggested that chronic ethanol exposure may lead to impaired hepatic autophagy (Dolganiuc, Thomes et al. 2012). This notion is supported by the observations that chronic ethanol exposure leads to hepatomegaly (Baraona, Leo et al. 1975), accumulated ubiquitin positive protein aggregates (Zatloukal, French et al. 2007; Harada 2010) and decreased protein degradation (Donohue, Zetterman et al. 1989). In the future, it will be interesting to determine whether increased bile acid after chronic ethanol exposure would contribute to impaired hepatic autophagy and alcohol-induced liver injury.

Emerging evidence supports that transcriptional regulation of the expression of autophagy related genes is critical in induction of autophagy in yeast (Williams, Primig et al. 2002; Bartholomew, Suzuki et al. 2012), *Caenorhabditis elegans* (Yang and Zhang 2014) and mammals (Pietrocola, Izzo et al. 2013; Fullgrabe, Klionsky et al. 2014). Among the several known transcriptional factors that regulate autophagy, the FoxO family members play critical roles in regulating expression of autophagy related genes

in skeletal muscles (Mammucari, Milan et al. 2007; Zhao, Brault et al. 2007), cardiomyocytes (Sengupta, Molkentin et al. 2011) and liver (Xiong, Tao et al. 2012). In addition, cytosolic FoxO proteins also showed to regulate autophagy independent of their transcriptional activity (van der Vos, Eliasson et al. 2012). We also recently demonstrated that FoxO3a is critical for ethanol-induced hepatic autophagy, and play a protective role against ethanol-induced liver injury (Ni, Du et al. 2013). In agreement with our previous findings, we found that ethanol induced FoxO3a-mediated expression of autophagy related genes in WT mouse livers. The activation of FoxO3a is mainly regulated by its post-translational modifications including phosphorylation, acetylation, ubiquitination and methylation. It has been well-documented that FoxOs are phosphorylated by the serine/threonine protein kinase Akt and become sequestered in the cytoplasm, where they are unable to regulate gene expression (Tzivion, Dobson et al. 2011). In WT mice, we found that acute ethanol treatment decreased the level of phosphorylated Akt and FoxO3a and in turn increased nuclear retention of FoxO3a. In contrast, acute ethanol treatment increased Akt activity resulting in the increased phosphorylation of FoxO3a and decreased nuclear retention FoxO3a in FXR KO mouse livers. Currently it is not clear how Akt was activated in FXR KO mouse livers. However, FXR KO mice develop spontaneous hepatocellular carcinoma (Yang, Huang et al. 2007), and increased AKT activity can promote tumorigenesis (Bellacosa, Kumar et al. 2005). In addition to post-translational modifications, FoxO family members including FoxO3a bind to a variety of nuclear hormone receptors to regulate either its own or nuclear hormone receptors' DNA binding affinity and transcriptional activity (van der Vos and Coffer 2008). FoxO3a contains LxxLL motif in the C-terminal of forkhead

DNA binding region, which may enable FoxO3a to interact with nuclear hormone receptors (van der Vos and Coffer 2008; Wang, Marshall et al. 2012). Thus it is possible that the decreased FoxO3a-mediated expression of autophagy related genes is due to the lack of a direct interaction between FoxO3a and FXR. However, the failure to detect the direct interaction between FXR and FoxO3a both *in vitro* and *in vivo* excludes this possibility. In addition, we also found that acute ethanol treatment increased the binding of FoxO3a to the promoter regions of *Map1lc3b* in WT but not in FXR KO mouse livers by the ChIP assay. While the increased nuclear retention of FoxO3a in ethanol-treated WT mouse livers could contribute to these observations, it is also likely that other FoxO3a co-factors/suppressors may influence the binding of FoxO3a to the promoter regions of *Maplc3b*. It is known that FoxO3a also interacts with other co-factors such as PGC1-α (Sandri, Lin et al. 2006; Olmos, Valle et al. 2009; Borniquel, Garcia-Quintans et al. 2010) and p300/CBP (van der Heide and Smidt 2005). Future studies will be needed to determine the changes of the interactions of FoxO3a with PGC1-α or p300/CBP after ethanol treatment in WT and FXR KO mice.

In addition to the defect of FoxO3a-mediated transcriptional regulation of autophagy in FXR KO mouse livers, increased bile acid levels may also contribute to the impaired autophagy in FXR KO mouse livers. As we previously demonstrated that bile acids themselves did not have any effects on the expression of autophagy related genes regardless of the conjugated or unconjugated bile acids (Manley, Ni et al. 2014). However, bile acids impaired the fusion of autophagosomes with lysosomes through the alteration of intracellular Rab7, a key protein that regulates the fusion of autophagosomes with lysosomes (Manley, Ni et al. 2014). The increased number of

autophagosomes in ethanol-treated FXR KO mouse livers may reflect the impaired fusion of autophagosomes with lysosomes. Thus it is likely that the combination of impaired FoxO3a-mediated expression of autophagy related genes and bile acid-mediated defect on the fusion of autophagosomes with lysosomes contribute to the exacerbated steatosis and liver injury in ethanol-treated FXR KO mice.

It is known that alcohol consumption can lead to increased oxidative stress and mitochondrial damage (Hoek, Cahill et al. 2002; Ding, Manley et al. 2011). Metabolism of alcohol may play a critical role in alcohol-induced oxidative stress and mitochondrial damage. Alcohol is mainly metabolized in the liver through cytosolic alcohol dehydrogenase (ADH) to acetaldehyde, a highly reactive molecule that is further metabolized through mitochondrial acetaldehyde dehydrogenase (ALDH) to acetate. Consequently, alcohol metabolism increases hepatic NADH/NAD+ ratio and oxidative stress. Moreover, alcohol can also induce hepatic Cyp2E1 expression to oxidize alcohol, which also leads to increased oxidative stress and mitochondrial damage (Wu and Cederbaum 1996). Previous works including ours have shown that the metabolism of alcohol either via ADH or Cyp2E1 is important for alcohol-induced changes on hepatic autophagy (Ding, Li et al. 2010; Wu, Wang et al. 2012; Thomes, Ehlers et al. 2013). Interestingly, we found that the levels of hepatic Cyp2E1 were much higher induced by acute ethanol treatment in FXR KO mice than wild type mice. It is likely that the higher levels of Cyp2E1 in FXR KO mouse livers may also contribute to the increased liver injury by acute ethanol treatment. Future studies will be needed to further determine the levels of hepatic oxidative stress after ethanol treatment in FXR KO mice.

We also previously demonstrated that at least one purpose of acute ethanol-induced autophagy is to remove damaged mitochondrial through mitophagy (Ding, Li et al. 2010; Ding, Li et al. 2011). However, increased evidence suggests that cells may use alternative pathways or activate other forms of autophagy (i.e. microautophagy or chaperone-mediated autophagy) to regulate cellular organelle homeostasis (Cuervo and Wong 2014). We recently reported that several mitochondrial damage stressors induce the formation of mitochondrial spheroids likely as an alternative pathway to regulate mitochondrial homeostasis in cultured cells or mouse livers in both WT and autophagy-deficient cells (Ding, Guo et al. 2012). Interestingly, we also found that acute ethanol treatment increased the number of mitochondrial spheroids in FXR KO but not WT mouse livers. Moreover, mitochondrial spheroid structures were also found in the control FXR KO mouse livers. It is likely that increased bile acid levels might induce mitochondrial damage in control FXR KO mouse livers, which was further exacerbated after ethanol treatment. Damaged mitochondria can normally be removed via mitophagy but this process was impaired in FXR KO mouse livers. The increased mitochondrial spheroid in ethanol-treated FXR KO mouse livers thus may also serve as an alternative pathway to regulate mitochondrial homeostasis in FXR KO mouse livers.

In conclusion, we demonstrated that FXR is a protective factor against ethanol-induced hepatotoxicity and steatosis. Furthermore, we also showed that FXR is associated with ethanol-induced FoxO3a activation and FoxO3a-mediated autophagy. Modulating FXR activity may be a promising novel therapeutic target for ALD.

CHAPTER 6:

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 General Conclusions

The present dissertation presents a novel insight in how bile acids mediate hepatic autophagy, and play a role in liver pathogenesis, in particular, alcohol-induced liver injury. We showed that bile acids induce hepatocyte apoptosis by initiating cleavage of caspase-3. We demonstrated that bile acids impair autophagy in hepatocytes via inhibiting the fusion of autophagosomes with lysosomes by altering intracellular Rab7 protein. Bile acids did not affect lysosomal functions or the ubiquitin-proteasome system. Furthermore, whole body FXR deficient mice displayed defective hepatic autophagy mediated by elevated hepatic bile acids, which could play a role in liver tumorigenesis found in FXR deficient mice. Using an acute alcohol mouse model, we further found that FXR deficiency exacerbated ethanol-induced hepatotoxicity and steatosis in mice. Moreover, FXR is required for ethanol-induced FoxO3a activation and FoxO3a-induced autophagy. Ethanol treatment increases Akt activity and subsequent phosphorylation and sequestration of FoxO3a in the cytosol, resulting in FoxO3a inactivation in FXR deficient mice. Impaired hepatic autophagy in FXR deficient mice may increase ethanol-induced mitochondria stress due to defective mitophagy, resulting in mitochondrial spheroid formation as a compensatory mechanism for mitochondria quality control. Altogether, we demonstrated how bile acids and FXR play a major role in liver physiology and pathogenesis by modulating autophagy and stress response, especially as it relates to ethanol. This dissertation shows how little we know about bile acids and hepatic autophagy, and that there is much more of liver biology left to be explored.

6.2 Future directions

Clarifying the Effects of Bile Acids on the Autophagosomal-Lysosomal Fusion Machinery

This dissertation demonstrated that bile acids inhibit autophagosomal-lysosomal fusion by altering Rab7 protein expression in hepatocytes. However, the molecular mechanism by which bile acids alter Rab7, and possibly other fusion machinery proteins, is not clear. Rab7 is a small GTPase protein that has been used as a late endosome marker (Bottger, Nagelkerken et al. 1996), and is demonstrated to have functions in late endosomal transport (Vitelli, Santillo et al. 1997; Press, Feng et al. 1998) and lysosomal biogenesis (Bucci, Thomsen et al. 2000). Furthermore, Rab7 is also required for completion of autophagosomal-lysosomal fusion (Jager, Bucci et al. 2004; Ganley, Wong et al. 2011). Thapsigargin is a sesquiterpene lactone that inhibits calcium-ATPase in the endoplasmic reticulum, resulting in a release of calcium from stores and subsequent increase in cytosolic calcium concentration (Thastrup, Cullen et al. 1990). Thapsigargin blocks autophagosomal-lysosomal fusion by blocking the recruitment of Rab7 to autophagosomes (Ganley, Wong et al. 2011). However, the mechanism of the blockage is not completely understood. On the other hand, bile acids have been shown to induce calcium release from the endoplasmic reticulum and intracellular calcium stores (Gerasimenko, Flowerdew et al. 2006). Interestingly, CDCA and TCA (at high doses) also increased intracellular calcium concentration in rat hepatocytes (Thibault and Ballet 1993). Moreover, exogenously introduced calcium using calcium phosphate precipitates induces autophagosome accumulation by blocking autophagosomal-lysosomal fusion at 24 hours (Sarkar, Korolchuk et al. 2009).

We also showed that bile acids inhibit autophagosomal-lysosomal fusion by blocking the recruitment of Rab7 to the autophagosomes. Therefore, it is possible that bile acids may alter calcium signaling and thus affect Rab7 activity and cellular localization. Future experiments are needed to further explore these possibilities.

Rab7 is one of the essential proteins for autophagosomal-lysosomal fusion. However, the autophagosomal-lysosomal fusion machinery is not completely elucidated yet. Multiple proteins and complexes have been shown to be involved in the fusion machinery, including the SNARE complexes and HOPS complexes. Syntaxin 17, a SNARE protein, has been identified as another required component for the fusion of autophagosomes with lysosomes by interacting with the following specific HOPS complex proteins: vacuolar sorting protein 33A (VPS33A), VPS16, and VPS39 (Itakura, Kishi-Itakura et al. 2012; Jiang, Nishimura et al. 2014). Currently, the effect of bile acids on fusion machinery other than Rab7 is unknown. Therefore, it will be interesting to see whether bile acids block interaction of the SNARE proteins with the HOPS complex proteins.

Role of autophagy and p62 in liver tumorigenesis

Autophagy is a *bona fide* tumor suppressor, and deletion or partial knockdown of autophagy genes, including Atg5 (Takamura, Komatsu et al. 2011), Atg7 (Inami, Waguri et al. 2011; Takamura, Komatsu et al. 2011), and Beclin-1 (Qu, Yu et al. 2003), results in spontaneous liver tumorigenesis. Deletion of autophagy results in p62 positive protein aggregates accumulation, persistent Nrf2 activation, disruption of mitochondria homeostasis, and elevated oxidative stress, which all together drive tumorigenesis. The

p62 protein is an autophagy receptor protein responsible for recruiting ubiquitinated protein aggregates and organelles to the autophagosomes for degradation, which results in degradation of p62 as well (Manley, Williams et al. 2013). Over expression of p62 enhances tumorigenesis in autophagy-deficient cells, whereas tumor progression is significantly stunted in Atg7/p62 double KO mice (Takamura, Komatsu et al. 2011). Moreover, p62 may drive tumorigenesis through persistent Nrf2 activation (Inami, Waguri et al. 2011). The p62 protein binds to Keap1 at the same site as Nrf2, and excessive p62 dissociates Nrf2 from Keap1, resulting in up regulation of Nrf2 activation (Jain, Lamark et al. 2010; Lau, Wang et al. 2010; Manley, Williams et al. 2013). Our research group has demonstrated that Atg5/Nrf2 double KO mice completely abolished liver tumorigenesis that was found in hepatocyte-specific Atg5 KO mice (Ni, Woolbright et al. 2014). Moreover, p62 also has been shown to inhibit phosphatase and tensin homolog (PTEN), a tumor suppressor in mammary tumors. The p62-mediated inhibition of PTEN results in activated Akt, beta-catenin stabilization and nuclear translocation, and subsequent cyclin D1 up regulation (Cai-McRae, Zhong et al. 2014). Altogether, p62 appears to play a pivotal role in tumorigenesis.

FXR is also a tumor suppressor, and FXR deficiency results in spontaneous hepatic tumorigenesis in mice (Kim, Ahn et al. 2007; Yang, Huang et al. 2007). The mechanism of tumorigenesis in FXR KO mice is not fully understood. Bile acid sequestrant, cholestyramine, has been shown to lower the bile acids pool and reduce the number of malignant tumors (Yang, Huang et al. 2007). Moreover, liver specific FXR-deficient mice display normal bile acids level and an absence of liver tumorigenesis (Kong, Li et al. 2012), and our work showed that hepatic autophagy is not

impaired in liver specific FXR KO mice. The results suggest that bile acids may drive tumorigenesis (Borude, Edwards et al. 2012; Kong, Wang et al. 2012). Cholic acid feeding also promotes N,N-diethylnitrosamine-induced tumorigenesis (Shiota, Oyama et al. 1999; Yang, Huang et al. 2007), and in the clinical cases, cholestasis results in liver tumorigenesis (Jansen 2007). Our research showed that bile acids impair autophagy and initiate p62 accumulation *in vitro*. Moreover, we found that p62 is accumulated in FXR KO mouse livers, suggesting that p62 may participate in bile-acid-driven liver tumorigenesis by inhibiting hepatic autophagy (Manley, Ni et al. 2014).

We demonstrated that ethanol treatment exacerbates p62 accumulation in FXR deficient mouse livers due to impaired autophagy. A small fraction of alcoholic liver disease patients subsequently progresses to hepatocellular carcinoma (Gao and Bataller 2011), and FXR activation may play a role in the protection against ALD progression. The relationship between FXR, alcohol, and p62 in alcohol-induced hepatocellular carcinoma progression currently is not known, and appears to be a promising field of study for future therapies. It also will be interesting to examine whether ethanol-induced p62 accumulation in FXR KO mice induces Nrf2 activation, reduces PTEN protein expression and activates Akt- GSK3β-beta-catenin signaling axis, resulting in possible subsequent tumor growth.

Modulating Autophagy and FXR in Cholestasis

The dissertation showed that FXR indirectly regulates autophagy through bile acid homeostasis, and whole body FXR deficiency results in impaired autophagy through the accumulation of hepatic bile acids. Cholestasic patients show markedly

reduced FXR activation (Chen, Ananthanarayanan et al. 2004; Cai, Gautam et al. 2009; Jonker, Liddle et al. 2012). Moreover, we demonstrated that cholestasic mouse models display p62 accumulation, possibly due to bile acid-mediated impaired hepatic autophagy. Mallory-Denk bodies (MDB) are p62-positive intracellular protein aggregates, and are the hallmark of liver pathogenesis, including alcoholic steatohepatitis (Manley, Williams et al. 2013). Furthermore, cholic acid feeding and common bile duct ligation result in the formation of MDBs in drug-primed mice, suggesting that bile acids may drive the formation of MDBs (Fickert, Trauner et al. 2002). The formation of MDBs is also observed in the liver tissues from cholestatic patients afflicted with either primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC) (Gerber, Orr et al. 1973; Fickert, Trauner et al. 2002; Strnad, Zatloukal et al. 2008). Interestingly, the liver tissues from PBC patients display LC3-II and p62 accumulation (Sasaki, Miyakoshi et al. 2012; Sasaki, Miyakoshi et al. 2012). It will be interesting to determine whether induction of autophagy and FXR using a pharmacological approach may alleviate cholestasic liver disease by the elimination of p62-positive protein aggregates and the restoration of FXR-mediated bile acid homeostasis.

The Role of FXR in Ethanol-Induced Hepatotoxicity and Steatosis

The dissertation demonstrated that ethanol-induced liver injury and steatosis are exacerbated in FXR KO mice due to impaired autophagy and the lack of FoxO3a activation. Currently, the protective mechanism of FXR activation is not clearly understood.

Chronic ethanol treatment has been reported to inhibit FXR activation and disrupt bile acid homeostasis by decreasing transcription of the FXR target gene, Bsep, and by abolishing SHP-mediated inhibition of transcription of the bile acid synthesis genes, CYP7A1 and CYP8B1 (Xie, Zhong et al. 2013; Wu, Zhu et al. 2014). Moreover, chronic ethanol treatment has been demonstrated to decrease Sirt1, a deacetylase, and increase p300, an acetyltransferase, resulting in acetylation of and subsequent inactivation of FXR (Ni, Du et al. 2013; Wu, Zhu et al. 2014). In accordance with previous reports, we demonstrated that acute ethanol treatment attenuates Sirt1 protein levels. However, the effects of acute ethanol treatment on FXR activation, bile acid metabolism, and hepatic transporters are not known.

Other possible protective mechanisms by FXR activation are reduced CYP2E1 activity and decreased generation of reactive oxygen species. Administration of the FXR agonist, WAY-362450, has been shown to repress chronic ethanol-mediated CYP2E1 induction resulting in decreased oxidative stress (Wu, Zhu et al. 2014). Another FXR agonist, 6-ECDCA, has been demonstrated to attenuate chronic ethanol-induced ROS generation (Livero, Stolf et al. 2014). However, this dissertation showed that WAY-362450 did not attenuate acute ethanol-induced CYP2E1 protein levels. Increased CYP2E1 activity has been suggested to be the culprit of ethanol-induced ROS generation in addition to ADH (Caro and Cederbaum 2004). Ethanol has been shown to increase CYP2E1 protein levels by protein stabilization (Lu and Cederbaum 2008). Moreover, impaired autophagy has been reported to promote CYP2E1-dependent toxicity *in vivo* and *in vitro* (Wu and Cederbaum 2013). Currently, it is not clear how FXR regulates CYP2E1 protein expression in response to ethanol treatment.

In addition to being the master regulator of bile acid homeostasis, FXR also regulates lipid metabolism by several mechanisms. FXR activation indirectly represses expression of sterol regulatory element-binding transcription factor 1 (SREBP1) through SHP, resulting in decreased transcription of lipogenic target genes (Zhang, Castellani et al. 2004). Paradoxically, FXR directly activates the transcription of fatty acid synthase (FAS), a key lipogenic gene, suggesting FXR may bypass SHP-mediated inhibition of lipogenesis (Matsukuma, Bennett et al. 2006). Ethanol has been reported to induce SREBP1-mediated lipogenesis (You, Fischer et al. 2002; Gao and Bataller 2011). Activation of FXR by 6-EDCA has been showed to repress chronic ethanol-induced transcription of the lipogenesis genes, SREBP1 and FAS (Livero, Stolf et al. 2014). Autophagy is important for hepatic lipid droplet removal and impaired autophagy exacerbates ethanol-induced steatosis (Ding, Li et al. 2010; Czaja 2011). Currently, the role of FXR in regulation of ethanol-induced steatosis is largely unknown, and the preliminary studies suggested that FXR activation might protect against ethanol-induced steatosis possibly through the inhibition of SREBP1-mediated lipogenesis and maintenance of autophagy.

Regulation of FoxO3a Activation in Ethanol-Induced Hepatotoxicity

This dissertation demonstrated that FXR deficiency indirectly inhibits ethanol-induced FoxO3a activation by up-regulating the Akt signaling pathway. Akt is a well-established canonical regulator of FoxO3a activation. However, emerging evidence demonstrates that multiple biochemical pathways are involved in the regulation of FoxO3a activation (Calnan and Brunet 2008; Tzivion, Dobson et al. 2011; Tikhanovich, Cox et al. 2013). Currently, the effects of acetylation on FoxO3a is not completely

understood, but it has been suggested that acetylated FoxO3a has decreased DNA-binding affinity (Calnan and Brunet 2008). Moreover, our group has reported that ethanol induces acetylation of FoxO3a. However, Sirt1 activation by resveratrol enhances ethanol-induced FoxO3a activation and transcription of autophagy related genes (Ni, Du et al. 2013). Surprisingly, we found that ethanol decreases FoxO3a acetylation and also decreases FoxO3a binding to the DNA in FXR KO mouse livers, suggesting that other post-translational modifications may be involved. We also found that ethanol increases Akt-mediated serine 253 phosphorylation of FoxO3a in FXR KO mouse livers, resulting in cytoplasmic sequestration of FoxO3a. These data suggest that cytoplasmic sequestration of FoxO3a is more important than acetylation in ethanol-induced liver injury in FXR KO mice. Moreover, total hepatic FoxO3a level was decreased in ethanol-treated FXR KO mice, which suggest that FoxO3a could be decreased due to either decreased FoxO3a mRNA level or increased degradation of FoxO3a. However, the mechanism of FoxO3a degradation was not elucidated. Interestingly, deacetylation of FoxO3a by Sirt1 may result in Skp2-mediated ubiquitination of FoxO3a and subsequent FoxO3a degradation (Wang, Chan et al. 2012). This mechanism may explain why FXR KO mice displayed both decreased acetylation of FoxO3 and hepatic FoxO3a levels, but further investigation is required. Moreover, the mechanism of how FoxO3a binds to DNA in response to ethanol needs to be further studied.

6.3 Why Do We Care about the Alcohol Abuse, a Self-Inflicted Malady?

Alcohol is ubiquitous in the United States. According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), almost 9 out of 10 adults reported that they had drunk alcohol in their lifetime (SAMHSA). Moreover, about 1 out of 4 adults reported that they had engaged in binge drinking (SAMHSA). Centers for Disease Control and Prevention also reported that alcohol is directly responsible for 88,000 deaths annually in the United States (CDC). Interestingly, alcohol misuses cost $223.5 billion per year in the United States alone, and around three-quarters of the cost is due to binge drinking (CDC). In 2009, alcohol liver disease was the primary reason for 1 in 3 liver transplantions in the United States (Singal, Guturu et al. 2013). Moreover, alcoholism is a self-inflicted disease that not only affects individuals, but also their families, in which 10% of children live with parents with alcohol problems in the United States (SAMHSA 2012). Despite the profound influence of alcoholism on the economy and family structure, very little progress had been made in ALD research and therapeutics. No novel treatment has been proposed for ALD since the 1970s, in which corticosteroids were used to treat alcoholic steatohepatitis. The lack of progression can be attributed to the limitations of animal models, and the difficulty in recruiting patients with active addiction for clinical trials (Gao and Bataller 2011). Rodent models have yielded some novel insights. However, the manifestations of alcohol-induced liver injury in rodent models are very mild and never progress past simple steatosis and mild inflammation (Bertola, Mathews et al. 2013). Our long-term goal is to identify novel therapeutic targets despite the limitations, so we can improve the quality of life for alcoholics, and reduce the need for liver transplantation as a treatment for end stage ALD.

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APPENDICES:

APPENDIX A:

The dissertation is based on the following publications:

1. **Manley S,** Ni HM, Williams JA, Kong B, DiTacchio L, Guo GL, and Ding WX. Farnesoid X Receptor Regulates Forkhead Box O3a Activaiton in Ethanol-Induced Autophagy and Hepatotoxicity. Redox Biology. In Press.

2. Williams, JA, **Manley S**, and Ding WX. New Progresses and Emerging Therapeutic Targets in Alcoholic Liver Diseases. World J Gastroenterol. In Press.

3. **Manley S**, Ni HM, Kong B, Apte U, Guo G, and Ding WX. Suppression of autophagic flux by bile acids in hepatocytes. Toxicol Sci. 2014;137(2):478-90. PMCID: 3908720

4. **Manley S**, Williams JA, Ding WX. Role of p62/SQSTM1 in liver physiology and pathogenesis. Exp Biol Med (Maywood). 2013;238(5):525-38.

5. Ding WX, **Manley S**, Ni HM. The emerging role of autophagy in alcoholic liver disease. Exp Biol Med (Maywood). 2011;236(5):546-56.

APPENDIX B:

Other publications including this author:

1. Ni HM, Bhakta A, Wang SG, Li Z, **Manley S**, Huang H, Copple B, and Ding WX. Role of hypoxia inducing factor in alcohol-induced autophagy, steatosis and liver injury in mice. PLoS One (Submitted).

2. Ding WX, Guo F, Ni HM, Bockus A, **Manley S**, Stolz DB, Eskelinen EL, Jaeschke H, and Yin XM. Parkin and mitofusins reciprocally regulate mitophagy and mitochondrial spheroid formation. J Biol Chem. 2012.

3. Sullivan BP, Kassel KM, **Manley S**, Baker AK, and Luyendyk JP. Regulation of transforming growth factor-beta1-dependent integrin beta6 expression by p38 mitogen-activated protein kinase in bile duct epithelial cells. J Pharmacol Exp Ther. 2011;337(2):471-8. PMCID: 3083106.

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5. Rafiee P, Nelson VM, **Manley S**, Wellner M, Floer M, Binion DG, and Shaker R. Effect of curcumin on acidic pH-induced expression of IL-6 and IL-8 in human esophageal epithelial cells (HET-1A): role of PKC, MAPKs, and NF-kappaB. Am J Physiol Gastrointest Liver Physiol. 2009;296(2):G388-98.

6. 2008 Floer M, Binion DG, Nelson VM, **Manley S**, Wellner M, Sadeghi S, Behmaram B, Sewell C, Otterson MF, Kucharzik T, and Rafiee P. Role of MutS homolog 2 (MSH2) in intestinal myofibroblast proliferation during Crohn's disease stricture formation. Am J Physiol Gastrointest Liver Physiol. 2008;295(3):G581-90. PMCID: 2536780.

APPENDIX C:

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Deciphering the nuclear bile acid receptor FXR paradigm

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Title: Farnesoid X receptor regulates forkhead Box O3a activation in ethanol-induced autophagy and hepatotoxicity

Author: Sharon Manley,Hong-Min Ni,Jessica A. Williams,Bo Kong,Luciano DiTacchio,Grace Guo,Wen-Xing Ding

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