NCOA5 HAPLO-INSUFFICIENCY RESULTS IN EARLY-ONSET GLUCOSE INTOLERANCE AND SUBSEQUENT HEPATOCELLULAR CARCINOMA

By

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ABSTRACT

NCOA5 HAPLO-INSUFFICIENCY RESULTS IN EARLY-ONSET GLUCOSE INTOLERANCE AND SUBSEQUENT HEPATOCELLULAR CARCINOMA

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The association between type 2 diabetes (T2D) and hepatocellular carcinoma (HCC) is of great public health concern, not only because T2D is associated with elevated risks for many cancers, but also due to increasing global T2D prevalence and limited therapies for HCC. We demonstrate that heterozygous deletion of the *Ncoa5* gene causes spontaneous development of HCC, exclusively in male mice. Tumor development is preceded by increased IL-6 expression, early-onset glucose intolerance, and progressive steatosis and dysplasia in livers. Blockading IL-6 overexpression averts glucose intolerance and partially deters HCC development. Moreover, reduced Ncoa5 expression is associated significantly with human HCC and HCC with comorbid T2D. These findings implicate the coexistence of T2D and HCC not as an epiphenomenon, but rather that these two diseases share a common pathogenic mechanism controlled by Ncoa5.

Our results demonstrate that Ncoa5 haplo-insufficiency activates a pathogenic pathway concomitantly leading to impaired glucose tolerance and HCC development in mice. These results reveal Ncoa5 haplo-insufficiency as a genetic link between T2D and HCC. Moreover, our *Ncoa5*$^{+/-}$ mouse model of glucose intolerance with comorbid HCC provides an ideal platform for studying the molecular basis and therapeutic

responsiveness of HCC with comorbid T2D. Thus, our findings open new avenues for developing therapeutic approaches to combat these diseases.

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KEY TO ABBREVIATIONS

Abbreviation Description

AFP Alpha fetoprotein

ALT Alanine amino transferase

AR Androgen receptor

CIA Coactivator independent of AF-2

ER Estrogen receptor

FAS Fatty acid synthase

FFA Free fatty acid

GTT Glucose tolerance test

HCC Hepatocellular carcinoma

H&E Hematoxylin and Eosin

HPF High power field

IHC Immunohistochemistry

IL-6 Interleukin 6

ITT Insulin tolerance test

KO Knockout

Ncoa5 Nuclear Receptor Coactivator 5

NF-κB Nuclear factor kappa-light-chain-enhancer of activated B cells

ORO Oil red o

PCNA Proliferating cell nuclear antigen

SOCS3 Suppressor of cytokine signaling 3

TAG Triacylglyceride

T2D Type 2 Diabetes

TNFα Tumor necrosis factor α

TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling

WT wildtype

CHAPTER 1 INTRODUCTION

1.1 Hepatocellular carcinoma

1.1.1 Epidemiology of HCC

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy in adults (Pons-Renedo and Llovet, 2003). HCC is the fifth most common and the third most lethal cancer worldwide with approximately 600,000 deaths every year (Alison et al., 2011; Parkin et al., 2005). The incidence of HCC has large geographical variations and is not distributed evenly throughout the world. While most cases occur in less developed areas including Eastern Asia or sub-Saharan Africa, China alone accounts for more than 50% of the world's cases (El-Serag and Rudolph, 2007). Due to the vaccination of all newborns against HBV in many high-rate Asian countries, the incidence of HCC in these areas has been growing slowly. On the other hand, the prevalence of HCC has been increasing in the developed countries during the past two decades.

In the United States, the incidence of HCC has doubled over the past three decades, with an increased incidence shifted toward the younger people (El-Serag, 2002b; El-Serag and Mason, 1999; El-Serag and Rudolph, 2007). The increased incidence is likely due to the HCV epidemic infection in the late 1960s and early 1970s and lack of effective vaccination as well as increased prevalence of other risk factors including obesity, fatty liver disease and diabetes mellitus (El-Serag, 2002a; Seeff, 2004).

1.1.2 Etiology of HCC

The risk factors of HCC include hepatic B and C viral infection (HBV and HCV), heavy alcohol consumption, toxin exposure, non-alcoholic fatty liver disease (NAFLD), obesity and diabetes (El-Serag and Rudolph, 2007; Farazi and DePinho, 2006). These risk factors could cause liver inflammation and repetitive liver damage, which results in compensatory liver regeneration and eventually leads to liver fibrosis and cirrhosis. Meanwhile, during this injury-inflammation-regeneration process, various genetic and epigenetic alterations accumulate and lead to loss of growth control and increased cell survival and proliferation, and finally HCC occurs (Figure 1).

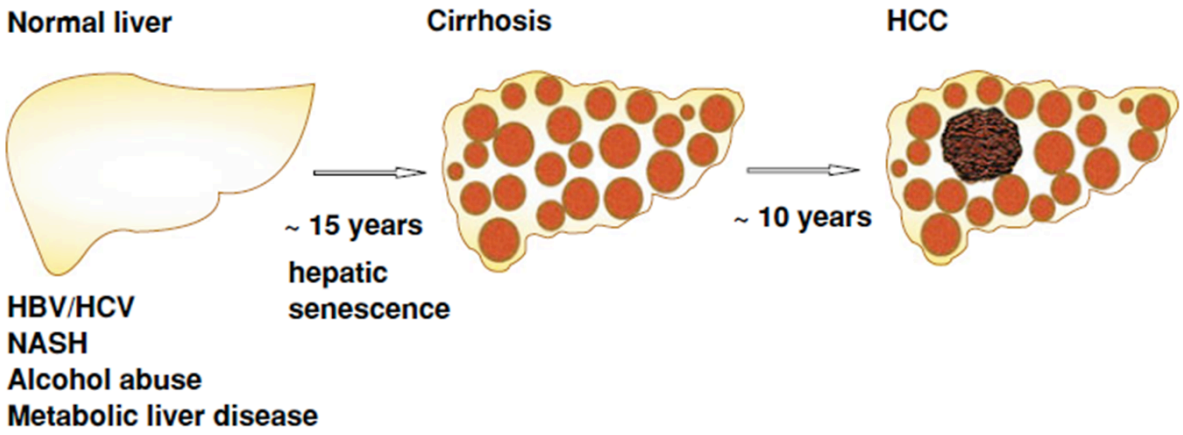

Figure 1.1 Simplified overview of events leading to HCC development.

Adapted from Alison etc. 2011

“For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation”.

1.1.3 HCC as an inflammation-associated disease

Carcinogen exposure and chronic inflammation are two important risk factors for tumor development. It is estimated that about 20% of human cancers are associated with infections and inflammation (Coussens and Werb, 2002). The best examples for inflammation- and infection-associated cancers are colon cancer, gastric cancer and HCC (Grivennikov et al., 2010; He and Karin, 2011). Persistent inflammation in the liver leads to continuous hepatocyte death and triggers long-lasting local infiltration and activation of inflammatory cells including Kupffer cells (resident liver macrophage), dendritic cells and T cells, which generate a large amount of cytokines, chemokines and growth factors, supporting increased hepatocyte proliferation. In addition, reactive oxygen species (ROS) and nitrogen intermediates (RNI) produced by both inflammatory cells and hepatocytes could cause oxidative damage to the host DNA, resulting in the activation of oncogenes and/or inactivation of tumor suppressor genes as well as various epigenetic alterations that favor HCC progression.

1.1.4 Signaling pathways linking liver inflammation and HCC development

Multiple signaling pathways have been recognized in the injury-inflammation-regeneration response and in human HCC development. Among them, nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) signaling pathways are two important ones that link liver inflammation and HCC development.

The activation and interaction between STAT3 and NF-κB play a pivotal role in mediating the dialog between malignant cells and their microenvironment, especially for the inflammatory/immune cells infiltrating tumors (Grivennikov and Karin, 2010).

1.1.4.1 NF-κB signaling pathway

NF-κB signaling pathway provides a mechanistic connection between inflammation and cancer development and progression (Karin, 2006; Karin and Greten, 2005). Before stimulation, most NF-κB dimers (p50/p65) are retained in the cytoplasm and bound to the inhibitors of NF-κB (IκB) (Moynagh, 2005). Upon stimulation, the IκB kinase (IKK) composed of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ/NEMO) will be activated. Activated IKKs phosphorylate the NF-κB-bound IκB, making it become target for polyubiquitination and proteasomal degradation. Released NF-κB dimers translocate to the nucleus where they modulate transcriptional activation of many target genes that have tremendous effects on the growth of hepatocytes. NF-κB signaling pathway could be activated by the downstream of Toll-like receptor (TLR) through the adaptor molecule, myeloid differentiation primary response gene 88 (MyD88), or by TNF-α or IL-1β.

NF-κB or IKK knockout mice suffer from extensive liver apoptosis and degeneration, which clearly demonstrated an anti-apoptotic role for IKK-dependent NF-κB signaling in hepatocytes (Beg et al., 1995; Li et al., 1999; Rudolph et al., 2000; Tanaka et al., 1999).

However, studies with different animal models have generated conflicting results about the role of hepatic NF-κB signaling in HCC development (Feng, 2012; He and Karin, 2011). In injury-promoted DEN hepatocarcinogenesis mouse models, hepatocyte IKK/NF-κB signaling was found to reduce liver damage, compensatory proliferation and cancer development by preventing ROS accumulation and maintaining hepatocyte survival (Luedde et al., 2007; Maeda et al., 2005). However, in low grade chronic inflammation mouse models, including *Mdr 2*$^{-/-}$ and lymphotoxin (LT) transgenic mouse (Haybaeck et al., 2009; Pikarsky et al., 2004), hepatic NF-κB signaling promotes HCC development through production of inflammatory cytokines that maintain the inflammatory microenvironment contributing to cancer development.

In contrast to the different roles NF-κB signaling plays in the hepatocytes in different animal models, NF-κB in liver myeloid cells has been demonstrated to promote HCC development. Activated Kupffer cells produce a group of inflammatory cytokines and growth factors in an IKK-NF-κB dependent pathway. Deletion of NF-κB in Kupffer cells removes the production of cytokines, reduces liver compensatory proliferation, and strongly inhibits DEN-induced HCC development (Maeda et al., 2005). One of the most important NF-κB dependent cytokines produced by Kupffer cells is IL-6 (Naugler et al., 2007) and IL-6 is a major STAT3 activator in the liver (Hodge et al., 2005).

1.1.4.2 STAT3 signaling pathway

STAT3 belongs to the signal transducer and activator of transcription (STAT) family of signal response transcription factors. STAT3 signaling is involved in immune responses, inflammation and tumorigenesis (Frank, 2007; Yu et al., 2009). Like NF-κB, STAT3 is inactive in the cytoplasm in unstimulated cells and is rapidly activated by various cytokines and growth factors including IL-6, EGF and hepatocyte growth factor (HGF) (Hirano et al., 2000; Zhong et al., 1994). STAT3 activation requires phosphorylation of a critical tyrosine residue (Tyr705), which is mediated by Janus kinase (JAKs). Phosphorylated STAT3 undergoes dimerization through phosphotyrosine-SH2 domain interaction. Once dimerized, STAT3 enters nucleus where it binds to the DNA and activates a broad array of target genes. Normally, STAT3 activity is tightly regulated by multiple feedback mechanisms including SHP phosphatases and suppressor of cytokine signaling 3 (SOCS3), which block STAT3 activation and ensure that cytokine-induced STAT3 activation is transient in normal cells (Kubo et al., 2003; Yoshimura et al., 2007).

However, constitutively activated STAT3 has been detected in many forms of cancers, including breast, colon, gastric, lung, head and neck, skin, prostate, liver cancer and others (Al Zaid Siddiquee and Turkson, 2008; Bromberg et al., 1999; Yu and Jove, 2004). STAT3 was found to be activated in about 60% of human HCC specimens examined and STAT3 activation is associated with more aggressive tumors (He et al., 2010). Consistently, STAT3 was found activated in most of human HCCs with poor prognosis but not in the adjacent non-cancerous tissue or in the normal liver (Calvisi et al., 2006). In human cancers, STAT3-activating mutations are rare; instead, most likely,

STAT3 in cancer cells is activated by cytokines and growth factors that are produced in the tumor microenvironment, as tumor cells with activated STAT3 rapidly lose STAT3 phosphorylation once put into culture without neighboring immune or stromal cells (Yu et al., 2007). IL-6 is one of the major STAT3-activating cytokines and has been shown to be elevated in human liver diseases and HCC. In addition, hepatocyte-specific deletion of STAT3 mice ($Stat3^{\Delta hep}$) developed fewer and smaller size of HCCs compared to wild type mouse after DEN treatment. Moreover, tumors arising in $Stat3^{\Delta hep}$ mice were much smaller, indicating STAT3 may play a critical role in HCC cell proliferation and/or survival (He et al., 2010).

1.1.5 Gender disparity of HCC and the mechanisms

The gender disparity of HCC between males and females has been widely characterized regardless of those etiologies mentioned above, with the incidence of HCC being two to four times higher in men than in women (De Maria et al., 2002; El-Serag and Rudolph, 2007). Similar male predominance of liver tumor development is also observed in the HCC rodent models induced with chemical carcinogens, which suggest an important role of sex hormones (estrogen and androgen) in hepatocarcinogenesis (Kalra et al., 2008).

1.1.5.1 Estrogen and estrogen receptor and HCC

Liver cells express estrogen receptor (ER) and response well to estrogens including estrone (E1), estradiol (E2), and estriol (E3). Recent studies using the chemical carcinogen diethylnitrosamine (DEN) treated rodent model discovered that E2 has a protective role against HCC by repressing IL-6 transcription through the interaction between estrogen receptor α (ERα) and the transcription factor NF-κB (Libermann and Baltimore, 1990; Naugler et al., 2007). The protection role of E2 against HCC could also be supported by the evidence that HCC incidence increases in postmenopausal women who have higher serum IL-6 level after menopause (Bosch et al., 2004).

Typically, ERα, which belongs to the steroid receptor family, functions as a transcription factor by binding directly to the promoter region of the target genes and recruiting cofactors to regulate gene expression (Shang et al., 2000). However, steroid receptors may also function as antagonist of transcription activators through direct protein-protein interactions to repress gene expression (Ray et al., 1994). One well-known example is the inhibition of transcription factor activator protein 1 (AP-1) activity by glucocorticoid hormones through a direct physical interaction between glucocorticoid receptor (GR) and AP-1 component Jun/Fos (Schule et al., 1990; Yang-Yen et al., 1990). It has also been reported that estrogenused in hormone replacement therapy to prevent postmenopausal osteoporosis in women might inhibit IL-6 production via the interaction between ERα and NF-κB (Stein and Yang, 1995). The mechanisms of how the protein-protein interaction represses gene transcription have been mainly studied i*n*

vitro. A mutual inhibition of DNA binding of transcription factors has been proposed (Schule et al., 1990; Yang-Yen et al., 1990). Unfortunately, it is still largely unknown how ERα's interaction with NF-κB represses IL-6 transcription *in vivo* (Libermann and Baltimore, 1990). It has been speculated that cofactors of ERα may have a role in this process (Shang et al., 2000).

1.1.5.2 Androgen and androgen receptor and HCC

Androgen and androgen receptor (AR), which have been mostly studied in prostate cancers (Heinlein and Chang, 2004), have also been implicated to play important roles in liver cancer development (Lee and Chang, 2003). For example, AR protein level has been detected higher in liver tumor compared to non-cancerous liver (Nagasue et al., 1992; Ohnishi et al., 1986). A recent study reported that the incidence of DEN-induced HCC decreased in AR liver conditional knockout mice compared to wild type mice, suggesting an important role of AR in promoting HCC (Ma et al., 2008). In addition, using HBV transgenic mice, Wu et al. found that AR could promote HBV-induced hepatocarcinogenesis through modulating HBV RNA transcription (Wu et al., 2010). However, AR's function and regulation in liver cancer are still elusive (Lee and Chang, 2003; Nagasue et al., 1992). For instance, it is largely unknown which cellular factors could affect AR's transcription and expression or whether there is a cross talk between ERα and AR in HCC.

1.1.6 Treatment of HCC

The standard treatments of HCC including surgical resection, local ablation and liver transplantation could cure a fraction of patients with early stages of HCC. However, most cases of HCCs present are in advanced stages which need systemic treatments. Unluckily, conventional chemotherapy with cytotoxic agents is unsuccessful and seems unable to modify the natural history of the disease. Thanks to the identification and the recognition of the roles of the specific signaling pathways in the pathogenesis of HCC, drugs that directed at specific targets have been successfully developed. One successful example is Sorafenib, a BRAF/VEGFR/PDGFR tyrosine kinase inhibitor, which has been shown to improve the survival in patients with advanced HCC (Alves et al., 2011; Llovet et al., 2008). This recent success has stimulated intensive research pointed at identifying aberrant activation of signaling pathways in the development of HCC, such as VEGF receptor signaling pathway, WNT/β-catenin pathway, HGF/c-MET signaling pathway and other pathways (Whittaker et al., 2010).

1.2 Diabetes, insulin resistance and HCC

1.2.1 Diabetes Mellitus

Diabetes mellitus (DM) is a group of complex metabolic diseases mainly characterized by insulin resistance, pancreatic β cell dysfunction, and associated hyperglycemia and is a serious and growing health problem worldwide. People with diabetes have increased risks of developing cardiovascular disease such as heart disease and stroke, retinopathy, and neuropathy. There are about 285 million people (6.6% of the world population) have diabetes with the age between 20 and 79 years globally (Giovannucci et al., 2010). In the United States, there were about 25.8 million, or 8.3% of the population in 2010 with diagnosed and undiagnosed diabetes, with an estimated 1.6 million new cases reported every year (Cowie et al., 2006). It should be noted that many people are prediabetic with blood glucose levels higher than normal but not high enough to be classified as diabetes. In 2005 to 2008, about 35% of U.S. adults aged 20 years or older had prediabetes. These people have an increased risk of developing T2D, heart disease, and stroke.

Diabetes is typically divided into two major subtypes, Type 1 and Type 2 diabetes. Type 1 diabetes is characterized by insulin deficiency due to a T-cell-mediated autoimmune attack and loss of the insulin-producing β cells of the islets of Langerhans in the pancreas. Type 1 diabetes can happen in children or adults, but was traditionally called "juvenile diabetes" as a majority of these diabetes cases were in children. Type 2 diabetes (T2D) is the most common form and accounts for approximately 95% of the prevalent cases (Giovannucci et al., 2010). T2D is characterized by hyperglycemia and hyperinsulinemia, which are often companied with insulin resistance and failure of pancreatic β cell compensation. According to the standards of World Health

Organization, a person with a fasting blood glucose higher than 126 mg/dL and a two hour after meal glucose more than 200 mg/dL will be diagnosed as T2D (Foti et al., 2005). In the early stage of T2D, hyperglycemia can be reversed by a variety of methods and medications that improve insulin sensitivity or reduce glucose production by the liver, such as Metformin (Bailey and Turner, 1996). Metformin activates AMP-activated protein kinase (AMPK), an enzyme that plays pivotal roles in insulin signaling, metabolism of glucose and lipids, and whole body energy balance (Towler and Hardie, 2007). In addition, Metformin could enhance peripheral glucose uptake and increase fatty acid oxidation (Collier et al., 2006).

1.2.2 Diabetes and cancer

Diabetes and caner are common diseases that have a remarkable influence on people's health globally. A large body of epidemiologic evidence has suggested that diabetes is associated with increased risks of cancers (Coughlin et al., 2004; Giovannucci et al., 2010; Amarapurkar et al., 2008; Strickler et al., 2001; Suh and Kim, 2011). Liver, pancreas and endometrium cancers are at the greatest risks increased by diabetes (about 2-fold or higher), whereas colon/rectum, breast and bladder cancers are at less risks (about 1.2-fold to 1.5-fold). It has been suggested that increased insulin level due to hyperinsulinemia plays an important role in promoting cancer initiation and progression in diabetic patients (Strickler et al., 2001). Insulin is a growth factor for a wide range of tissues. Briefly, increased insulin could result in an increase of bio-

available IGF1. Insulin and IGF1 share approximately 40% amino acid sequence homology and can cross-bind with each other's receptor: insulin receptors and IGF1 receptors, the latter has more mitogenic effects than IRs, to enhance cellular proliferation, inhibit apoptosis and promote tumorigenesis in various tissues. Moreover, increased levels of reactive oxygen species, lipid peroxidation, as well as chronic inflammation in diabetic patients, may promote oxidative damage to DNA and promote carcinogenesis by DNA damage (Dandona et al., 1996).

1.2.3 Inflammation and insulin resistance

Inflammation is the body's protective reaction responding to an injury or infection. Acute phase of inflammation generally protects the body back to good health condition; however, chronic inflammation could be harmful to the health. Insulin resistance is a pathophysiological feature of T2D. As a matter of fact, chronic inflammation has been recognized as an important feature of obesity that is linked with insulin resistance and T2D (Kim et al., 2009). Obesity-derived circulating factors with systemic effects such as TNFα, IL-1β, IL-6, and various other adipose-secreted factors (known as adipokines), have been shown to play major roles in the development of insulin resistance.

Under the physiological condition, after having a meal, increased levels of insulin stimulate glucose uptake in the peripheral tissues, including muscle and adipose tissue, and reduce hepatic glucose production through decreased gluconeogenesis and

glycogenolysis, and increased glycogenesis. At the molecular level, insulin binds to the transmembrane insulin receptor (IR), composed of two α and two β subunits, activates the intrinsic tyrosine kinase activity of the receptor, and results in the autophosphorylation of IR and the recruitment and phosphorylation of insulin receptor substrates (IRSs). This in turn activates PI3K and Akt signaling pathway, leading to the phosphorylation and inactivation of glycogen synthase kinase 3 (GSK3) and initiate the synthesis of glycogen in the liver (White, 2003). Skeletal muscle accounts for about 75% of the whole body insulin-stimulated glucose uptake, facilitated by the translocation of the glucose transporter 4 (GLUT4) to the plasma membrane, which is mediated by the activation of Akt and atypic protein kinase C (aPKC) (Bjornholm and Zierath, 2005).

However, under some conditions, such as chronic inflammation, insulin resistance occurs when insulin-induced signaling pathway is affected (Samuel and Shulman, 2012). For instance, SOCS3 plays an important role in the pathogenesis of insulin resistance and connects insulin signaling with cytokine signaling (Krebs and Hilton, 2000; Krebs and Hilton, 2003). The expression of SOCS3 could be enhanced by various inflammatory cytokines including IL-6. Studies have demonstrated that overexpression of SOCS3 suppressed insulin-induced glycogen synthesis in myotubes and glucose uptake in adipocytes (Ueki et al., 2004). Hepatocyte-specific SOCS3 deletion improved insulin sensitivity in the liver (Torisu et al., 2007). Mechanistically, SOCS3 inhibit insulin-stimulated signaling through affecting IR activation, blocking IRS activation or inducing IRS degradation (Howard and Flier, 2006; Senn et al., 2003; Ueki et al., 2004).

1.3 Interleukin 6 in insulin resistance and HCC

1.3.1 IL-6 biological functions

The interleukin (IL)-6 gene was first cloned in 1986. IL-6 is a pro-inflammatory cytokine that has multiple and pleiotropic functions. It is produced by different types of cells, including lymphoid cells, such as T and B lymphocytes, monocytes, and non-lymphoid cells, including fibroblasts, endothelial cells, adipose tissues, skeletal muscle and several tumor cells. IL-6 has been known as the third member of the trio cytokines (tumor necrosis factor (TNF)-α and IL-1β) that mediate the acute inflammatory response. IL-6 could stimulate B cell maturation and secretion of immunoglobulins. Moreover, IL-6 functions as a hepatocyte stimulatory factor which could modulate hepatic protein synthesis and plays an important role in liver regeneration (Cressman et al., 1996). Recently, a large number of studies have shown that dysregulation of IL-6 signaling is associated with chronic inflammatory diseases, including obesity and insulin resistance, auto-immune disease such as sepsis and inflammatory-bowel diseases (IBDs), as well as cancer development (Ataie-Kachoie et al., 2013; Naugler and Karin, 2008; Ramadori et al., 1988).

1.3.2 IL-6 receptors and signaling pathways

IL-6 exerts its diverse functions through its unique receptor system (Ataie-Kachoie et al., 2013). It interacts with a receptor complex composed of the ligand-binding glycoprotein (gp) IL-6Rα, and the signal-transducing component gp130. There are two types of receptors for IL-6, cell membrane IL-6 receptor (IL-6R) and soluble IL-6 receptor (sIL-6R). While gp 130 ubiquitously expressed by different cells, IL-6R only exists on selected cells such as hepatocytes, neutrophils, monocytes, macrophages, and T and B lymphocytes. After binding with IL-6, IL-6R forms a complex with gp130 to initiate the intracellular signal (classical signaling). sIL-6R, generated by shedding from membrane-bound IL-6R or by alternative splicing, binds with IL-6 and then with the membrane receptor β chain gp130, leading to the signal transduction (trans-signaling) in cells such as neural cells, smooth muscle cells and endothelial cells that do not express IL-6R on their surfaces.

The signal transduction of IL-6 involves activation of Janus kinase (JAK) tyrosine kinase family members. The IL-6-JAK-STAT3 pathway regulates the expression of a group of genes leading to the induction of cell growth, differentiation and survival. In addition to STAT3, the Ras protein is also activated in response to IL-6. Ras activation leads to hyperphosphorylation of mitogen activated protein kinases (MAPK) and an increase in its serine/threonine kinase activity. MAPK then activates transcription factors which mediate diverse effects depending on cell type, including cell growth stimulation, acute phase protein synthesis and immunoglobulin synthesis. A third pathway activated

by IL-6 is phosphoinositol-3 kinase (PI3K)–protein kinase B (PkB)/Akt pathway as JAK can phosphorylate and activate PI3K. Activated Akt phosphorylates several downstream targets to up-regulate cellular survival signal pathways.

1.3.3 IL-6 in insulin resistance

The role of IL-6 in insulin resistance has been studied and debated for years. Numerous studies of human populations have revealed a positive correlation between elevated serum IL-6 levels and insulin resistance (Hu et al., 2004; Pradhan et al., 2001). Increased plasma IL-6 could also be used to predict the onset of T2D, especially in combination with increased levels of IL-1β (Spranger et al., 2003). However, there has been debate regarding the role of IL-6 in insulin resistance in different tissues or at different physiologic/pathophysiologic states (Kristiansen and Mandrup-Poulsen, 2005).

Based on the literature, generally, physiologic level of IL-6 could enhance insulin sensitivity and prevent diet induced obesity as IL-6 deletion mice developed obesity and insulin resistance (Matthews et al., 2010; Wallenius et al., 2002). However, increased systemic IL-6 produced by adipose tissue contributes to the insulin resistance observed in obesity (Bastard et al., 2000; Lazar, 2005; Vozarova et al., 2001; Wellen and Hotamisligil, 2005). On the other hand, during exercise, contracting skeletal muscle generates a large amount of IL-6, which has been shown to be beneficial for insulin-

regulated glucose metabolism (Ellingsgaard et al., 2011; Steensberg et al., 2000; Wojtaszewski and Richter, 2006).

Despite the different effects of IL-6 on insulin resistance in skeletal muscle and adipocytes, similar findings have been consistently reported within the liver that excessive IL-6 has a negative impact on hepatic insulin action. Both in vitro and in vivo studies have demonstrated that increased IL-6 expression in the liver can promote hepatic insulin resistance (Fernandez-Real et al., 2001; Johnson et al., 2012; Klover et al., 2005; Klover et al., 2003; Liu et al., 2007; Pang et al., 2011). One of the mechanisms is due to the activated IL-6-STAT3-SOCS signaling pathway that causes insulin resistance. Therefore, the available evidence has indicated that the effect of increased IL-6 expression on insulin action is highly tissue-specific, dependent on the physiological state and influenced by whether it is present acutely or chronically (Kim et al., 2009). It also reveals the complex roles IL-6 plays in glucose regulation in different tissues and the complicated crosstalk among liver, muscle, adipose tissue, and the brain. In hence, the effects of IL-6 on insulin action are better considered in a tissue specific manner.

1.3.4 IL-6 in HCC

Numerous clinical studies have demonstrated the association between elevated serum IL-6 level and increased risk or incidence of HCC in human (Porta 2008; Pang

2011; Ohishi 2013). It has been reported that elevated serum levels of IL-6 are associated with increased risk of HCC in female chronic hepatitis C patients (Nakagawa et al., 2009). Studies also revealed that combination of serum levels of IL-6 and alpha-fetoprotein improves sensitivity in diagnosing HCC or predicting future HCC development in chronic hepatitis B patients (Wong et al., 2009). Moreover, it has been reported that high levels of serum IL-6 are correlated positively with tumor size and with poor prognosis in HCC patients (Pang et al., 2011).

In addition to these clinical findings, experimental studies with different mouse models have demonstrated the role of IL-6 in the hepatocarcinogenesis. Studies using DEN-induced mouse models of HCC suggested that IL-6 production by Kupffer cells is critical for HCC development in male mouse and contributes to the gender disparity of HCC between male and female mouse (Naugler et al., 2007). Studies on obesity-induced mouse HCC model uncovered that obesity-promoted HCC development is associated with elevated production of the tumor-promoting cytokines, including IL-6 and TNFα (Park et al., 2010), which cause hepatic inflammation and activation of the oncogenic transcription factor STAT3. Importantly, it was recently reported that aberrant activation of the IL-6-STAT3 signaling pathway is a characteristic of HCC development in mice and humans (Bard-Chapeau et al., 2011). Interestingly, recent studies on human inflammatory hepatocellular adenoma revealed a frequent somatic gain-of-function mutation of *IL-6ST* gene which encodes the signal transducing beta chain-gp130, causing in-frame deletions of gp130 binding site for IL-6 and constitutively

activation of IL-6-STAT3 pathway in the absence of IL-6 (Rebouissou et al., 2009). Together, these findings pointed out the important role of IL-6 in hepatocarcinogenesis.

1.4 Nuclear receptor coactivator 5

Nuclear receptor coactivator 5 (Ncoa5), also known as Coactivator Independent of AF-2 function (CIA) was discovered by Vincent Giguere and colleagues with yeast two-hybrid screening (Sauve et al., 2001). They used orphan receptor RVR which lacked AF-2 domain as bait and found an interaction between Ncoa5 and RVR. Ncoa5 has 620 amino acids, with an arginine-(R) and aspartic acid (D)-rich region in its N-terminus and an overlapping nuclear receptor coactivator and corepressor binding motif LXXLL and ΦXXΦΦ in its C-terminus. Ncoa5 is ubiquitously expressed in all examined tissues and cell types at various levels (Sauve et al., 2001). Interestingly, several unique characteristics of Ncoa5 have been discovered. First, Ncoa5 specifically interacts with estrogen receptor and increases its transcriptional activity. This coactivator activity was not observed with any other nuclear receptors including thyroid receptor, GR or progesterone receptor. In addition, Ncoa5 could be recruited by ERα to the promoter region of c-myc gene to form a pre-initiation complex, but it was not released from the gene during transcription elongation. Moreover, Ncoa5 is able to form a complex with SAM68, hnRNP-G and transcription factors ZAP3, ILF2 and ILF3 (Ulke-Lemee et al., 2007), and it may also regulate transcription of genes targeted by other transcription factors such as ILF2 and ILF3.

Accumulative evidence has suggested that chromosome 20q12-13.1 region, where the Ncoa5 gene locates, is linked to T2DM in European Americans (Bowden et al., 1997; Ji et al., 1997; Permutt et al., 2001). Recently, Bento group has finished a 5.8 Mb dense SNP map across the chromosome 20q13.1 region in two European American case control populations and revealed that Ncoa5, along with two other nearby genes, cadherin 22 (CDH22) and phosphatidylinositol 3, 4, 5-triphosphate-dependent Rac Exchanger 1 (PREX1), were associated with T2D (Bento et al., 2008; Lewis et al., 2010). However, whether these T2D susceptibility genes are authentic remains unknown.

1.5 Hypothesis

Based on the characteristics of Ncoa5, I hypothesized that Ncoa5 may play a role in the development of T2D and HCC. In the next chapters, I investigated the roles of Ncoa5 in the development of T2D and HCC using a genetically-engineered mouse model and detected the expression of Ncoa5 in human HCC specimens.

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CHAPTER 2 Ncoa5 Haplo-insufficiency Leads to Spontaneous Development of Early-onset Glucose intolerance and Subsequent HCC Exclusively in Male Mouse

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This Chapter has been included in a manuscript that has been accepted by *Cancer Cell* and is currently in press.

2.1 Abstract

Type 2 Diabetes (T2D) and male gender are associated with hepatocellular carcinoma (HCC) development. We demonstrate that heterozygous deletion of the *Ncoa5* gene causes spontaneous development of HCC, exclusively in male mice. Tumor development is preceded by increased IL-6 expression, early-onset glucose intolerance, and progressive steatosis and dysplasia in livers. Blockading IL-6 overexpression averts glucose intolerance and partially deters HCC development. These findings suggest that Ncoa5 is a haplo-insufficient tumor suppressor in mouse, and Ncoa5 deficiency increases susceptibility to both glucose intolerance and HCC, partially by increasing IL-6 expression. Thus, our findings provide an ideal platform to study the molecular basis of HCC and HCC comorbid with glucose intolerance and open new avenues for developing therapeutic approaches to combat these diseases.

2.2 Introduction

Hepatocellular carcinoma (HCC) is the fifth most common and the third most lethal cancer worldwide, with increasing incidence in many developed countries including the United States (El-Serag and Mason, 1999; El-Serag and Rudolph, 2007). The incidence of HCC is two to four times higher in men than women. The risk factors of HCC include hepatitis B and C viral infection, aflatoxin-B exposure, alcohol consumption, inborn

metabolic diseases and diabetes (Coleman, 2003; Coughlin et al., 2004; Donadon et al., 2008; Staib et al., 2003). While hepatitis viral infection currently remains the major risk factor for HCC globally, diabetes is the second most common risk factor for HCC (36% of HCC cases) in the United States, topped only by nonalcoholic fatty liver disease (59% of HCC cases) between 2002 and 2008 (Sanyal et al., 2010). Furthermore, the incidence of HCC in diabetic patients increases with male gender and duration of diabetes (El-Serag et al., 2009; El-Serag and Mason, 1999; Lai et al., 2012; McGlynn and London, 2011). With growing global prevalence of diabetes and declining prevalence of hepatitis virus B and C infections, T2D may become an even more important risk factor for HCC in the future (McGlynn and London, 2011). However, molecular mechanisms underlying the association between these two diseases are largely unknown (Donadon et al., 2008; Feng, 2012).

Inflammation is known as a common pathogenic condition leading to both T2D and HCC (Donath and Shoelson, 2011; Giovannucci et al., 2010; Kalra et al., 2008; Olefsky and Glass, 2010). Of particular interest is the implication of inflammatory cytokine IL-6 in the pathogenesis of these two diseases. Even though the role of IL-6 in insulin resistance has been debated, the available evidence has clearly indicated that the effect of increased IL-6 expression on insulin action is highly tissue-specific and dependent on physiological state (Kim et al., 2009). It is generally accepted that IL-6 released from skeletal muscle during exercise can improve insulin sensitivity (Ellingsgaard et al., 2011) and global deletion of the IL-6 gene promotes insulin resistance in mice (Matthews et al.,

2010). On the other hand, increased IL-6 expression in the liver, induced by chronic inflammation, can promote hepatic insulin resistance and HCC (Fernandez-Real et al., 2001; Johnson et al., 2012; Klover et al., 2005; Klover et al., 2003; Liu et al., 2007; Pang et al., 2011). Consistent with this, inhibition of elevated IL-6 signaling increases insulin sensitivity in mice and humans with diabetes and/or rheumatoid disease (Klover et al., 2005; Klover et al., 2003; Ogata et al., 2011; Schultz et al., 2010). Moreover, studies on obesity or diethylnitrosamine (DEN)-induced mouse models of HCC demonstrated that IL-6 production from macrophages, in liver and adipose tissues, is necessary for HCC development in male mice (Naugler et al., 2007; Park et al., 2010). Importantly, it was recently reported that aberrant activation of the IL-6-Stat3 signaling pathway is a characteristic of HCC development in mice and humans (Bard-Chapeau et al., 2011). Therefore, unraveling the regulation of IL-6 expression in T2D and HCC would be important for the understanding of mechanisms underlying the association between the two diseases.

Estrogen signaling is another regulatory pathway that plays important roles in the pathogenesis of both T2D and HCC (Naugler et al., 2007; Tiano et al., 2011). It is well documented that estrogen and estrogen receptor α (ERα) can regulate inflammatory cytokine expression, glucose and lipid homeostasis, and pancreatic β cell survival (Nadal et al., 2009), thereby providing protection from T2D and HCC development. ERα modulates transcription of genes through interaction with coactivators and corepressors, as well as other transcription factors. The nuclear receptor coactivator 5 (Ncoa5), also

called coactivator independent of AF2 (CIA), is a unique coactivator that contains both coactivator and corepressor domains, and is known to modulate ERα-mediated transcription (Jiang et al., 2004; Sauve et al., 2001). Recent linkage analysis revealed that Ncoa5, along with two, nearby genes in the 20q13.1 region were associated with T2D, implying Ncoa5 as a possible T2D susceptibility gene (Bento et al., 2008; Lewis et al., 2010). In the present study we investigate the role of Ncoa5 in the development of T2D and HCC.

2.3 Experimental procedures

2.3.1 Generation of *Ncoa5*$^{+/-}$ and *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ mice

Details for constructing Ncoa5 targeting vector and generation of Ncoa5 knockout mouse on a mixed 129 × C57BL/6 background were listed in the supplemental information. All mice were and housed in microisolator cages at Michigan State University animal facility. B6.129S6-IL-6^{tm1Kopf} mice of C57BL/6 or Balb/c were purchased from Jackson Laboratory (Bar Harbor, ME). To generate *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ mice, *IL-6*$^{-/-}$ C57BL/6 or Balb/c male mice were mated with *Ncoa5*$^{+/-}$ female mice of mixed 129 × C57BL/6 or Balb/c genetic background to obtain an F1 generation *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$, respectively. *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ F1 male and female mice were

subsequently mated to derive *Ncoa5*$^{+/-}$ *IL-6*$^{+/-}$ and *Ncoa5*$^{+/-}$ *IL-6*$^{+/+}$ mice of mixed 129 × C57BL/6 or Balb/c genetic background. All experimental procedures on mice were approved by the Michigan State University Institutional Animal Care and Use Committee.

2.3.2 Antibodies and reagents

Ncoa5 (SC-86178), ERα (SC-542), CBP (SC-369), IL-6 (SC-1265), TNF-α (SC-52746), AR (C-19), PCNA (SC-56), IRβ (SC-711), pIRβ (SC-81500), IRS1 (SC-7200) and Ep-CAM (SC-66020) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Insulin (#3014S), Akt (# 4691), pAkt (#4060), STAT3 (#4904), pSTAT3 (#9145S), TGF-β (#3711), FAS (#3180S), AFP (#3903S) and pTyrosine (#9411) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-Mouse MAC-2 monoclonal antibody (CL 8942 AP) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Anti-phosphor serine Pol II (ab5095) was purchased from Abcam (Cambridge, MA, USA). Mouse anti-β-actin (AC-15) was from Sigma-Aldrich (St. Louis, MO, USA). DAPI, Biotin conjugated goat anti-rabbit, anti-mouse second antibodies, and ABC kits for immunohistochemistry were from Vector Laboratories (Burlingame, CA, USA). Donkey anti-goat Alexa-488, donkey anti-rabbit Alexa-594 were from Invitrogen (Eugene, Oregon, USA). Lipopolysaccharide (LPS) was purchased from InvivoGen (Ultra-pure LPS-EB, San Diego, California, USA). Human interleukin 6 (8904SF) was purchased from Cell Signaling Technology (Danvers, MA,

USA). Insulin solution (U-100) was purchased from Eli Lilly and Company (Indianapolis, IN, USA).

2.3.3 Histology and immunostaining

Mouse tissues were fixed in 4% formalin, sectioned and H&E stained in the histology laboratory at Michigan State University. Histopathology of mouse liver sections was reviewed according to the classification and criteria recommended by mouse models of human cancers consortium by board-certified pathologists. Slides were subjected to Masson's trichrome staining (Fisher scientific) to review collagens. Immunohistochemistry and immunofluorescent analysis of insulin, PCNA, IL-6, TNFα, Mac2 and FAS were performed according to the protocol supplied by Cell Signaling Technology (Danvers, MA, USA). 0.3% Sudan black B was used to reduce the auto-fluorescence of the liver tissue. Apoptotic cells in mouse livers were visualized using a TUNEL assay kit (Roche, Basel, Switzerland) according to the manufacturer's instructions.

2.3.4 Blood Glucose level, glucose tolerance test (GTT) and insulin tolerance test (ITT)

Mice were fasted for 12 hours and then measured blood glucose and insulin levels. For GTT experiments, mice were injected intraperitoneally with 2g/kg D (+)-glucose. For ITT experiments, mice were fasted for 5 hours and then treated with 1U/kg insulin.

Blood drop was obtained from tail vein and glucose levels were measured by a glucometer Accu-chek (Roche, Basel, Switzerland) at different time points as described previously (Lam et al., 2005). Serum insulin levels were assessed using a mouse insulin ultrasensitive ELISA kit (#90080, Crystal Chem, Chicago, IL).

2.3.5 Serological analyses

Mouse serum IL-6 was measured using Mouse IL-6 ELISA Kit, OptEIA^{TM} (BD Biosciences, Franklin Lakes, NJ, USA). Mouse serum ALT and AFP were measured by ELISA kits (M7832 for ALT and M7534 for AFP) from Biotang (Waltham, MA).

2.3.6 Colorimetric assays

Mouse liver triglycerides and serum triglycerides were measured by a triglyceride colorimetric assay kit (item No. 10010303) purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Mouse serum free fatty acids were measured using a free fatty acid assay kit (KA1667) purchased from Abnova (Taipei, Taiwan).

2.3.7 Insulin signaling

Mice were anesthetized with avertin (2, 2, 2-Tribromoethanol, Sigma-Aldrich, St. Louis, MO, USA) and injected intravenously with insulin (300 ng) or carrier (0.9% NaCl

and 0.1% BSA). Mice were killed 45 s after injection. Livers were placed in liquid nitrogen within 15 s and tissue extracts were subjected to Western blotting.

2.3.8 RNA preparation and quantitative Real-time PCR

Total RNA from mouse, human liver tissues or cultured cells was prepared using RNeasy Mini Kit (Qiagen, Valencia, CA). Quantitative real time PCRs were performed using SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA) on Bio-Rad iQ5 machine. Mouse RNA polymerase II RPB1 or human β-actin mRNA was used for data normalization, respectively. The primers for amplifying mouse Ncoa5, IL-6, TNF α, SOCS3, AFP, ALT , AR and FASN are:5'- CGTGATCTAAGAGACTCTAGAG-3' and 5'-CAAAGCTATCTCTGTACCGGTC-3'; 5'- AGAGGAGACTTCACAGAGGAT -3' and 5'-TACTCCAGGTAGCTATGGTAC-3'; 5'- GCCTATGTCTCAGCCTCTTCT-3' and 5'-TTGAGATCCATGCCGTTGGCC-3'; 5'- ACTTCACGGCTGCCAACATCT-3' and 5'-TGGTACTCGCTTTTGGAGCTG-3'; 5'- TATGAAGTGTCAAGGAGGAAC-3' and 5'-AGCGAGTTTCCTTGGCAACACT-3'; 5'-TCCGTGAAGATGTGGCAGCCTT-3' and 5'-GGAGCTCGTCCACATTCAAAG-3'; 5'-GTGGAGTTGTGAACAGAGTAC-3' and 5'-ACCTTGCAGCTGCCACAAGT-3';5'-CCTCAAGGCCTGCGTAGACAC-3' and 5'-GCCACATCATGCTGCTGCAGT-3', respectively. The primers for amplifying human IL-6 and AR are: 5'-CTCACCTCTTCAGAACGAATTG-3' and 5'-AGCTCTGGCTTGTTCCTCACT-3'; 5'- ATTCCTGTGCATGAAAGCACTG-3' and 5'-GATGATCTCTGCCATCATTTCC-3', respectively. The upstream primer used to amplify Ncoa5 or sNcoa5 was 5'-TCTCTGCCTGGCCCGATTTCCCG-3' or 5'-

TCTCTGCCTGGTGAGCTACGT-3', respectively. The common downstream primer was 5'-CTGGCTGTTTGCTGCTGTGGA-3'.

2.3.9 Cell culture and lentiviral knockdown

Mouse macrophage RAW 264.7 (ATCC, Manassas, VA, USA) and human liver cancer cell line PLC/PRF/5 (ATCC, Manassas, VA, USA) were cultured in 10% fetal bovine serum (FBS) DMEM medium (Invitrogen, Eugene, Oregon, USA) with 100 units/ml penicillin and streptomycin. Human monocyte THP1 (ATCC, Manassas, VA, USA) was cultured in RPMI 1640 medium supplemented with 10% FBS, 0.05mM β-mercaptoethanol and 1% P/S. For E2 starvation, cells were cultured in phenol-red free DMEM with 5% charcoal-stripped serum (Invitrogen, Eugene, Oregon, USA) and 1% P/S for 48 h. For lentiviral vector-based knockdown, Ncoa5 shRNA constructs pLKO.1-Ncoa5 shRNA or pLKO.1-TRC control, packaging plasmid psPAX2 and enveloping plasmid pMD2.G (Sigma-Aldrich, St. Louis, MO, USA) were co-transfected into HEK 293T cells and prepared viruses according to manufacturer's instructions. Cells were selected with puromycin for two days and pooled for further experiments.

2.3.10 Plasmids and transient transfection

The IL-6 promoter-reporter plasmid pXP2-IL-6 (Spooner et al., 2007) containing murine IL-6 promoter (-250 to +1) and expression vector pCMV-p65 were kindly provided by Dr. Richard Schwartz (Michigan State University). Expression plasmid

pCDNA3.1(+)-Ncoa5 was previously constructed by Chao Jiang (Jiang et al., 2004). The transfection assays were performed as described previously (Jiang et al., 2004). RAW 264.7 or PLC/PRF/5 cells plated in 12-well plates were transfected with Invitrogen Lipofectamine 2000 (Eugene, Oregon, USA) according to manufacturer's instructions. Twenty-four hours after transfection, RAW 264.7 cells were treated with or without 500 ng/mL LPS for 4 hours before harvesting. PLC/PRF/5 cells were cultured with or without 10^{-8} M E2. Cells were harvested, lysed and analyzed for luciferase activity using Promega's dual luciferase reporter assay system. In all experiments, a plasmid pRL-CMV for expressing Renilla luciferase was used as a control for transfection efficiency. Data were normalized and expressed as relative luciferase light units.

2.3.11 Immunoprecipitation and Western-Blot Analysis

Immunoprecipitation with liver lysates was performed according to the protocol provided by Cell Signaling Technology (Danvers, MA, USA). Western blots were performed and developed by Odyssey infrared scanner according to the manufacturer's protocols (Li-COR Biosciences, Lincoln, NE, USA) as described previously (Zhang et al., 2010).

2.3.12 Chromatin immunoprecipitation (ChIP) Assay

ChIP assays were performed using Imprint Chromatin Immunoprecipitation kit purchased from Sigma-Aldrich (St. Louis, MO, USA) according to the manufacturer's protocol. RAW 264.7 cells were cultured in phenol red free DMEM with 10% CSS and 1% P/S for two days for E2 starvation. Ten nM E2 or ethanol vehicle was added to starved cells. Anti-RNA polymerase II largest subunit and normal mouse IgG were used as the positive and negative controls, respectively. Anti-Ncoa5, anti-ERα and anti-CBP were used to immunoprecipitate DNA-protein complexes. For tissue ChIP experiments, liver tissues from age-matched wild type and *Ncoa5*$^{+/-}$ male mice were prepared. Anti-RNA polymerase II, anti-phosphor RNA polymerase II and anti-ERα were used to immunoprecipitate DNA-protein complexes. The final DNA preparations were amplified by quantitative PCR. The primers for amplifying DNA fragments of IL-6 promoter from -225 to +11 are 5'-CTAGCCTCAAGGATGA-CTTAAG-3' and 5'-CCAGAGCAGAATGAGCTACAG-3'.

2.3.13 Statistical analysis

The differences between groups were analyzed using Student's 2-tailed t test. Survival curves were compared using a Log-Rank (mantelcox) test. Tumor incidences were compared using Chi-square test. Values are expressed as mean ± SEM or SD. P ≤0.05 is considered statistically significant.

2.4 Results

2.4.1 Ncoa5 haplo-insufficiency results in late-onset HCC, exclusively in male mice

To assess the role of Ncoa5 in mouse development and tumorigenesis, we generated genetically engineered *Ncoa5*$^{+/-}$ mice (Figures S2.1A and S2.1B). Ncoa5 expression was detected in all mouse tissues examined, but with variable levels that were lowest in liver (Sauve et al., 2001). *Ncoa5*$^{+/-}$ mice were found to have approximately 50% decrease in Ncoa5 expression within the liver (Figures S2.1C and S2.1D). *Ncoa5*$^{+/-}$ mice appeared indistinguishable from their wild-type littermates at the age of 8 weeks and have similar body weight and liver to body weight ratio as the *Ncoa5*$^{+/+}$ male mice (Figures S2.1E and S2.1F) at ages of 2, 6 or 10 months. However, *Ncoa5*$^{+/-}$ male mice suffered from a severe fertility defect, while *Ncoa5*$^{+/-}$ female mice were fertile (S. Gao, F. Chen, G. Perez and H. Xiao, unpublished data). Consequently, *Ncoa5*$^{-/-}$ homozygous embryos and mice were not generated.

We monitored a cohort of wild-type and *Ncoa5*$^{+/-}$ mice for tumor development for 18 months. Mice were euthanized and subjected to complete necroscopy when they were moribund or reached 18 months of age. We observed that 94% of *Ncoa5*$^{+/-}$ male mice spontaneously developed tumors in the liver at 10-18 months of age, whereas

Ncoa5$^{+/-}$ female and *Ncoa5*$^{+/+}$ male mice did not (Figures 2.1A-2.1C). In a cohort of wild-type and *Ncoa5*$^{+/-}$ mice of Balb/c genetic background, a liver tumor incidence of 71% was observed in *Ncoa5*$^{+/-}$ males (Figures S2.2).

Histological analysis revealed that tumors were well to moderately differentiated HCCs, often with a more than two cell-thick trabecular (Figures 2.1D-2.1F) or pseudoglandular pattern (Figure 2.1G), occasionally with lung metastasis (Figures 2.1H and 2.1I) and necrosis (Figure 2.1J). Tumor cells had morphological resemblance to hepatocytes, however, they displayed nuclear pleomorphism, at times with prominent nucleoli and vacuolation (Figures 2.1F and 2.1K). Some of the tumor cells were α-fetal protein (AFP) or Ep-CAM positive (Figure S2.1G). Ncoa5 expression was detectable using Western blot analysis (Figure S2.1H) and RT-PCR, and no mutations were found in Ncoa5 cDNAs of two tumors that were examined (Data not shown). These results suggest that Ncoa5 is haplo-insufficient to suppress HCC development in male mice.

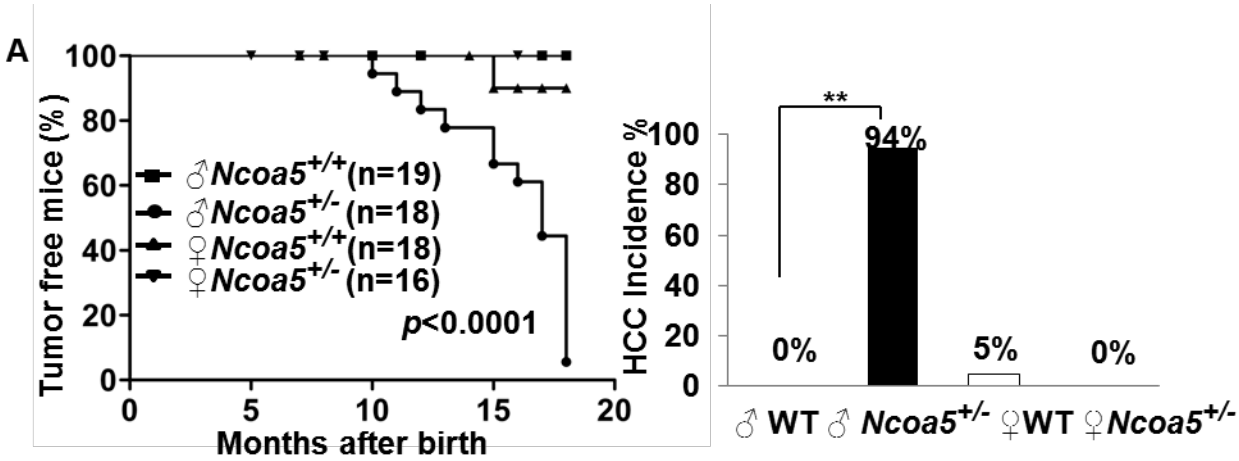
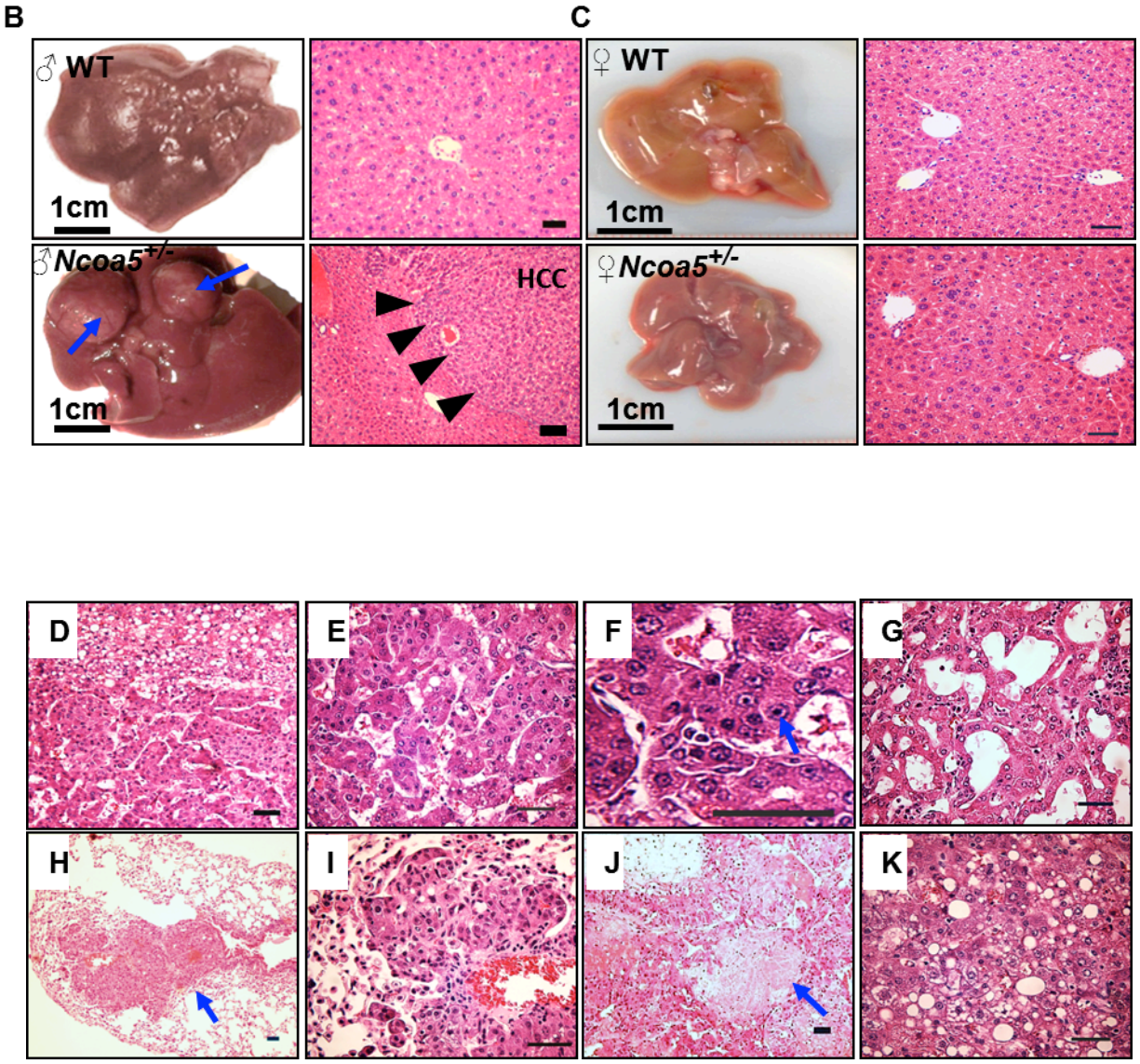


Figure 2.1 *Ncoa5*$^{+/-}$ male mice spontaneously develop HCC

Figure 2.1 (Cont'd)



(A) Kaplan Meier curves showing tumor-free survival of WT and *Ncoa5*$^{+/-}$ mice. Results are expressed as percentage of mice free of liver tumors (n=16-19; *P*<0.0001; log-rank test). The Bar graph shows liver tumor incidence of male and female WT and *Ncoa5*$^{+/-}$

mice (**$P \leq 0.01$). (B and C) Representative macroscopic appearance of livers and H&E stained liver sections derived from 18-month-old WT and *Ncoa5*$^{+/-}$ male (B) and female (C) mice. Blue arrows indicate tumors. Arrowheads indicate the edges of tumors. (D-F) H&E stained HCCs in *Ncoa5*$^{+/-}$ male mice showed thicker trabeculae and steatosis in lower magnification (D and E) and higher magnification (F). (G) H&E stained *Ncoa5*$^{+/-}$ HCC with a pseudo-glandular pattern. (H and I) A well-differentiated HCC metastasized to lung in *Ncoa5*$^{+/-}$ mice in lower (H) and higher magnification (I). The blue arrow indicates the metastatic tumor. (J) H&E stained *Ncoa5*$^{+/-}$ HCC with necrosis. The blue arrow indicates a necrotic area. (K) H&E stained *Ncoa5*$^{+/-}$ HCC with macrovesicular steatosis. Bars: 50 μm.

2.4.2 Ncoa5 haplo-insufficiency results in early-onset glucose intolerance in male mice

Given the finding that the human Ncoa5 gene is a possible T2D susceptibility gene (Bento et al., 2008; Lewis et al., 2010), blood glucose tests, glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) were performed in 6-week-old *Ncoa5*$^{+/+}$ and *Ncoa5*$^{+/-}$ mice. Six-week-old *Ncoa5*$^{+/-}$ male mice showed significantly elevated levels of fasting blood glucose, as well as markedly decreased glucose tolerance and insulin sensitivity compared to *Ncoa5*$^{+/+}$ littermates (Figures 2.2A-2.2C). Similar results were

obtained in *Ncoa5*$^{+/-}$ mice in a Balb/c genetic background (Figures S2.3A-S2.3C). Elevated fasting blood glucose levels and glucose intolerance were continuously present in *Ncoa5*$^{+/-}$ male mice at the age of 20 weeks (Figures S2.4A-S2.4C). Interestingly, these differences were not observed between *Ncoa5*$^{+/-}$ and *Ncoa5*$^{+/+}$ female mice (Figure 2.2D). Consistent with impaired insulin signaling, insulin-stimulated phosphorylation of IR-β, IRS-1 and Akt was reduced in livers of *Ncoa5*$^{+/-}$ male mice, whereas total IR-β, IRS-1 and Akt protein levels were not affected (Figure 2.2E). These results indicate the impairment of insulin signaling in *Ncoa5*$^{+/-}$ mouse livers.

Surprisingly, no statistically significant difference in serum insulin levels, in the fasting state, following an intraperitoneal glucose load, was detected between these two groups of mice (Figure 2.2F). This suggests a partial failure of functional β cell compensation in *Ncoa5*$^{+/-}$ male mice. Consistently, there was no significant difference in pancreas size between *Ncoa5*$^{+/-}$ and *Ncoa5*$^{+/+}$ male mice; the mass and number of islets were not expanded, but rather significantly reduced in both 8- and 24-week-old *Ncoa5*$^{+/-}$ male mice relative to *Ncoa5*$^{+/+}$ male littermate controls (Figures 2.2G-2.2I). Thus, Ncoa5 haplo-insufficiency results in the onset of glucose intolerance in male mice at the age of 6 weeks through inhibition of both hepatic insulin signaling and pancreatic β cell compensation.

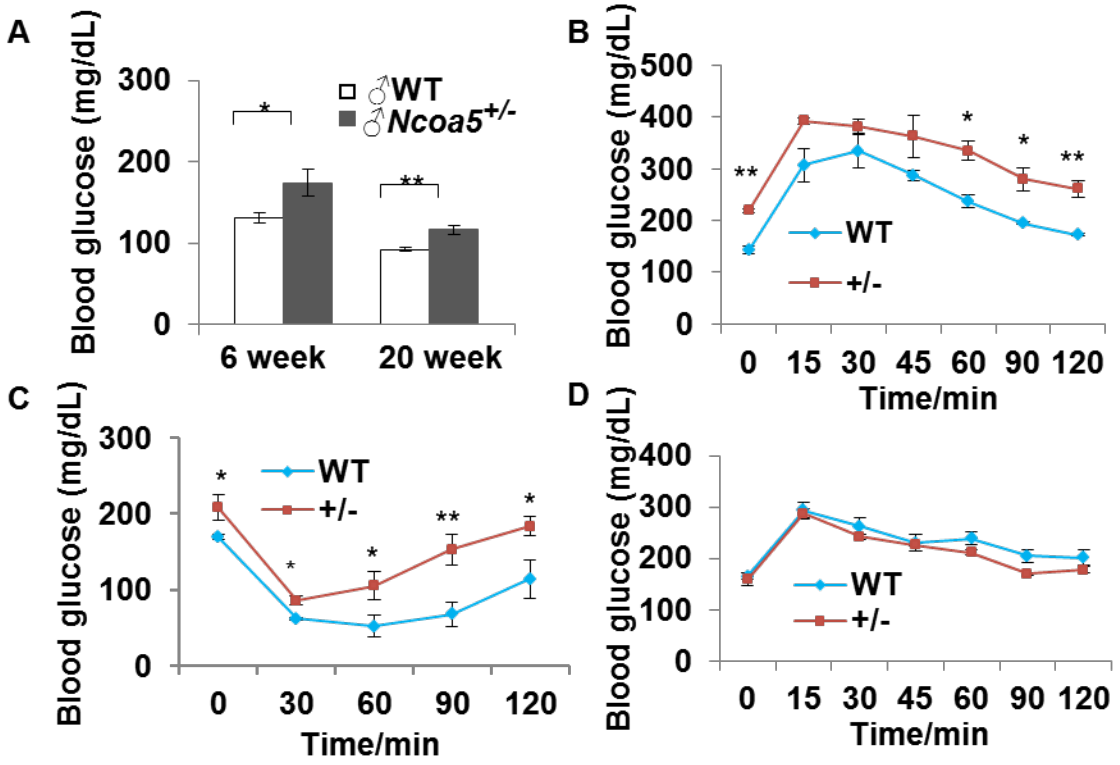
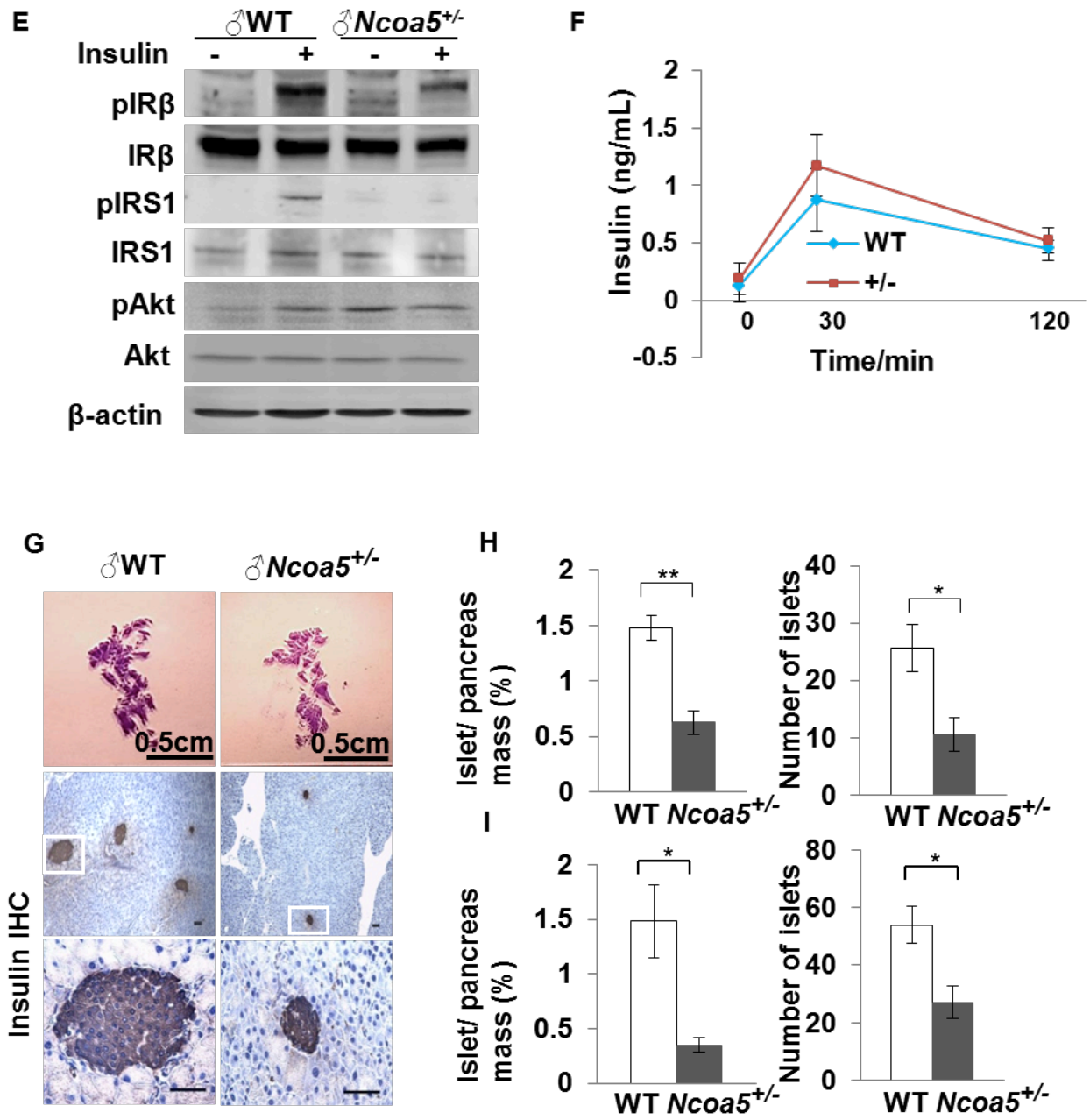


Figure 2.2 *Ncoa5*$^{+/-}$ male mice develop glucose intolerance at the age of 6 weeks

Figure 2.2 (Cont'd)



(A) Blood glucose levels of fasted 6-week-old and 20-week-old male mice with indicated genotypes (n=7). (B) GTT of 6-week-old male mice with indicated genotypes (n=3). Blood glucose levels were determined at indicated times. (C) ITT of 6-week-old male mice with indicated genotypes (n=4). Blood glucose levels of male mice with indicated genotypes at the indicated time. Note: Similar changes in blood glucose levels and GTT were observed in *Ncoa5*$^{+/-}$ male mice in Balb/c genetic background (Figure S2). (D) GTT of 6-week-old female mice with indicated genotypes (n=4; P>0.05). (F) Serum insulin concentrations were determined at the indicated times after glucose challenge (P>0.05). Western-blot analysis of liver extracts from WT and *Ncoa5*$^{+/-}$ male mice at 6 months of age after portal vein injection of insulin using antibodies for pIRβ (Tyr 1150/1151), IRβ, pAkt (Ser 473) and Akt. For pIRS1, lysates were immunoprecipitated with anti-IRS1 antibody and then immunoblotted with anti-phospho-tyrosine antibody. Results represent three independent experiments with different pairs of littermates. (F) Serum insulin concentrations were determined at indicated times after glucose challenge (*P*>0.05). (G) Representative H&E staining of pancreases from 6-month-old WT and *Ncoa5*$^{+/-}$ male mice (upper panel). Representative IHC staining of insulin of pancreases from 6-month-old WT and *Ncoa5*$^{+/-}$ male mice, lower magnification (middle panel) and higher magnification (bottom panel). (H and I) Quantification of the proportion of islet mass to the pancreas area and the numbers of islets counted from 10 sections of each mouse from 8-week-old (H) and 24-week-old (I)

WT and *Ncoa5*$^{+/-}$ male mice. Results are shown in proportion to the pancreas (%; n=3-4). All values are mean ± SEM. *$P \leq 0.05$, **$P \leq 0.01$.

2.4.3 *Ncoa5*$^{+/-}$ male mice developed apparent hepatic inflammation, steatosis and dysplasia after the onset of glucose intolerance and prior to the formation of HCC

To investigate the effects of Ncoa5 on preneoplastic lesion development, we carried out histological comparisons between the livers from *Ncoa5*$^{+/+}$ and *Ncoa5*$^{+/-}$ littermates at various ages. We found that the hepatocellular architecture of 2-month-old *Ncoa5*$^{+/-}$ mice is comparable to wild-type littermates. The livers of *Ncoa5*$^{+/-}$ male mice at ages of 6 or 10 months, but not the livers of age-matched *Ncoa5*$^{+/+}$ male (Figure 2.3A) and *Ncoa5*$^{+/-}$ female mice (Data not shown), displayed characteristic features of hepatic dysplasia and steatosis such as architectural disorganization, cytological atypia, enlarged nuclear, vacuolated hepatocytes and increased lipid deposition as revealed by Oil-Red-O staining (Figure 2.3A). Consistent with these notions, hepatic triglyceride levels were elevated in *Ncoa5*$^{+/-}$ male mice compared to *Ncoa5*$^{+/+}$ male mice, whereas serum triglyceride and free fatty acid levels were comparable in the two groups (Figures S2.5A-S2.5C).

In addition, *Ncoa5*$^{+/-}$ but not the wild type male mice exhibited signs of chronic hepatic inflammation including immune cell infiltrations around the bile ducts and in the

portal areas as well as focal aggregates of lymphocytes, neutrophils and macrophages (Figure 2.3B). Masson's trichrome staining showed fibrosis with connective tissues fibers in the periportal and periductular areas in livers of 10-month-old *Ncoa5*$^{+/-}$ male mice (Figure 2.3B). In parallel with these morphologic changes, serum levels of alanine aminotransferase (ALT) and α-fetal protein (AFP) were significantly increased in 6- and 12-month-old *Ncoa5*$^{+/-}$ male mice compared to age-matched wild type males, but not in 2-month-old mice (Figures 2.3C and 2.3D). Moreover, TUNEL assays detected more cell death in the livers of *Ncoa5*$^{+/-}$ male mice (Figure 2.3E), while PCNA staining revealed more proliferation in the livers and liver tumors of *Ncoa5*$^{+/-}$ male mice (Figure 2.3F). These results suggest that Ncoa5 haplo-insufficiency causes development of hepatic inflammation, steatosis and dysplasia prior to HCC development in male mice.

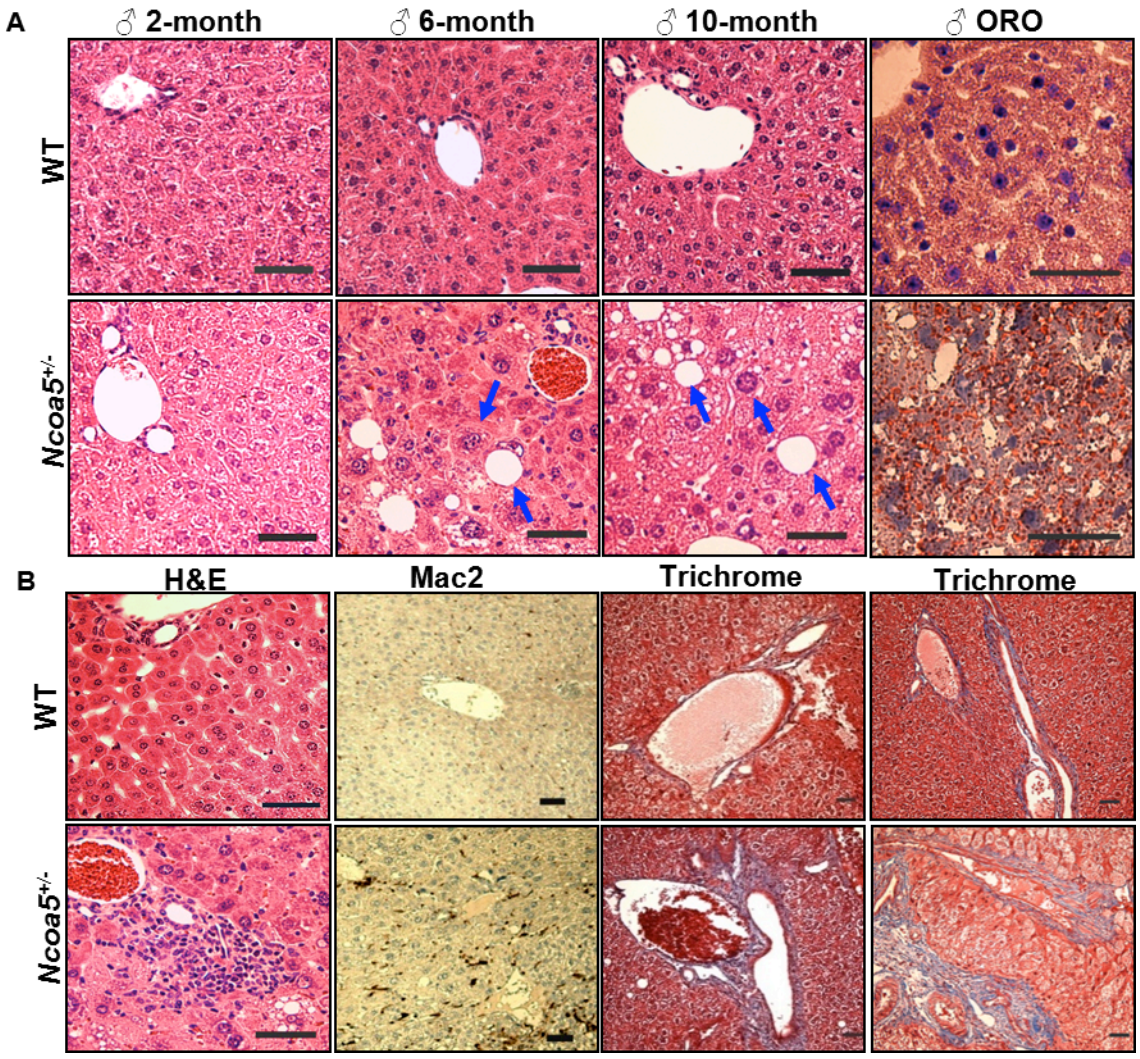
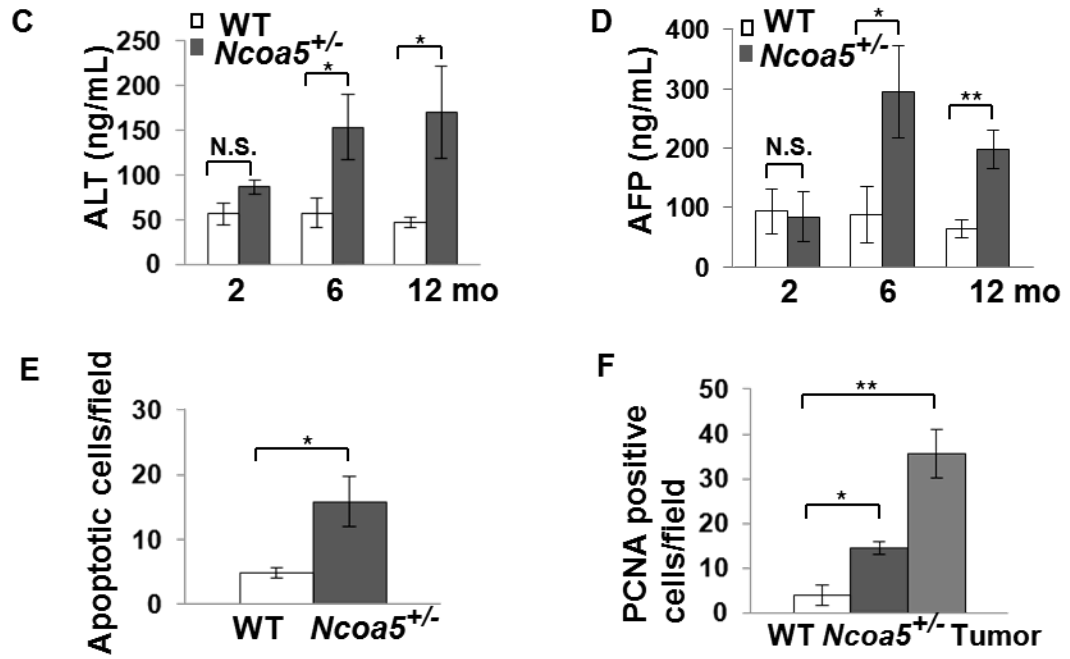


Figure 2.3 HCC development in *Ncoa5*$^{+/-}$ male is preceded by hepatic dysplasia and steatosis

Figure 2.3 (Cont'd)



(A) Representative H&E stained liver sections from male mice with indicated genotypes and ages. Arrows indicate vacuolated or enlarged hepatocytes. Representative Oil-red-O stained sections show accumulated lipids in the liver of *Ncoa5*$^{+/-}$ male mice (Bars: 50 μm). (B) Representative H&E stained liver sections from male mice showing infiltration of inflammatory cells in livers of *Ncoa5*$^{+/-}$ mice. Representative immunostaining of Mac2 and trichrome staining of livers from male mice with indicated genotypes (Bars: 50 μm). (C) Serum levels of ALT of male mice with indicated ages and genotypes (n=4-8; * $P \leq 0.05$; N.S: no significance). Values are mean ± SEM. (D) Serum levels of AFP of male mice with indicated ages and genotypes (n=4-5; * $P \leq 0.05$; N.S: no significance). Values are mean ± SEM. (E) The graph bar shows mean ± SEM of TUNEL-positive cells in the liver sections of 18-month-old WT and *Ncoa5*$^{+/-}$ male mice (n=3; * $P \leq 0.05$). (F) The

graph bar shows mean ± SEM of PCNA-positive cells in the liver sections of 18-month-old WT and *Ncoa5*$^{+/-}$ male mice and liver tumors from *Ncoa5*$^{+/-}$ male mice (n=3; * $P \leq 0.05$).

2.4.4 Ncoa5 deficiency increased the transcription of IL-6 by enhancing RNA Pol II assembly on the IL-6 promoter

Pro-inflammatory cytokines play important roles in hepatic inflammation and preneoplastic lesions (He and Karin, 2011; Johnson et al., 2012). We therefore examined the expression of inflammatory cytokines IL-6 and TNF-α in *Ncoa5*$^{+/-}$ and *Ncoa5*$^{+/+}$ mice. The mRNA levels of IL-6 and TNF-α in the livers were significantly increased in *Ncoa5*$^{+/-}$ male mice at age of 8 and 24 weeks compared to wild type controls, whereas the serum IL-6 levels were not significantly changed, indicating that Ncoa5 exerts its regulation of IL-6 expression in the liver but not in circulating IL-6 (Figures 2.4A, 2.4B and S2.6A). We next asked which cells displayed higher expression of IL-6 and TNF-α in the liver. As shown by immunohistochemical (IHC) staining of IL-6 in liver sections, IL-6 was positively stained in non-parenchymal cells in livers (Figure 2.4C) and the number of positively stained IL-6 cells is significantly increased in *Ncoa5*$^{+/-}$ male livers compared to *Ncoa5*$^{+/+}$ male livers (Figure 2.4D). In addition, dual immunofluorescent (IF) staining of liver macrophage (Kupffer cells) by Mac2 and IL-6 or TNF-α antibodies showed a significant increase in numbers of dual IL-6/Mac2-positive

and TNF-α/Mac2-positive macrophages in *Ncoa5*$^{+/-}$ male mouse livers at the age of 10 months compared with age-matched *Ncoa5*$^{+/+}$ male mouse livers (Figures 2.4E, 2.4F and S2.6B), indicating increased activation of Kupffer cells. Strikingly, IL-6 and TNF-α expression in hepatocytes was not apparently changed in *Ncoa5*$^{+/-}$ male mice (Figures 2.4C and S2.6B). In agreement with the effects of IL-6 on Stat3 and its canonical target SOCS-3 (Senn et al., 2003; Yu et al., 2007), phospho Stat3 (Try 705) protein levels and SOCS-3 mRNA levels were significantly increased in livers of *Ncoa5*$^{+/-}$ male mice compared with livers of wild-type male mice (Figures 2.4G and 2.4H). Increased pStat3 was more pronounced in tumors compared with their adjacent tissues, as the total protein levels of Stat3 were also markedly increased in tumors (Figure 2.4G). Moreover, we also demonstrated that knockdown of Ncoa5 resulted in an increased IL-6 expression in human monocyte/macrophage THP1 cells (Figures S2.7A and S2.7B). These results suggest that Ncoa5 haplo-insufficiency enhances expression of IL-6 and TNF-α in Kupffer cells, which in turn activates Stat3-SOCS3 signaling, leading to the inhibition of insulin signaling in hepatic cells through a paracrine mechanism.

Ligand-bound ERα represses NF-κB-mediated transcriptional activation of the IL-6 gene in macrophages through direct interaction with NF-κB, which binds to the IL-6 promoter responsive elements (Libermann and Baltimore, 1990; Naugler et al., 2007; Ray et al., 1994; Stein and Yang, 1995). We performed quantitative chromatin-immuoprecipitation (qChIP) and luciferase reporter assays to examine molecular mechanism by which Ncoa5 regulates IL-6 expression. qChIP analysis of cultured

mouse macrophages indicated that Ncoa5 assembly on the IL-6 promoter (Figure 2.4I) was increased upon estrogen stimulation, suggesting that Ncoa5, along with ERα, is recruited to the IL-6 promoter. In contrast, the assembly of coactivator CREB-binding protein (CBP) on the promoter was not enhanced after estrogen treatment. Moreover, luciferase reporter assay of mouse IL-6 promoter revealed that Ncoa5 could repress lipopolysaccharide (LPS) induced IL-6 transcription (Figure 2.4J). Consistent with the inhibitory effect of Ncoa5 on IL-6 transcription, mouse liver tissue qChIP analysis revealed that recruitment of RNA polymerase II (Pol II) and the phosphorylated form of Pol II on the IL-6 promoter was significantly increased in *Ncoa5*$^{+/-}$ livers when compared with *Ncoa5*$^{+/+}$ livers (Figure 2.4K), while the assembly of ERα on the promoter was not changed in *Ncoa5*$^{+/-}$ livers. These data indicate that Ncoa5 acts as a negative co-regulator of IL-6 transcription *in vivo* and Ncoa5 haplo-insufficiency increases IL-6 expression through enhancing recruitment of RNA Pol II to the IL-6 promoter.

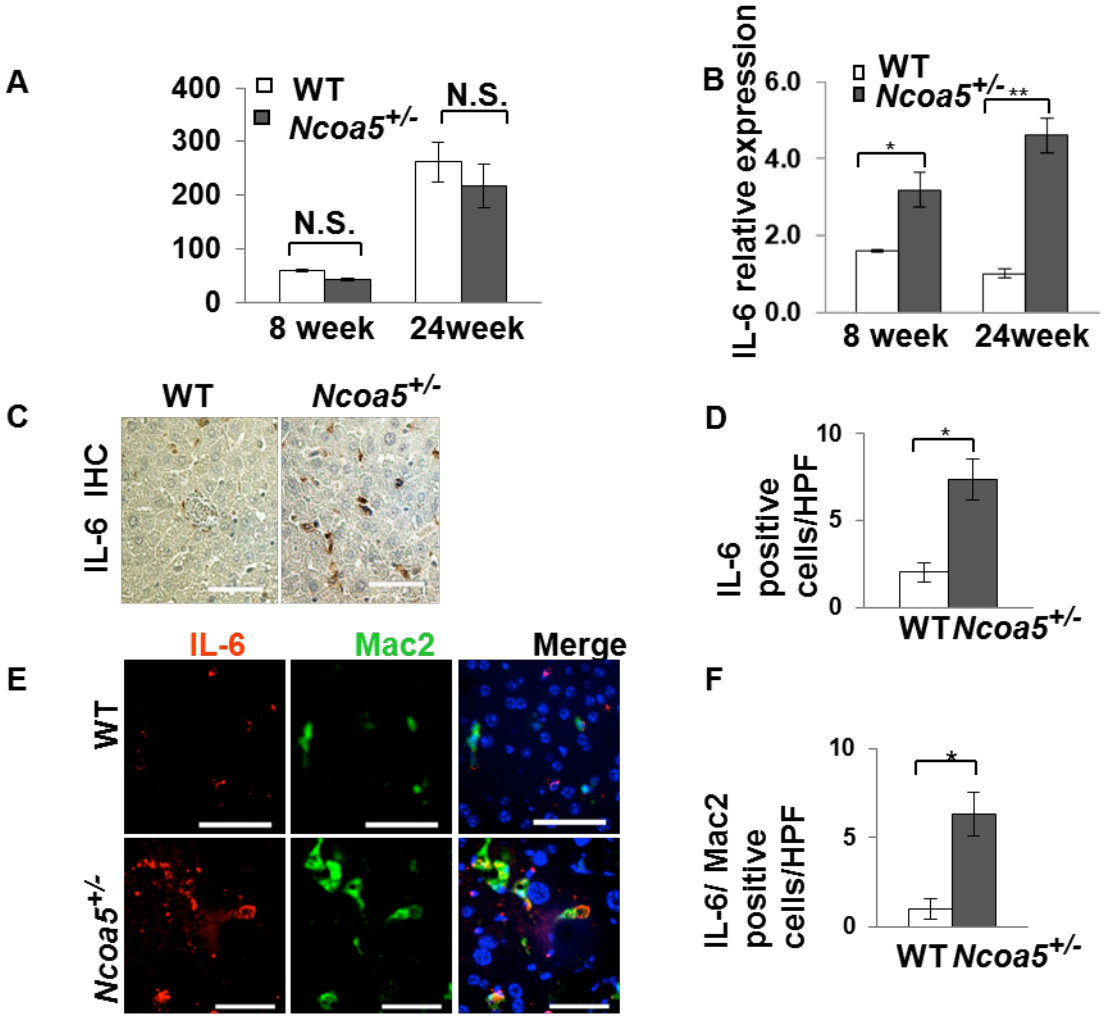
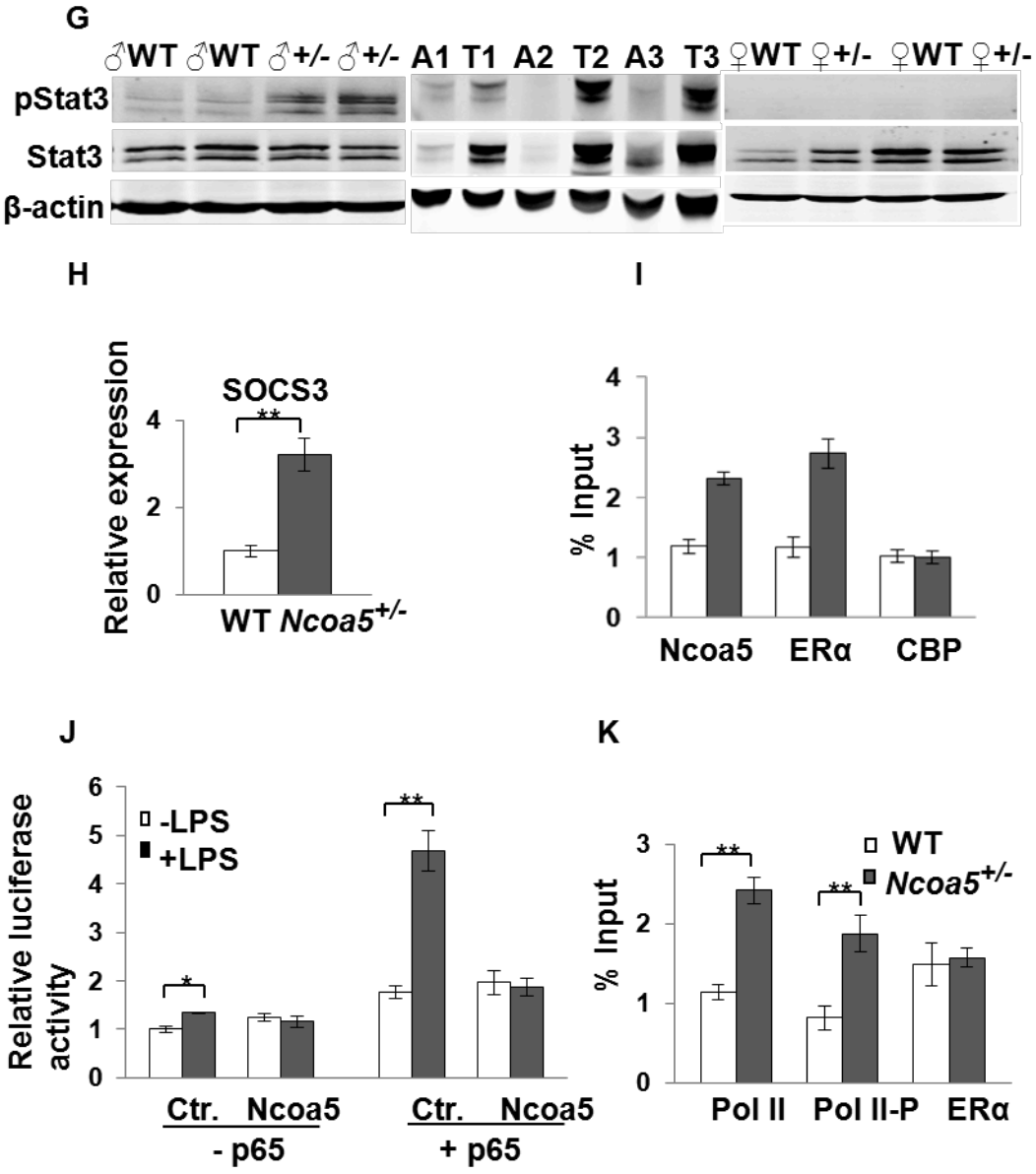


Figure 2.4 Ncoa5 deficiency increases IL-6 expression in macrophages of male mouse livers through inhibition of RNA Pol II assembly on IL-6 promoter

Figure 2.4 (Cont'd)



(A) The bar graph showing serum IL-6 concentration in WT and *Ncoa5*$^{+/-}$ male mice (n=3) with indicated ages. Values are mean ± SEM; N.S. No significance. (B) Quantitative RT-PCR of IL-6 mRNA levels in WT and *Ncoa5*$^{+/-}$ mouse livers with indicated ages (n=4). Values are mean ± SEM; *P≤0.05; ** P≤0.01. (C) Representative IHC stained sections of IL-6 in livers from 6-month-old WT and *Ncoa5*$^{+/-}$ male mice. (D) Quantification of the numbers of IL-6 positive cells per high power field (HPF) (n=3). Five HPFs per section were counted. Values are mean ± SEM; * P≤0.05. (E) Representative dual IF staining of IL-6 (red) and Mac-2 (green) in livers from 10-month-old WT and *Ncoa5*$^{+/-}$ male mice. Nuclei (blue) were stained with DAPI. (F) Quantification of the numbers of IL-6/Mac2 positive cells per high power field (HPF) (n=3). Five HPFs per section were counted. Values are mean ± SEM; * P≤0.05. (G) Western-blot analysis of lysates from 10-month-old WT and *Ncoa5*$^{+/-}$ male and female liver tissues, and liver tumors and adjacent liver tissues from *Ncoa5*$^{+/-}$ male mice with antibodies against phospho-Stat3 (Tyr 705) and total Stat3. β-actin serves as loading control. (H) qRT-PCR of SOCS3 mRNA of 10-month-old WT and *Ncoa5*$^{+/-}$ male mouse livers (n=3). Values are mean ± SEM; ** P≤0.01. (I) qChIP assay for Ncoa5 and ERα binding on IL-6 promoter in cultured mouse macrophages RAW 267.4 cells in the absence and presence of E2. Antibodies for Ncoa5, ERα and coactivator CBP were used for precipitated DNA-protein complexes. qRT-PCR analysis of precipitated IL-6 promoter DNA was performed to quantify the recruitment of indicated proteins on IL-6 promoter. Fold enrichment of qChIP sample relative to input sample is shown.

Experiments were repeated three times. Values are mean ± SEM; *$P \leq 0.05$; ** $P \leq 0.01$. (J) The bar graph shows relative luciferase activity of IL-6 promoter in RAW 267.4 cells transfected with control or Ncoa5 plasmids without or with the transfected p65 before and after LPS treatment. Experiments were repeated three times. Values are mean ± SEM; *$P \leq 0.05$; ** $P \leq 0.01$. (K) qChIP analysis of RNA Pol II assembly on IL-6 promoter in livers of 6-month-old WT and *Ncoa5*$^{+/-}$ male mice. Antibodies for RNA Pol II, phospho-Pol II serine 2 of C-terminal domain, and ERα were used to precipitate DNA-protein complex. The levels of protein-bound DNA were measured by qRT-PCR and expressed in fold enrichment of ChIP sample over input sample. A representative of two independent experiments is shown. Values are means ± SD; ** $P \leq 0.01$.

2.4.5 Ncoa5 deficiency increases AR expression in the livers of male mice and human HCC cells

Previous work has demonstrated androgen receptor (AR) as a key regulator of HCC development through both androgen dependent and independent pathways (Kalra et al., 2008; Ma et al., 2008; Nagasue et al., 1992). Since IL-6 is able to increase AR expression in prostate cancer cells (Lin et al., 2001), we wondered whether Ncoa5 deficiency increased AR expression in the liver. Firstly, we observed that recombinant IL-6 was able to increase AR expression in HCC HepG2 cells *in vitro* (Figure 2.5A). Next, we found that levels of AR mRNA and protein were significantly increased in *Ncoa5*$^{+/-}$ livers compared to livers of *Ncoa5*$^{+/+}$ littermates (Figures 2.5B and 2.5C). Moreover, the protein levels of AR and an AR downstream target TGF-β1 were

significantly elevated in HCCs arising in *Ncoa5*$^{+/-}$ mice compared with their adjacent non-tumorous liver tissues (Figure 2.5D). Interestingly, knockdown of Ncoa5 without IL-6 treatment also increased mRNA level of AR, but not the level of IL-6 mRNA in cultured human HCC PLC/PRF/5 cells (Figures 2.5E-2.5G). These results indicate that elevated AR expression in *Ncoa5*$^{+/-}$ livers might be due to both intrinsic effects of Ncoa5 deficiency and extrinsic effects of Kupffer cell-derived IL-6 on hepatocytes.

In addition, by using mouse signal transduction pathway PCR array, we identified multiple genes in the NF-κB, androgen and insulin pathways, which expression might be altered in *Ncoa5*$^{+/-}$ livers (Figure S2.8). Notably, fatty acid synthase (FAS/Fasn) mRNA was about 6 fold higher in *Ncoa5*$^{+/-}$ male mouse livers than in *Ncoa5*$^{+/+}$ control mice (Figure 2.5H). IHC staining for FAS protein confirmed that FAS expression was increased in *Ncoa5*$^{+/-}$ male mouse livers relative to wild-type control livers (Figure 2.5I). FAS was previously found to contribute to hepatocarcinogenesis and hepatic insulin resistance (Kalra et al., 2008; Kubota et al., 2000; Ma et al., 2008; Menendez et al., 2009; Nagasue et al., 1992; Postic and Girard, 2008). Thus, other factors such as increased expression of AR, TNF-α and FAS might also contribute to the development of T2D and HCC in *Ncoa5*$^{+/-}$ male mice.

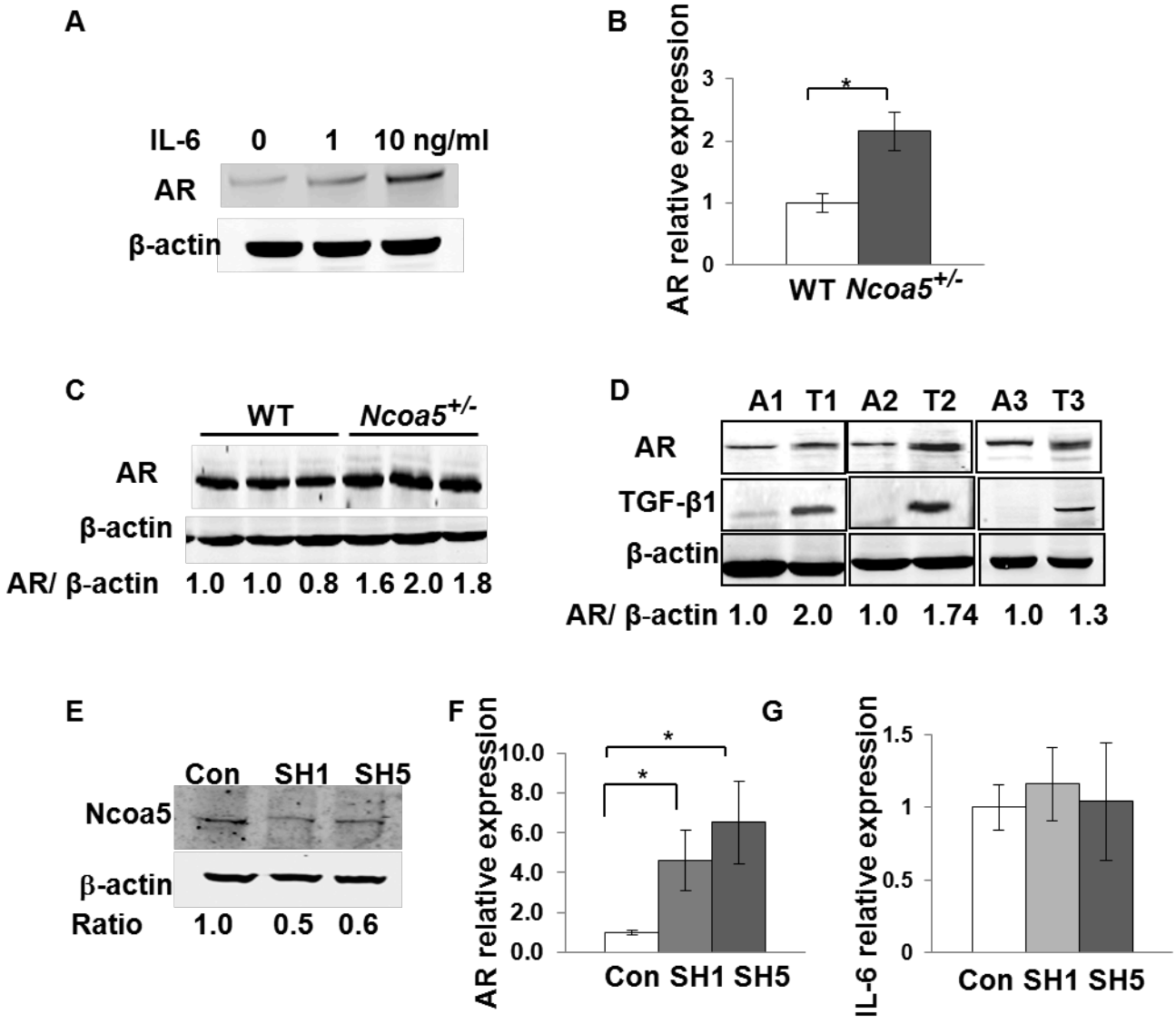
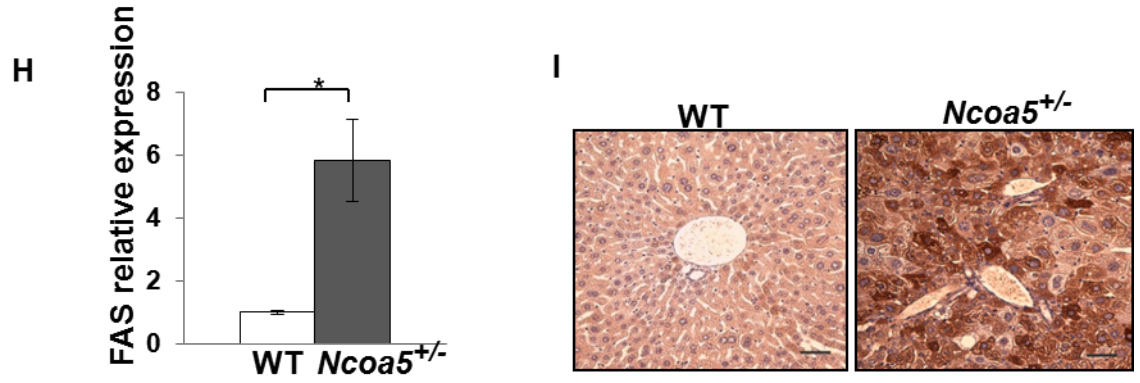


Figure 2.5 Ncoa5 deficiency results in elevated AR expression in mouse livers and cultured human HCC cells.

Figure 2.5 (Cont'd)



(A) Western-Blot analysis of AR protein levels in the cell extracts from HepG2 cell lines after treated with 0, 1 ng/mL and 10 ng/mL IL6, respectively. β-actin serves as loading control. (B) qRT-PCR analysis of AR mRNA levels in WT versus *Ncoa5*$^{+/-}$ livers derived from 5-month-old male mice (n=4). Values are mean ± SEM; * $P \leq 0.05$. (C) AR protein levels in WT versus *Ncoa5*$^{+/-}$ liver tissue lysates derived from 10-month-old male mice. Results were quantified and normalized to β-actin. (D) AR and TGF-β1 protein levels in liver tumors versus their adjacent tissues. The ratios of AR/β-actin were listed. Tissue lysates from three pairs of HCC and adjacent tissues from *Ncoa5*$^{+/-}$ mice were subjected to Western blotting with anti-AR and TGF-β1 antibodies. (E-G) Knockdown of Ncoa5 results in increased AR mRNA levels in human HCC PLC/PRF/5 cells. Whole cell lysates were made from pooled cells expressing a scramble shRNA-CON, Ncoa5-SH1 and Ncoa5-SH5 that specifically target Ncoa5, and then subjected to Western blotting with antibodies for Ncoa5 and β-actin (E). The bar graphs show qRT-PCR

analysis of AR (F) and IL-6 (G) mRNA levels in indicated cells. Experiments were repeated two times. Data represent means ± SD of triplicates from a representativeexperiment (* $P \leq 0.05$). Quantitative RT-PCR of FAS mRNA levels in 10-month-old WT and *Ncoa5*$^{+/-}$ male mouse livers (n=4). Values are mean ± SEM; *$P \leq 0.05$. (C) Representative IHC staining of FAS in livers from 10-month-old WT and *Ncoa5*$^{+/-}$ male mice.

2.4.6 Heterozygous deletion of IL-6 prevents glucose intolerance and partially deters HCC development in *Ncoa5*$^{+/-}$ male mice

To determine whether increased IL-6 expression is responsible for the phenotypes observed in *Ncoa5*$^{+/-}$ mice, we generated mice bearing dual heterozygous deletions of *IL-6* and *Ncoa5* genes by crossing *Ncoa5*$^{+/-}$ mice with *IL-6*$^{-/-}$ (B6.129S6-*IL-6*^{tm1Kopf}) mice. The level of IL-6 mRNA in livers of *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ males was decreased by approximately 50% compared to livers of *Ncoa5*$^{+/-}$*IL-6*$^{+/+}$ male littermates (Figure 2.6A). Notably, heterozygous IL-6 deletion in *Ncoa5*$^{+/-}$ male mice profoundly improved their fertility, as double *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ male mice became fertile. However, no *Ncoa5*$^{-/-}$ *IL-6*$^{+/-}$ pup was generated (S. Gao, F. Chen, G. Perez and H. Xiao, unpublished data). We found that *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ male mice exhibited a significant improvement in fasting

blood glucose levels, GTTs and ITTs compared with their *Ncoa5*$^{+/-}$*IL-6*$^{+/+}$ male littermates at the age of 6 weeks (Figures 2.6B-2.6D). Improved fasting blood glucose levels and GTTs were also observed in *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ Balb/c mice (Figures S2C-S2D).

Moreover, heterozygous IL-6 deletion did not block tumor initiation, as HCC was observed in four out of five (80%) *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ male mice by the age of 18 months. However, the number of tumors per mouse and the tumor volumes are significantly reduced compared to tumors arising in *Ncoa5*$^{+/-}$*IL-6*$^{+/+}$ male mice (Figures 2.6E-2.6G). Western blot analysis revealed that the levels of pStat3 and total Stat3 as well as IL-6 levels were reduced in tumors and their non-tumorous liver tissues in *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ male mice compared with those in *Ncoa5*$^{+/-}$*IL-6*$^{+/+}$ male mice (Figure 2.6H). Together, these results suggest that Ncoa5 deficiency-induced glucose intolerance and HCC development in male mice are dependent, at least in part, on increased IL-6 expression in livers.

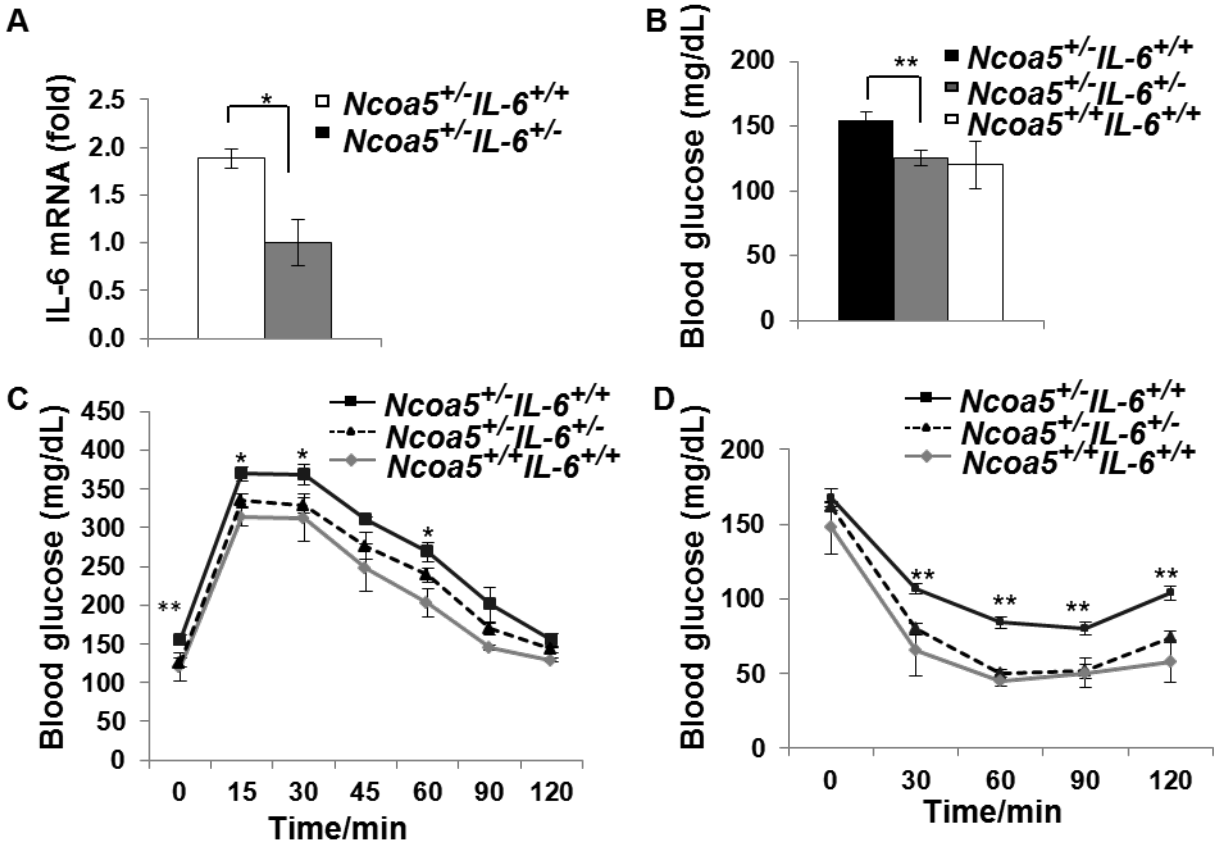
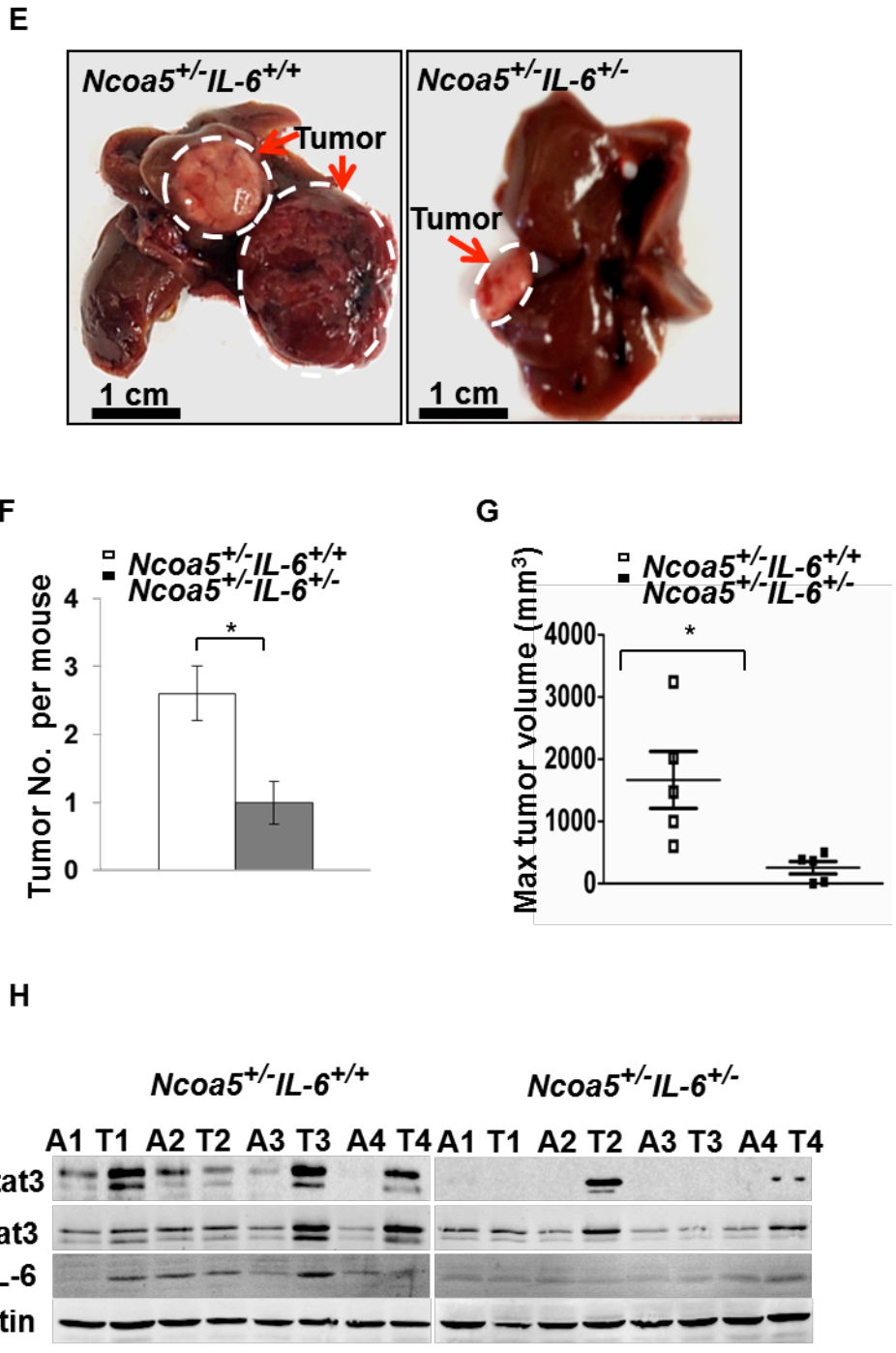


Figure 2.6 Heterozygous deletion of IL-6 prevents the onset of glucose intolerance and partially deters HCC development in *Ncoa5*$^{+/-}$ male mice

Figure 2.6 Cont'd



(A) qRT-PCR analysis of IL-6 mRNA levels in livers from 8-week-old *Ncoa5*$^{+/-}$*IL-6*$^{+/+}$ and *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ male mice (n=3). Values are mean ± SEM; * *P*≤0.05. (B) Blood glucose levels of 12-hour-fasted 6-week-old *Ncoa5*$^{+/-}$*IL-6*$^{+/+}$, *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ and *Ncoa5*$^{+/+}$*IL-6*$^{+/+}$ male mice (n=3-5). Values are mean ± SEM; ***P*≤ 0.01. (C) GTT of 6-week-old *Ncoa5*$^{+/-}$*IL-6*$^{+/+}$, *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ and *Ncoa5*$^{+/+}$*IL-6*$^{+/+}$ male mice (n=3-5). Values are mean ± SEM; **P*≤0.05; ***P*≤ 0.01 *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ versus *Ncoa5*$^{+/-}$*IL-6*$^{+/+}$. (D) ITT of 8-week-old *Ncoa5*$^{+/-}$*IL-6*$^{+/+}$, *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ and *Ncoa5*$^{+/+}$*IL-6*$^{+/+}$ male mice (n=3-5). Values are mean ± SEM; ***P*≤ 0.01 *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ versus *Ncoa5*$^{+/-}$*IL-6*$^{+/+}$. (E) Representative macroscopic appearance of livers derived from 18-month-old *Ncoa5*$^{+/-}$*IL-6*$^{+/+}$ and *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ male mice (n=5 per group). Dash circle lines and arrows indicate tumors. (F-G) The bar graphs show the numbers (F) and the maximal volume (G) of liver tumors arising in 18-month-old *Ncoa5*$^{+/-}$*IL-6*$^{+/+}$ and *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ male mice (n=5 per group). Values are mean ± SEM; * *P*≤0.05. (H) Western blot analysis of pStat3 (Tyr 705), Stat3 and IL-6 levels in four pairs of liver tumors (T) and their adjacent non-tumorous liver tissues (A) in 18-month-old *Ncoa5*$^{+/-}$*IL-6*$^{+/+}$ and *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ male mice. β-actin serves as loading control.

2.5 Discussion

In this Chapter, we describe for the first time that Ncoa5 plays a critical role in suppressing development of glucose intolerance, a pre-diabetic status, and HCC in mice, in part by regulating IL-6 expression in a male gender-specific fashion. Our results suggest Ncoa5 deficiency as a risk factor for both HCC and T2D, which triggers a common pathogenic mechanism in the promotion of both diseases.

Previous studies have demonstrated that IL-6 and TNF-α in Kupffer cells play key roles in HCC development in mice that are induced by chemical carcinogen (DEN), dietary or genetic obesity (Naugler et al., 2007; Park et al., 2010). It has been proposed that the protective effect of estrogens on HCC is due to the inhibition of IL-6 expression in Kupffer cells by estrogen-bound ERα that assembles on the IL-6 promoter through interaction with NF-κB (Libermann and Baltimore, 1990; Naugler et al., 2007; Ray et al., 1994; Stein and Yang, 1995). However, the molecular mechanisms underlying increased IL-6 expression in HCC remain largely unidentified. Our results suggest Ncoa5 as a critical regulator in controlling IL-6 expression and HCC development in mice, which is consistent with previous models of hepatocarcinogenesis (Farazi and DePinho, 2006; Feng, 2012; Naugler et al., 2007). Thus, not only do our results support the previous findings that Kupffer cell-derived IL-6 contributes to the gender disparity of HCC (Naugler et al., 2007), but also point out the mechanism of IL-6 regulation by Ncoa5 and its role in the suppression of HCC development.

Our data, nevertheless, does not suggest that increased IL-6 expression is the only mediator promoting hepatocarcinogenesis in *Ncoa5*$^{+/-}$ male mice. In fact, the correction of increased IL-6 expression in *Ncoa5*$^{+/-}$ male mice with deletion of one IL-6 allele reduces, but not completely blocks, HCC growth. In addition, our on-going experiment showed that one of two *Ncoa5*$^{+/-}$*IL-6*$^{-/-}$ male mouse did develop three small HCCs (≤108 mm^3), indicating that deletion of both IL-6 alleles does not completely block HCC development in *Ncoa5*$^{+/-}$ male mice (Data not shown). Therefore, our results suggest that other downstream targets of Ncoa5 may also contribute to hepatocarcinogenesis. Supporting this idea, we found that Ncoa5 haplo-insufficiency resulted in aberrant expression of other genes including TNF-α, FAS/Fsan and AR, that were previously reported to contribute to the development of T2D and HCC (Kalra et al., 2008; Ma et al., 2008; Naugler et al., 2007; Pikarsky et al., 2004; Postic and Girard, 2008). Since Ncoa5 could regulate ERα-targeted genes via a direct interaction with ERα (Jiang et al., 2004), it is possible that Ncoa5 may regulate other ERα-targeting genes in Kupffer cells as well as in hepatocytes. Alternatively, Ncoa5 may regulate genes independent of ERα in glucose homeostasis and HCC development, as our current evidence does not prove that the action of Ncoa5 is dependent on ERα. Indeed, Ncoa5 is able to form a complex with SAM68, hnRNP-G and transcription factors ZAP3, ILF2 and ILF3 (Ulke-Lemee et al., 2007), and it may also regulate transcription of genes targeted by other transcription factors such as ILF2 and ILF3, which also warrants further exploration. Therefore, we envision Ncoa5 as a transcriptional co-regulator that concomitantly controls expression of a set of genes in both Kupffer cells and/or hepatocytes that play key roles in hepatic

inflammation, apoptosis and proliferation, to influence the development of HCC. Thus, it will be interesting for future studies to determine whether Ncoa5 deficiency-induced HCC is dependent on ERα. Studies using mice bearing a cell-specific knockout of Ncoa5 and/or compound knockout of its downstream targets will help to clarify the mechanism of Ncoa5 deficiency induced HCC, and provide more mechanistic insights into HCC development.

Our study also indicates that Noca5 haplo-insufficiency causes glucose intolerance, a pathophysiological feature of T2D in mice through increased hepatic IL-6-Stat3 signaling. There is current evidence supporting both beneficial and detrimental effects of IL-6-Stat3 signaling on insulin sensitivity in animals and humans, thus leading to a debate regarding the role of IL-6 in insulin resistance and T2D. Evidently, IL-6 knockout mice display insulin resistance (Matthews et al., 2010), suggesting an essential role for IL-6 in insulin sensitivity. Conversely, genetically engineered mice with activation of NF-κB in the liver resulted in elevated serum levels of IL-6 and TNFα, and displayed insulin resistance (Naugler and Karin, 2008). Moreover, although the IL-6-Stat3 signaling in the liver can promote insulin resistance by inhibiting insulin signaling through SOCS3, this signaling is also critical for suppressing hepatic glucose production through the regulation of insulin action in the brain (Inoue et al., 2006). Thus, hepatic activation of IL-6-Stat3 signaling may act to promote or ameliorate insulin resistance. Our data here, however, suggests that a persistently increased IL-6 in the liver is necessary for the glucose intolerance observed in *Ncoa5*$^{+/-}$ male mice, as *Ncoa5*$^{+/-}$*IL-6*$^{+/-}$ mice show a

significant improvement in their insulin sensitivity. Notably, *Ncoa5*$^{+/-}$ male mice are not obese and no elevated serum IL-6 level was observed, neither the serum insulin level was significantly elevated in *Ncoa5*$^{+/-}$ male mice, indicating that Ncoa5 deficiency does not cause a systemic elevation of IL-6 expression and sufficient compensatory insulin production. Presumably, in the absence of compensatory serum insulin, activation of IL-6-Stat3 signaling in the liver is unable to suppress hepatic glucose production in *Ncoa5*$^{+/-}$ male mice through insulin action in the brain. This may explain the development of hepatic insulin resistance and glucose intolerance in *Ncoa5*$^{+/-}$ male mice in the presence of activated hepatic IL-6-stat3 signaling. Thus, we suggest that Ncoa5 deficiency mainly inhibits hepatic insulin signaling through elevated IL-6 in the liver, while accompanied with the inhibition of compensatory insulin production by pancreatic β cells, leading to impaired glucose homeostasis. Clearly, further studies, including experiments to measure hepatic pancreatic β cell function and hyperinsulinemic-euglycemic clamp assays to assess hepatic glucose production, insulin sensitivity in adipocytes and muscles in *Ncoa5*$^{+/-}$ and WT mice will be a priority.

In summary, our work uncovers Ncoa5 deficiency as a common risk factor in glucose intolerance and HCC and raises many questions about the upstream regulatory genes and downstream targets of Ncoa5 in hepatocytes and Kupffer cells that contribute to the pathogenesis of glucose intolerance and HCC.

2.6 Acknowledgements

We thank Jill Pecha for the construction of Ncoa5 targeting vector, Chao Jiang for initial screening of targeted ES clones, Ying Qin for histological analysis of the sections, Karl Olson for helpful suggestions, Irene Warner and Mingang Wang for technical supports. This work was supported by American Cancer Society Research Scholar Grant for Beginning Investigators (RSG0216501GMC) and NCI grant (1R21CA158539) to H.X.

2.7 Author Contributions

S. G conducted most of the experiments in this study. A.L. and F.C performed IHC and IF analyses. C. Z., M. W. and Z. K performed mouse breeding, analysis of gene expression in cultured cells. C-L. W. analyzed histology of tissue sections. S.G and H.X. wrote the manuscript. R.L. and H. X. supervised the project.

APPENDIX

Supplemental information

Generation of *Ncoa5*$^{+/-}$ mice

The Ncoa5 targeting vector contained a 2304-bp 5' homology arm comprising Ncoa5 intron 2 and a 4670-bp 3' homology arm comprising Ncoa5 intron 4 on each side of the neomycin cassette, which could delete exons 3 and 4 through homologous recombination. This vector was constructed by first inserting a 4,670-bp Xho-SmaI DNA fragment containing Ncoa5 intron 4 derived from BAC clone 432J9 into the plasmid pPNTLoxP (a gift from Dr. Guo-Hua Fong) adjacent to the neomycin cassette, which resulted in pPNTLoxP-4.7kb. A 2,304-bp fragment was then cloned into pPNTLoxP-4.7kb between the thymidine kinase selection marker and neomycin cassette, resulting in the Ncoa5 targeting vector. BAC clone 432J9 was identified from a 129/SvJ mouse BAC library (RPCI22) with the Ncoa5 cDNA probe and purchased from Invitrogen. The 2.304-bp fragment was obtained through PCR amplification of the genomic DNA of 129/SvJ embryonic stem cells. Linearized Ncoa5 targeting vector was electroporated into 129SVJ embryonic stem cells at InGenious Targeting Laboratory, Inc., Stony Brook, NY. Drug-resistant ES cells were screened for homologous recombination by PCR using two sets of primers: 5'-GGCCAGCTCATTCCTCC-CACTCATGATCTATAG-3' and 5'-CTAAGGAGAGGCTGTGTTCC-CAGAACATTTTGCTG-3'; 5'-CTCGTCCTGCAGTTCAT-TCAG-3' and 5'-GTACCTTGAACCC-CCTTGTGT-3'. Two correct ES clones were injected into C57BL/6 blastocysts. Male chimaeras were bred with C57BL/6 mice to obtain germ-line transmitted chimeras. Mice were genotyped by PCR using the following primers: 5'-GACCGTTATCTGAGGGTGGA-3' (Ncoa5) for the

wild type allele, 5’-GCCAGAGGCCACTTGTGTAG-3’ (neo) for the mutant allele and 5’-TTCACCTGCAACCTTCTCCT-3’ (common) for both wild type and mutant allele.

Supplemental Figures

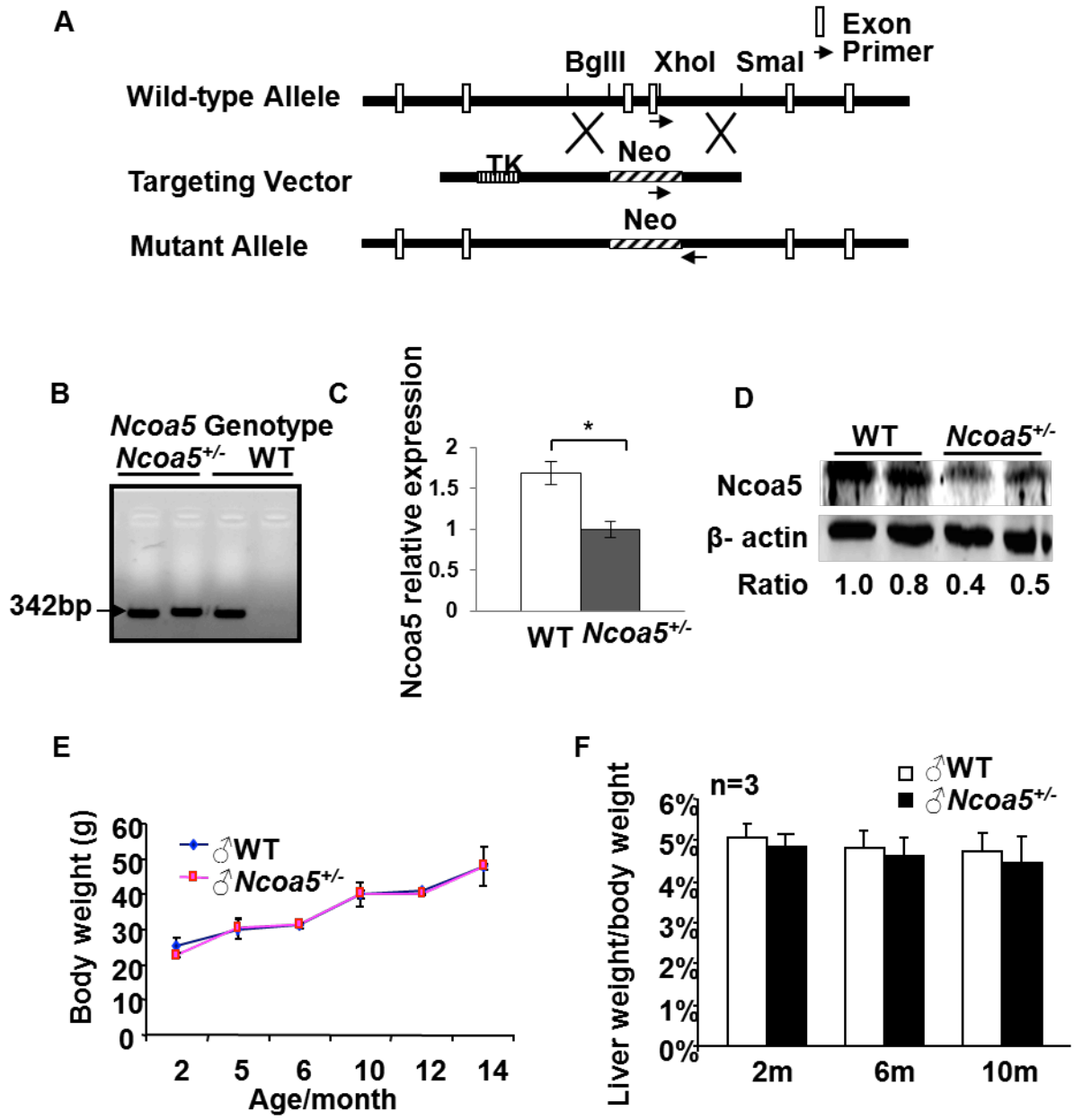
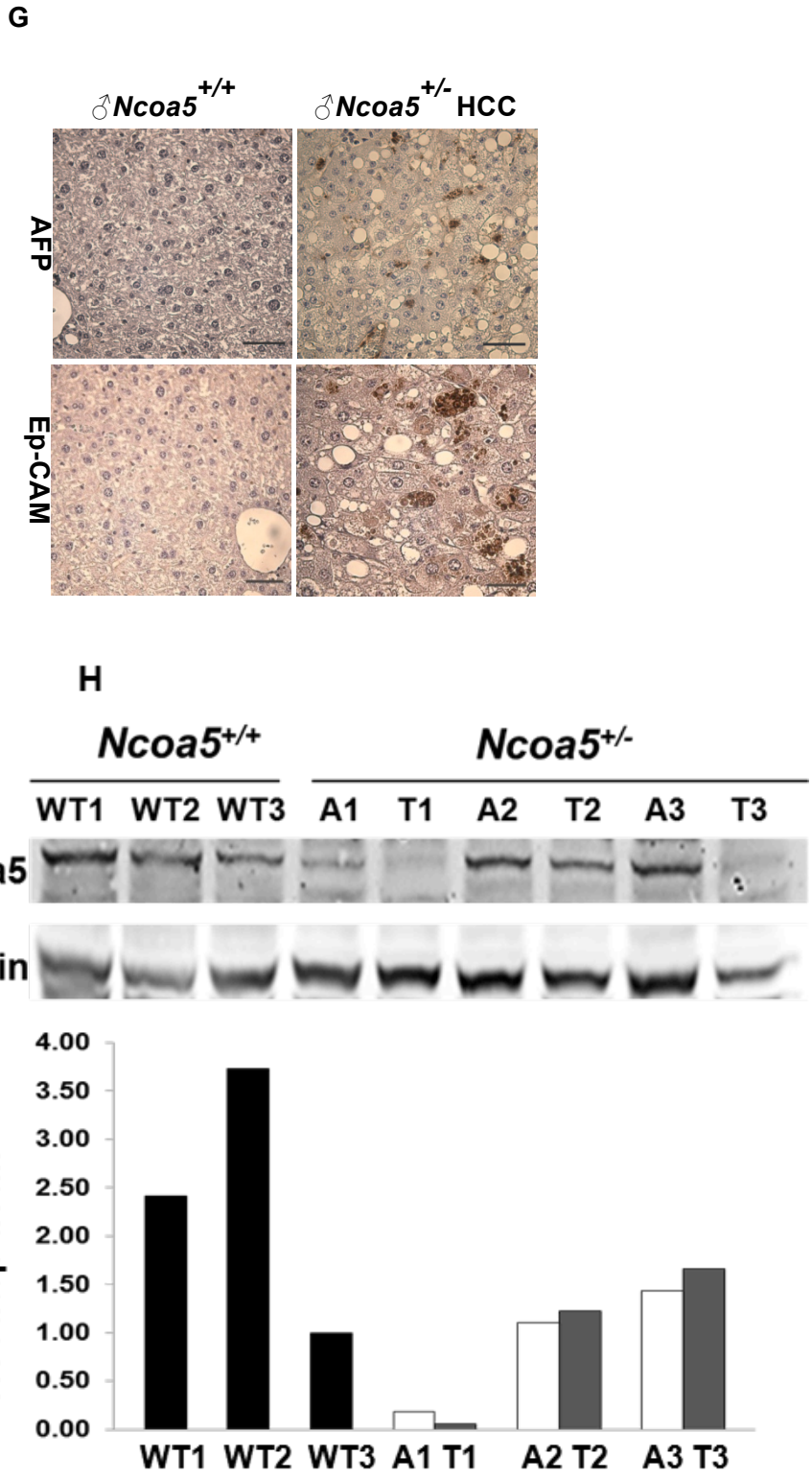


Figure S2.1 Generation of Ncoa5 gene knockout mice. Relative to Figure 2.1.

Figure S2.1 Cont'd



(A) A schematic diagram of the wild-type (WT) *Ncoa5* locus (top), targeting vector (middle), and the predicted targeting locus (bottom) is shown. The genomic sequences are shown as a line, with open boxes representing exons 1-6. DNA sequences of the targeting vector are displayed as a line with shaded boxes of the neomycin resistance cassette (neo) and the thymidine kinase cassette (TK), as indicated. Arrows indicate the positions of primers specific for genotyping. (B) Representative PCR genotyping results showing WT and *Ncoa5*$^{+/-}$ mice. (C) Quantitative RT-PCR analysis of Ncoa5 mRNA in WT and *Ncoa5*$^{+/-}$ mouse livers (n=3). Results are means ± SEM. * $P \leq 0.05$. (D) Western-Blot analysis of Ncoa5 protein levels in WT and *Ncoa5*$^{+/-}$ mouse livers (n=2). (E) Body weight of WT and *Ncoa5*$^{+/-}$ male mice at indicated ages (n=3). Values are mean ± SEM. (F) Ratio of liver weight to body weight of WT and *Ncoa5*$^{+/-}$ male mice at indicated ages (n=3). Values are mean ± SEM. (G) Representative immunohistochemical staining of AFP and Ep-CAM in 18-month-old WT liver and *Ncoa5*$^{+/-}$ HCC tissues. Scale bar: 50 μm. (H) Western Blot analysis of Ncoa5 in liver lysates from WT male mice, *Ncoa5*$^{+/-}$ male liver tumor (T) and adjacent non-tumorous tissues (A) (n=3). The bar graph shows the ratio of Ncoa5 to β-actin in each liver tissue analyzed by Western Blot.

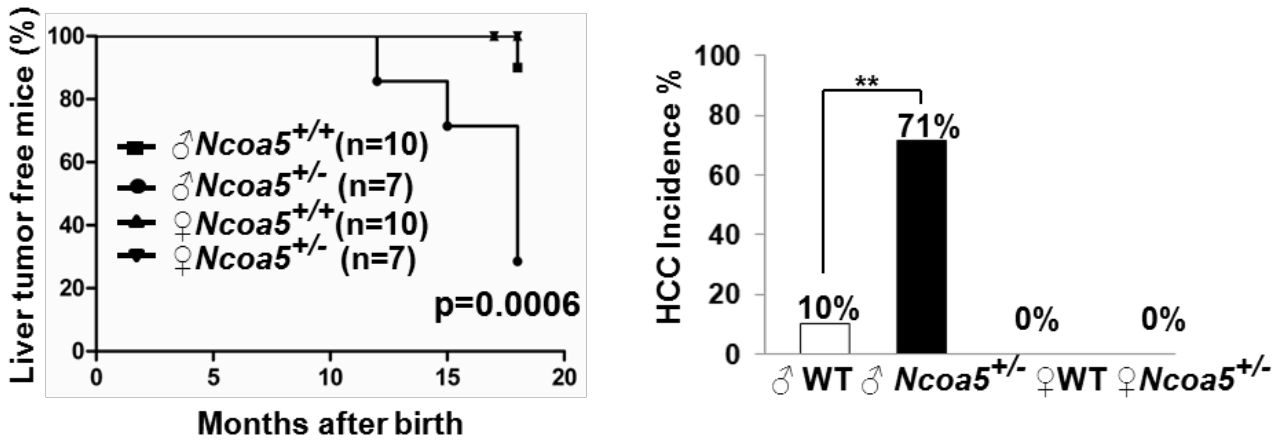


Figure S2.2 *Ncoa5*$^{+/-}$ male mice, in a Balb/c background, develop HCC at 12-18 months. Relative to Figure 2.1.

Ncoa5$^{+/-}$ mice in Balb/c genetic background were generated by backcrossing the Ncoa5 deletion allele for seven generations from the C57BL6/129svj to the Balb/c genetic background. Kaplan Meier curves showing liver tumor-free survival of WT and *Ncoa5*$^{+/-}$ mice. Results are expressed as percentage of mice free of liver tumors (n=7-10; *P*=0.0006; log-rank test). The Bar graph shows liver tumor incidences of male and female WT and *Ncoa5*$^{+/-}$ mice (**$P \leq 0.01$, Pearson Chi-square test).

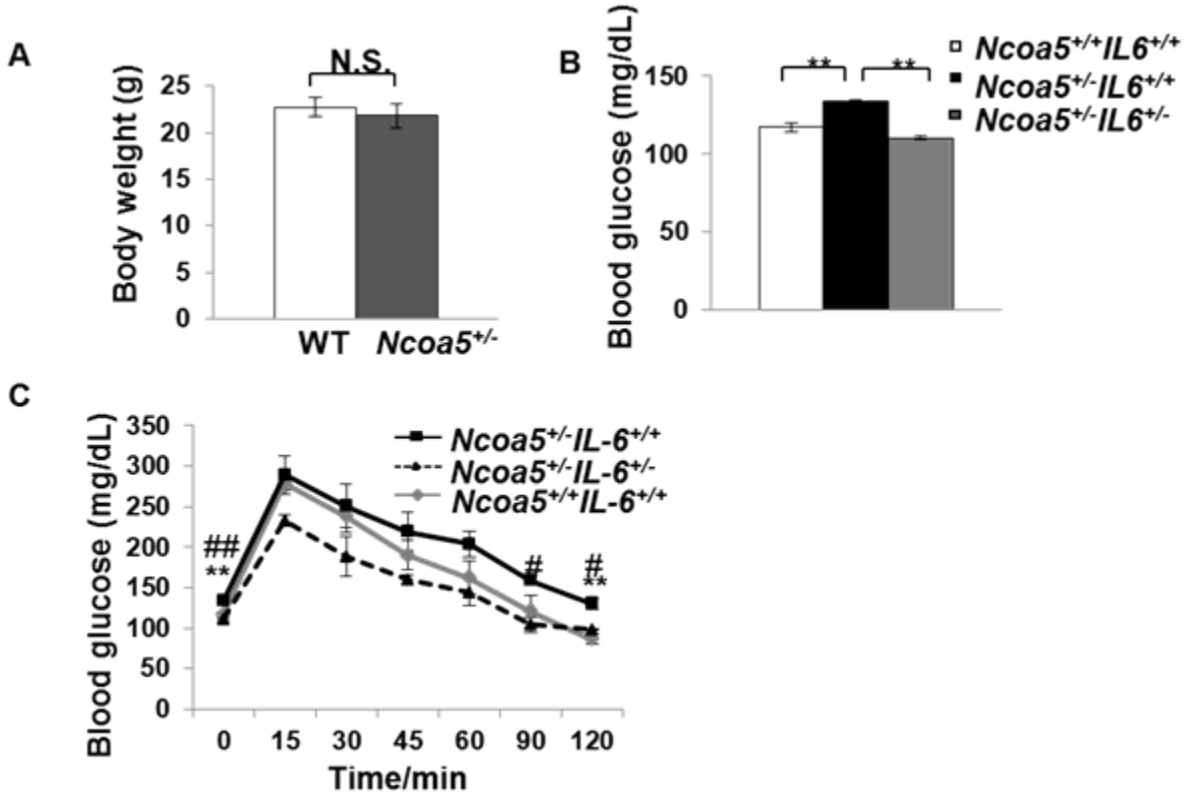


Figure S2.3 *Ncoa5*$^{+/-}$ male mice, in a Balb/c background, develop glucose intolerance at 6 weeks and heterozygous deletion of IL-6 prevents the onset of glucose intolerance in *Ncoa5*$^{+/-}$ male mice. Relative to Figure 2.2 and 2.6.

(A) Body weight of 6-week-old WT and *Ncoa5*$^{+/-}$ male mice in Balb/c genetic background. Mice were weighed after overnight fasting (n=3). Values are mean ± SEM. N.S.: no significant difference. (B) Fasting blood glucose levels of male mice at 6 weeks from *Ncoa5*$^{+/+}$ *IL-6*$^{+/+}$ (n=3), *Ncoa5*$^{+/-}$ *IL-6*$^{+/+}$ (n=3) and *Ncoa5*$^{+/-}$ *IL-6*$^{+/-}$ (n=2) male mice. Values are mean ± SEM. ***P*≤ 0.01. (C) GTT tests for 6-week-old *Ncoa5*$^{+/+}$ *IL-6*$^{+/+}$ (n=3), *Ncoa5*$^{+/-}$ *IL-6*$^{+/+}$ (n=3) and *Ncoa5*$^{+/-}$ *IL-6*$^{+/-}$ (n=2) male mice. Values are mean ± SEM. **

$P \leq 0.01$ *Ncoa5*$^{+/+}$ *IL-6*$^{+/+}$ versus *Ncoa5*$^{+/-}$ *IL-6*$^{+/+}$. $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ *Ncoa5*$^{+/-}$ *IL-6*$^{+/+}$ and *Ncoa5*$^{+/-}$ *IL-6*$^{+/-}$.

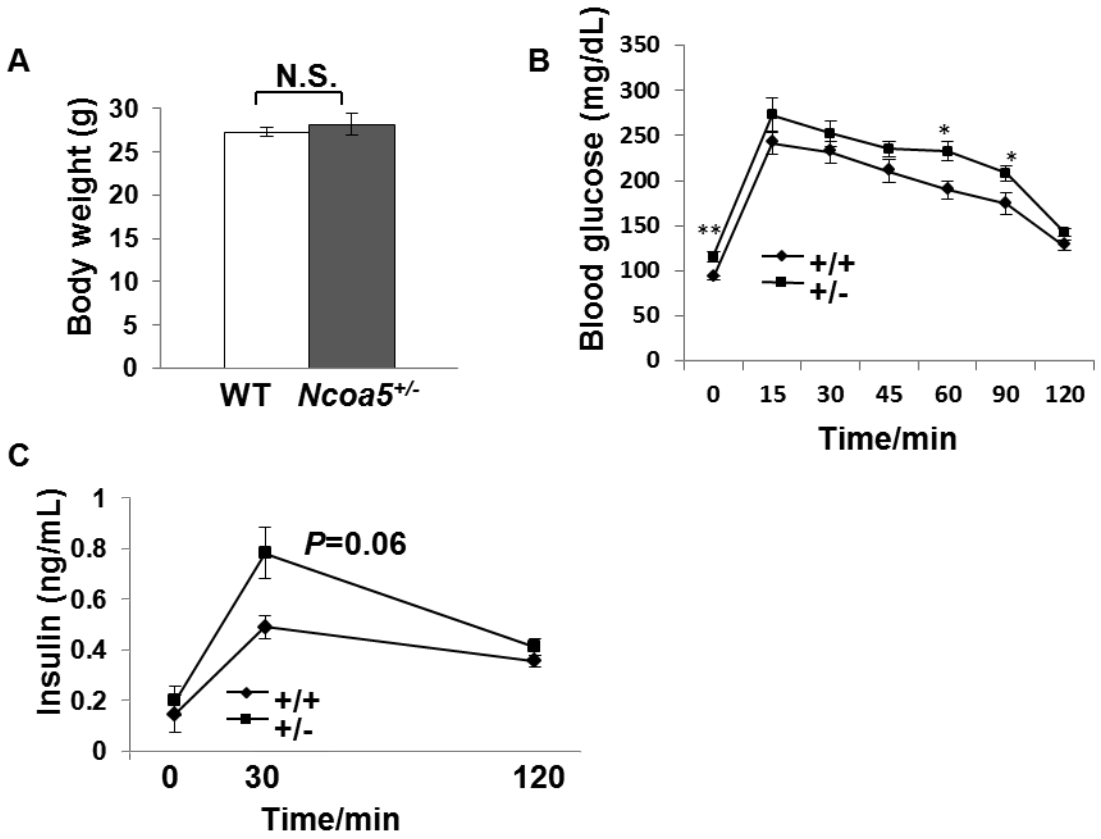


Figure S2.4 Glucose tolerance is continuously observed in *Ncoa5*$^{+/-}$ male mice at the age of 20 weeks. Relative to Figure 2.2.

(A) Body weight of 20-week-old WT and *Ncoa5*$^{+/-}$ male mice in Balb/c genetic background. Mice were weighed after overnight fasting (n=6). Values are mean ± SEM. N.S.: no significant difference. (B) GTT test for 20-week-old WT and *Ncoa5*$^{+/-}$ male mice (n=6).Values are mean ± SEM. *P≤0.05 **, P≤ 0.01. (C) Serum insulin levels were measured in mice with indicated genotypes at indicated times after glucose challenge (n=3). Values are mean ± SEM; P >0.05.

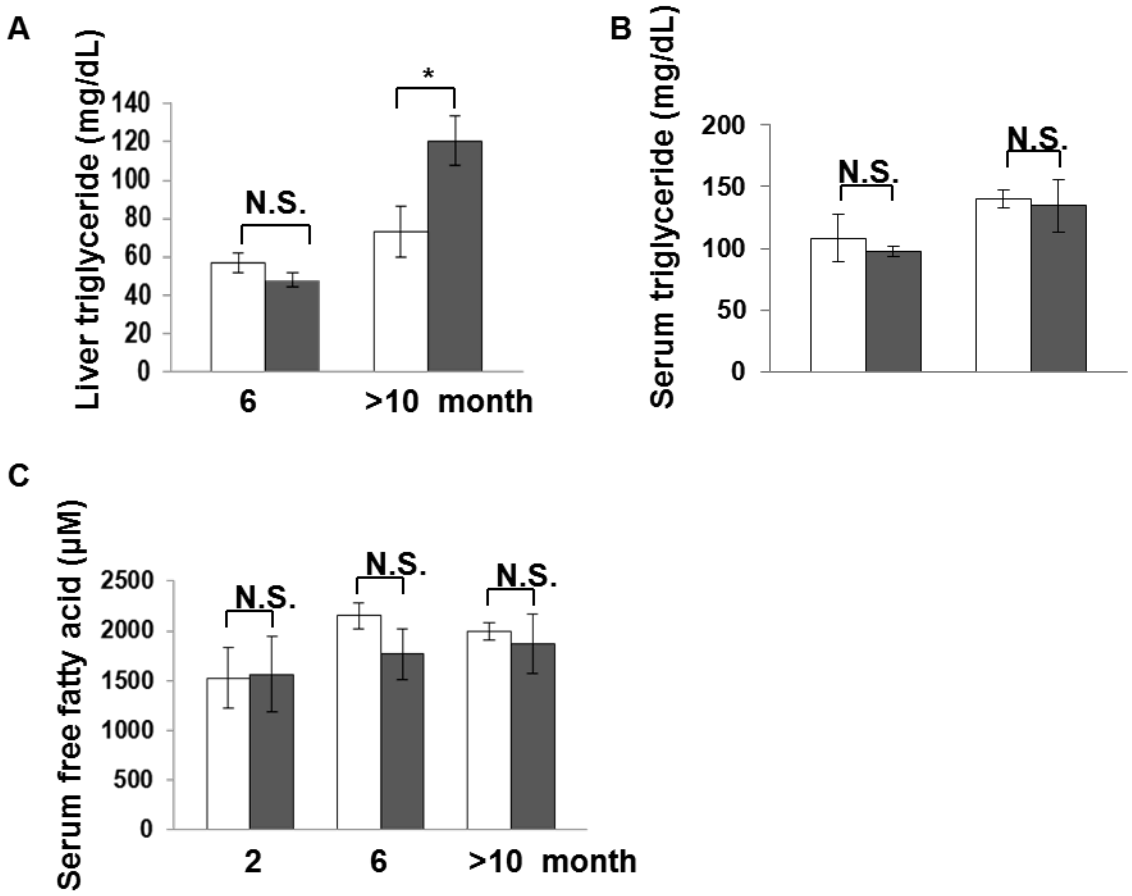


Figure S2.5 Liver triglyceride, serum triglyceride and free fatty acid levels in WT and *Ncoa5*$^{+/-}$ male mice. Relative to Figure 2.3

(A-C) Liver triglycerides (A), serum triglycerides (B) and serum free fatty acids (C) were measured in WT and *Ncoa5*$^{+/-}$ male mice at indicated ages (n=3-4). Values are mean ± SEM. *$P \leq 0.05$, N.S.: no significant difference.

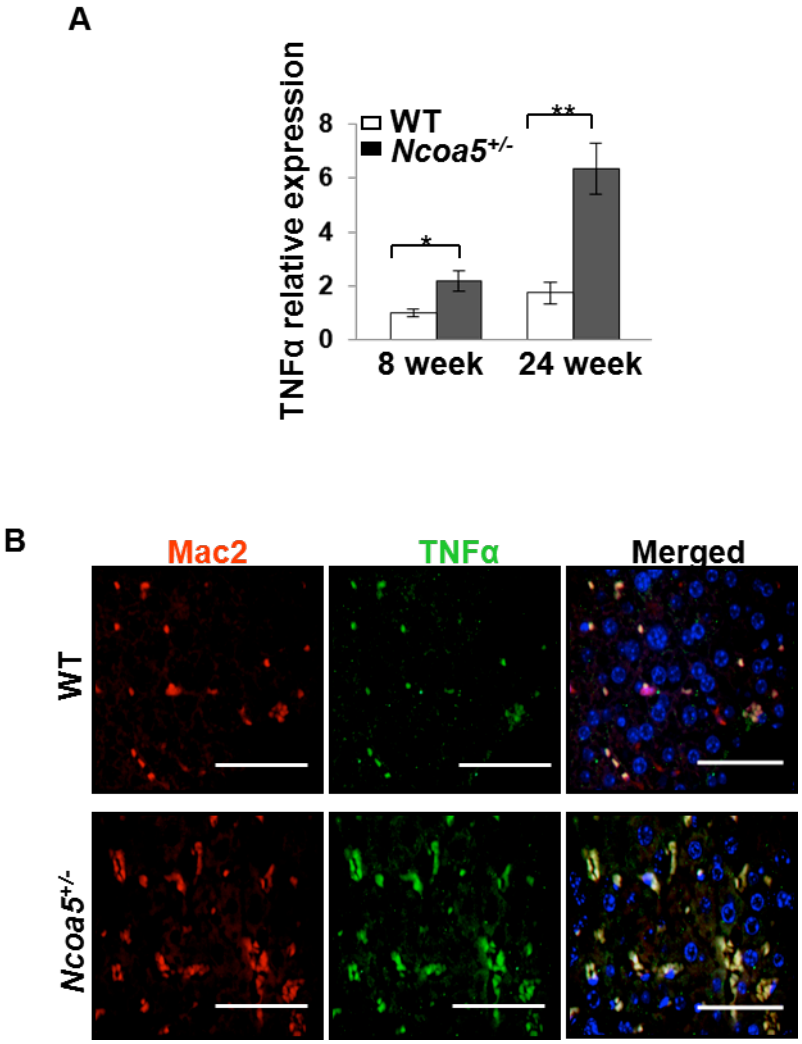


Figure S2.6 Kupffer cell-derived TNF-α production is increased in *Ncoa5*$^{+/-}$ male mice. Relative to Figure 2.4.

(A) Quantitative RT-PCR of TNFα mRNA levels in WT and *Ncoa5*$^{+/-}$ mouse livers with indicated ages (n=3). Values are mean ± SEM. *$P \leq 0.05$, ** $P \leq 0.01$. (B) Representative immunofluorescence staining of TNF-α (green) and Mac-2 (red) on liver sections of WT

or *Ncoa5*$^{+/-}$ male mice at the age of 10 months (n=3), as indicated, followed by counterstaining with DAPI. Scale bar, 50 μm.

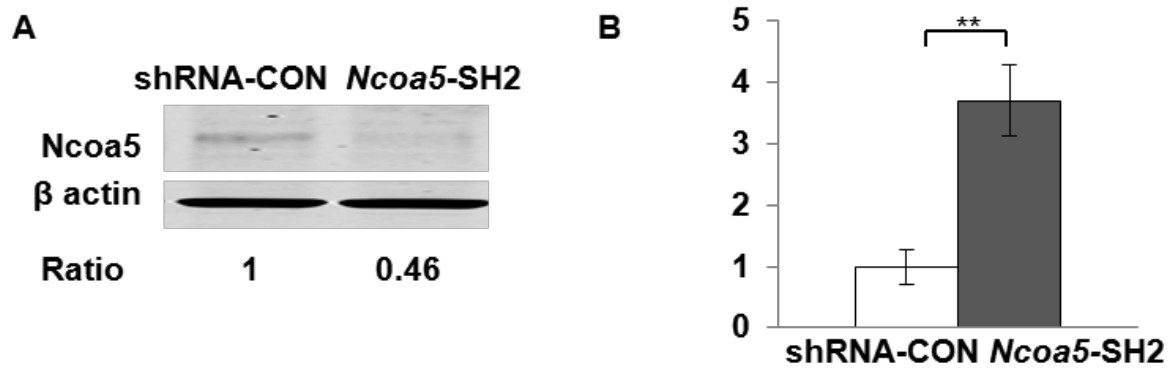


Figure S2.7 Knockdown of Ncoa5 increases the level of IL-6 mRNA in human monocyte/macrophage THP1 cells. Relative to Figure 2.4

(A) Western blot analysis of Ncoa5 levels in shRNA-control or Ncoa5-shRNA-SH2 knockdown THP1 cells. Whole cell lysates were made from pooled cells expressing a scramble shRNA-CON or *Ncoa5*-SH2 that specifically target *Ncoa5*. β-actin serves as loading control. Ncoa5/β-actin ratios were listed. (B) qRT-PCR of IL-6 mRNA in Ncoa5 knockdown cells versus shRNA control cells. Values are mean ± SD of triplicates from one representative experiment. * $P \leq 0.05$.

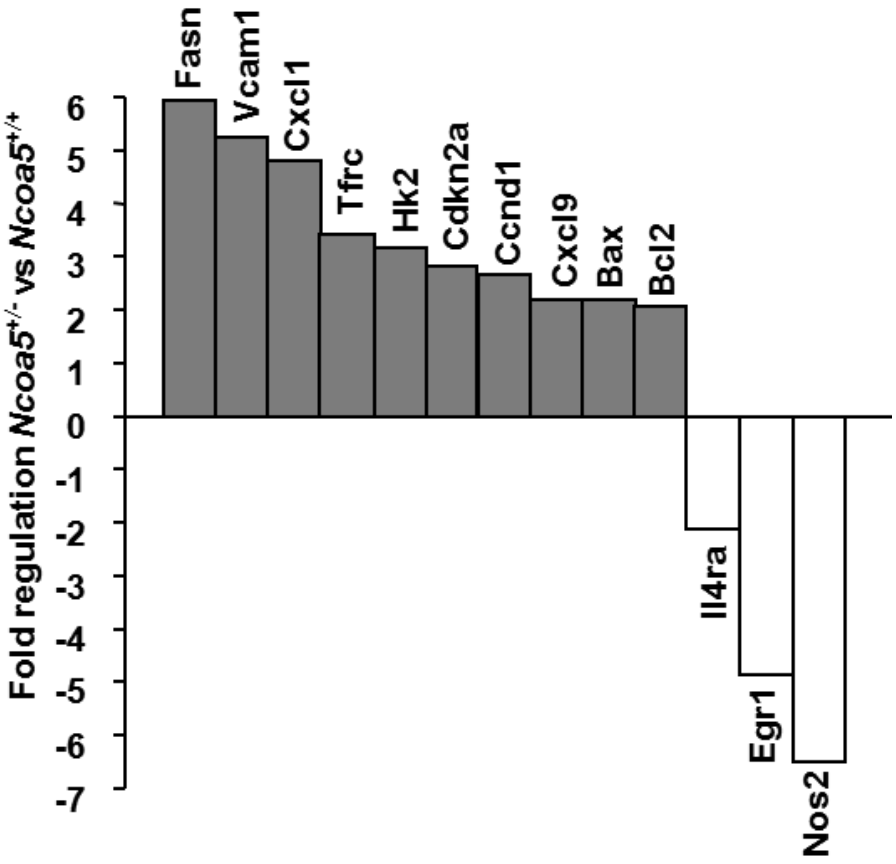


Figure S2.8 Analysis of gene expression between WT and *Ncoa5*$^{+/-}$ mouse livers. Relative to Figure Figure 2.5.

A RT^{2} Profiler PCR Array (Mouse Signal Transduction pathway Finder RT2 Profiler PCR array, PAMM-014A, SA Biosciences, QIAGEN) was used to analyze the expression levels of 84 key genes involved in different signal transduction pathways.

Total mRNAs were isolated from 10-month-old WT and *Ncoa5*$^{+/-}$ male mouse livers (n=3) and then measured by qRT-PCR. Graph shows fold change (fold ≥2.0) of genes.

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CHAPTER 3 Decreased expression of Ncoa5 and overexpression of sNcoa5 are frequently associated with human HCC

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This Chapter has been included in a manuscript that has been accepted by *Cancer Cell* and is currently in press.

3.1 Abstract

Ncoa5 is a haplo-insufficient tumor suppressor in mouse. Human HCC specimens sequencing did not find any Ncoa5 mutations; however, an alternatively-spliced form of Ncoa5 mRNA which encodes a shortened Ncoa5 (sNcoa5) with 406 amino acids due to a frame-shifting was identified. Interestingly, it was found that 63% (19/30) of HCC specimens showed over 50% reduction in Ncoa5 mRNA level in adjacent non-cancerous tissues compared to normal human liver tissue controls; among them, three of four specimens from diabetic patients showed remarkable reduction in Ncoa5 mRNA level. In addition, Ncoa5 expression was found reduced in 40% (12/30) of HCC specimens when compared with adjacent non-cancerous liver tissue. Moreover, sNcoa5 expression was significantly increased in 43% (13/30) of HCC specimens compared with adjacent non-cancerous tissues. Luciferase reporter assays revealed sNcoa5 lacks transcriptional co-activation of ER responsive element but retains transcriptional co-repression of IL-6 promoter compared to Ncoa5. Our findings suggest that reduced Ncoa5 expression and increased sNcoa5 expression are associated with human HCC development.

3.2 Introduction

3.2.1 Pathogenesis of HCC

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with an increasing incidence in the United States (El-Serag and Rudolph, 2007). The risk factors of HCC including hepatic viral infection, alcohol consumption, aflatoxin exposure, non-alcoholic fatty liver disease, obesity and T2D could cause chronic liver inflammation and liver injury, which in turn activates liver repair and regeneration. Continuous rounds of liver damage and regeneration result in stellate cells activation and expansion of collagens in the liver and form regenerative hepatic nodules, when liver cirrhosis occurs. With the contribution of genetic and epigenetic alterations caused by these risk factors, the regenerative nodules could develop to hyperplastic, dysplastic nodules, and finally becomes HCC (Farazi and DePinho, 2006; Thorgeirsson and Grisham, 2002). Especially, activation of key oncogenes such as β-catenin, c-myc and inactivation of tumor suppressor genes such as p53 and retinoblastoma (Rb), contribute to the initiation and progression of human HCC (Martin and Dufour, 2008).

3.2.2 The tumor suppressor genes

Cell destiny is controlled by division, differentiation, and death. The balance between these commitments is determined by negative and positive regulations mainly through two groups of genes: tumor suppressor genes and the proto-oncogenes, respectively (Vogelstein and Kinzler, 2004). Tumor suppressor genes function as a car brakes which regulates its speed, appropriately working tumor-suppressor genes act as

brakes to the cycle of cell growth, DNA replication and division. When these genes fail to function correctly, uncontrolled growth, an important feature of cancer cells, happens (Vogelstein et al., 2000). The function of tumor suppressor genes could be impaired by mutations, loss of chromosome region or silencing by promoter methylation (Martin and Dufour, 2008).

3.2.3 Haplo-insufficiency

The majority of tumor suppressor genes follow the “two-hit hypothesis”, which means requiring mutation or loss of both alleles to inactivate the gene and promote tumorigenesis. However, in case of haplo-insufficiency, only missing of one allele is sufficient to disable the synthesis of the gene product and lose the normal function of the gene. Haplo-insufficiency is associated with a few tumor suppressor genes such as p53 and phosphatase and tensin homologue deleted from chromosome 10 (PTEN) (Santarosa and Ashworth, 2004).

3.2.4 The p53 tumor suppressor gene in HCC

The tumor protein (TP) 53 gene, located on chromosome 17p13.1, encodes a 53 kDa DNA-binding transcription factor p53. The p53 protein functions as an important cell cycle checkpoint and suppressor of tumor development. Cells with damaged DNA or

under stress will activate p53 and undergoes cell cycle arrest for repairing before entering into the division; however, mutations of p53 lose this cell cycle control and result in aberrant cell proliferation and progression to malignancy (Levine and Oren, 2009; Meek, 2009). p53 is a haplo-insufficient tumor suppressor (Lynch and Milner, 2006; Venkatachalam et al., 1998). Mutations of p53 have been identified in different types of human tumors (Nigro et al., 1989). In human HCC, it has been detected mutations in p53 only in larger HCCs, suggesting that genetic inactivation of p53 is associated with HCC progression to late-stage disease (Teramoto et al., 1994). Moreover, studies on the HBx transgenic mouse model have revealed that HBx protein could functionally inactivate p53 through interaction and therefore sequestration p53 in the cytoplasm (Feitelson et al., 2002; Kim et al., 1991; Ueda et al., 1995).

3.2.5 Ncoa5, a possible tumor suppressor in human HCC?

In the previous chapters, we have demonstrated that Ncoa5 is a haplo-insufficient tumor suppressor in mouse HCC development, partly through inhibition of Kupffer cell-derived IL-6 production. This finding in mice drives us to ask whether Ncoa5 functions as a tumor suppressor in human HCC. Meanwhile, epidemiologic studies have demonstrated that females develop less HCC than males (De Maria et al., 2002); however, the incidence of HCC increases in postmenopausal women, suggesting estrogen and ER have a protection role against HCC. Therefore, it is possible that

Ncoa5, as an ER's transcriptional co-regulator, might play a role in human HCC development.

3.3 Experimental procedures

3.3.1 Human tissue samples

Total mRNAs from a pooled five normal male human autopsy liver tissues (N1) and a pair of HCC and adjacent tissues from a male patient were purchased from Biochain Inc, CA. Twenty-nine pairs of frozen human HCC and non-cancerous adjacent liver tissues from patients (25 males, 4 females) with HCC and two frozen human liver tissues (N2, N3) from female patients with hepatic hemangioma or gallstones were collected after hepatectomy, respectively, in Nanfang Hospital, Southern Medical University, China. The age range was 39-76 years. The experimental procedures were approved by the Research Ethics Committee of Southern Medical University.

3.3.2 Human liver cDNA sequencing

RNA was extracted from human HCC sample using RNAiso Reagent (TAKARA BIO INC, Otsu, Shiga, Japan). Ncoa5 cDNAs were reverse transcribed and PCR amplified with the primers (5'-TGGCCAGAAATTATGAGCG-TT-3' and 5'-CTGGCTGTTTGCTGCTG TGGA-3'), followed by sequencing with primer (5'-TGGCCAGAAATTATGAGCGTT-3').

3.3.3 Plasmids

sNcoa5 was amplified from human HCC specimen and inserted into pCR2.1 vector, and then inserted to pCDNA3.1(+) vector through EcoRI and XhoI sites to generate pCDNA3.1(+)-sNcoa5.

3.3.4 Statistical analysis

The differences between groups were analyzed using Student's 2-tailed t test. Values are expressed as mean ± SEM or SD. P ≤0.05 is considered statistically significant.

3.4 Results

3.4.1 Decreased expression of Ncoa5 and overexpression of the alternatively-spliced form of Ncoa5 (sNcoa5) are frequently associated with human HCC

To extend our findings to human HCC, we sequenced Ncoa5 cDNAs amplified from nine pairs of HCC and adjacent non-cancerous tissue samples from male patients as well as a pooled mRNA sample from five normal male human livers. We did not detect any mutations among these Ncoa5 cDNAs; however, we identified a novel alternatively-

spliced form of Ncoa5 mRNA in all of the samples, which encodes a shortened Ncoa5 (sNcoa5) with 406 amino acids due to a frame-shifting insertion caused by an extended exon 7 containing the first 23 nucleotides of intron 7 (Figure 3.1A).

Next, we compared expression levels of Ncoa5 and sNcoa5 among human HCC specimens with their adjacent non-cancerous tissues and normal liver tissues. The mRNA levels of Ncoa5 and sNcoa5 in 30 pairs of frozen HCC and adjacent non-cancerous tissue specimens (four pairs are from diabetic patients) were examined with quantitative RT-PCR analysis. The results showed that at least 63% (19/30) of HCC specimens exhibited over 50% reduction in Ncoa5 mRNA level in adjacent non-cancerous tissues compared to normal human liver tissue controls (Figure 3.1B); among them, three of four specimens from diabetic patients showed remarkable reduction in Ncoa5 mRNA level.

In addition, we detected a statistically significant reduction in Ncoa5 expression in 40% (12/30) HCC specimens when compared with adjacent non-cancerous liver (Figure 3.1B). In contrast, sNcoa5 expression was significantly increased in 43% (13/30) of HCC specimens compared with their adjacent non-cancerous tissues (Figure 3.1C). Western blot analysis confirmed the protein level of sNcoa5 was significantly increased in two of four tested HCC specimens (Figure S3.1). This inverse correlation between low Ncoa5 expression and high sNcoa5 expression in human HCC specimens indicates that Ncoa5 deficiency may contribute to human HCC development.

Furthermore, the microarray data of pancreatic islets from seven normal and five T2D patients reported previously has been examined (Gunton et al 2005). Results identified that two out of five patients with T2D displayed a 70-80% reduction of Ncoa5 expression in pancreatic islets relative to normal control subjects (Data not shown). These results imply a potential association of reduced Ncoa5 expression with human T2D.

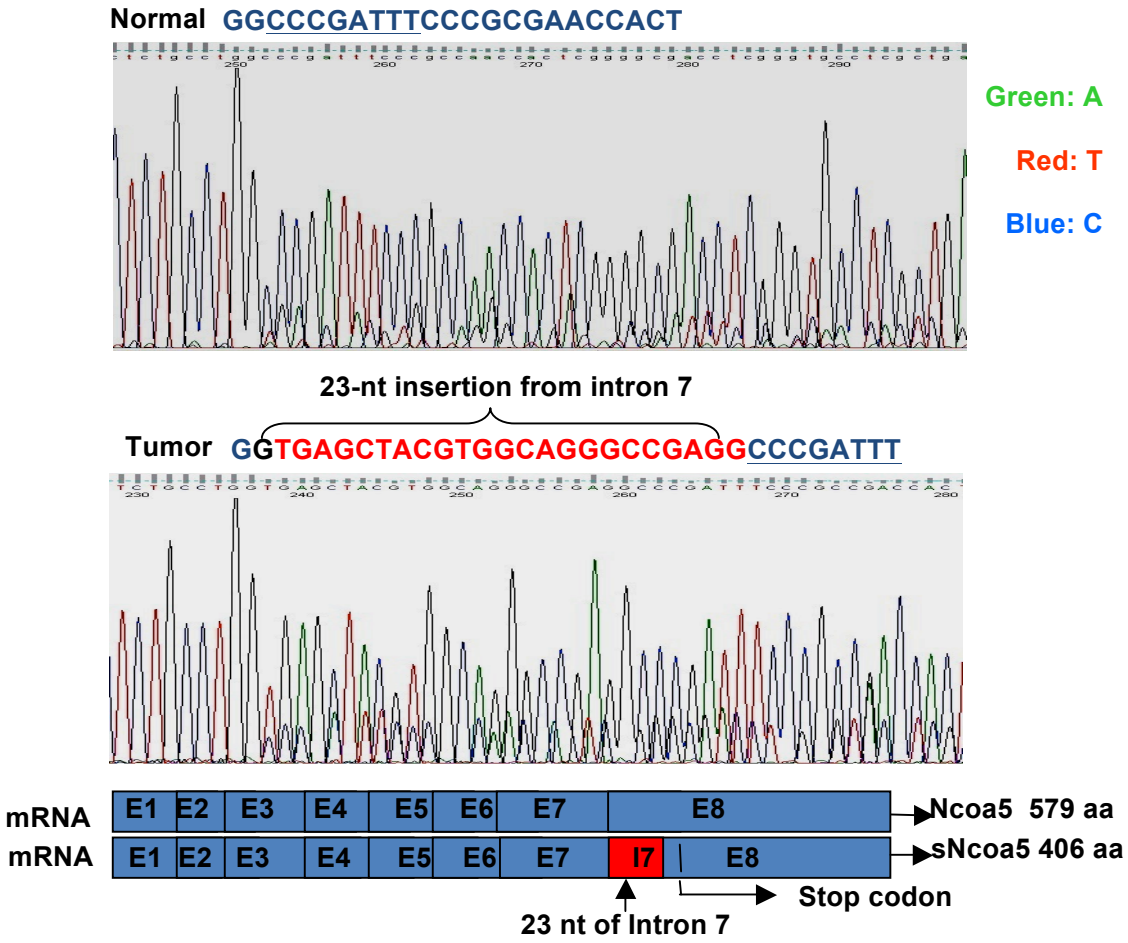


Figure 3.1 Decreased expression of Ncoa5 and overexpression of sNcoa5 in human HCC specimens

Figure 3.1 Cont'd

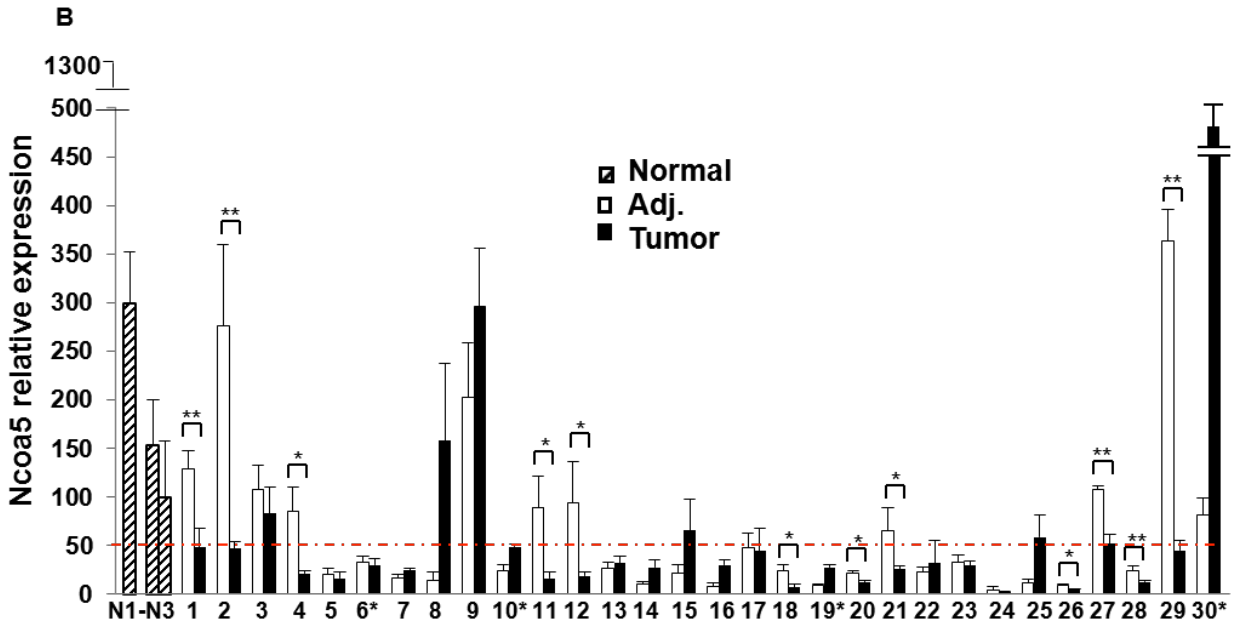
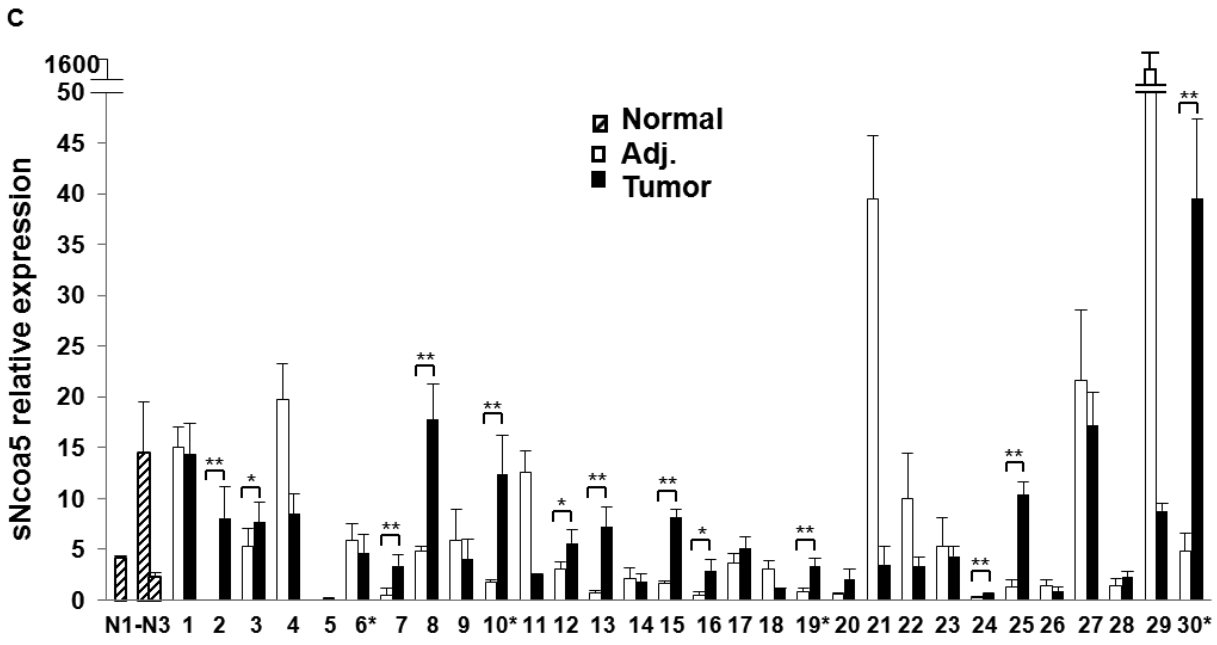


Figure 3.1 Cont'd



(A) Representative sequence traces showing Ncoa5 and sNcoa5 cDNA sequences amplified from five pooled normal human liver tissues or human HCC tumor specimens (n=9) as indicated. Diagrams show exons of Ncoa5 and sNcoa5, as indicated. (B and C) qRT-PCR showing Ncoa5 (B) or sNcoa5 (C) mRNA levels in pairs of human HCC and tumor adjacent tissues (n=30) versus normal human liver tissues (N1-N3) as indicated. Red dotted line indicates at least 50% reduction of Ncoa5 mRNA levels compared to normal human livers. Primers specific for Ncoa5 or sNcoa5 mRNA were used to perform qRT-PCR. Patients 6,10,19,30 have T2D and are marked with asterisk (*). Values are mean ± SD. *$P \leq 0.05$, **$P \leq 0.01$.

3.4.2 sNcoa5 lacks transcriptional activation function and retains transcriptional repression function

Based on the previous studies (Sauve et al., 2001), Ncoa5 has a transcriptional activation domain at its carboxyl-terminus and a transcriptional repression domain at its amino-terminus (Figure 3.2). Compared to the wild type Ncoa5 sequence, sNcoa5 lacks 199 amino acids at its carboxyl-terminus and it is possible that sNcoa5 loses transcriptional activation function. In order to investigate the transcriptional function of sNcoa5, luciferase reporter assays of estrogen response element (ERE) and IL-6 promoter were performed. Luciferase reporter assay with IL-6 promoter revealed that sNcoa5 remains transcriptional repression activity of IL-6 promoter (Figures 3.3A and 3.3B) as sNcoa5 retains amino-terminal transcription repression domain (Figure 3.2). However, luciferase reporter assay with ERE promoter showed that sNcoa5 lacks transcriptional activation and fails to enhance ERα-mediated transcriptional activation as it lacks the transcriptional activation domain at the carboxyl-terminus (Figures 3.3C and 3.3D).

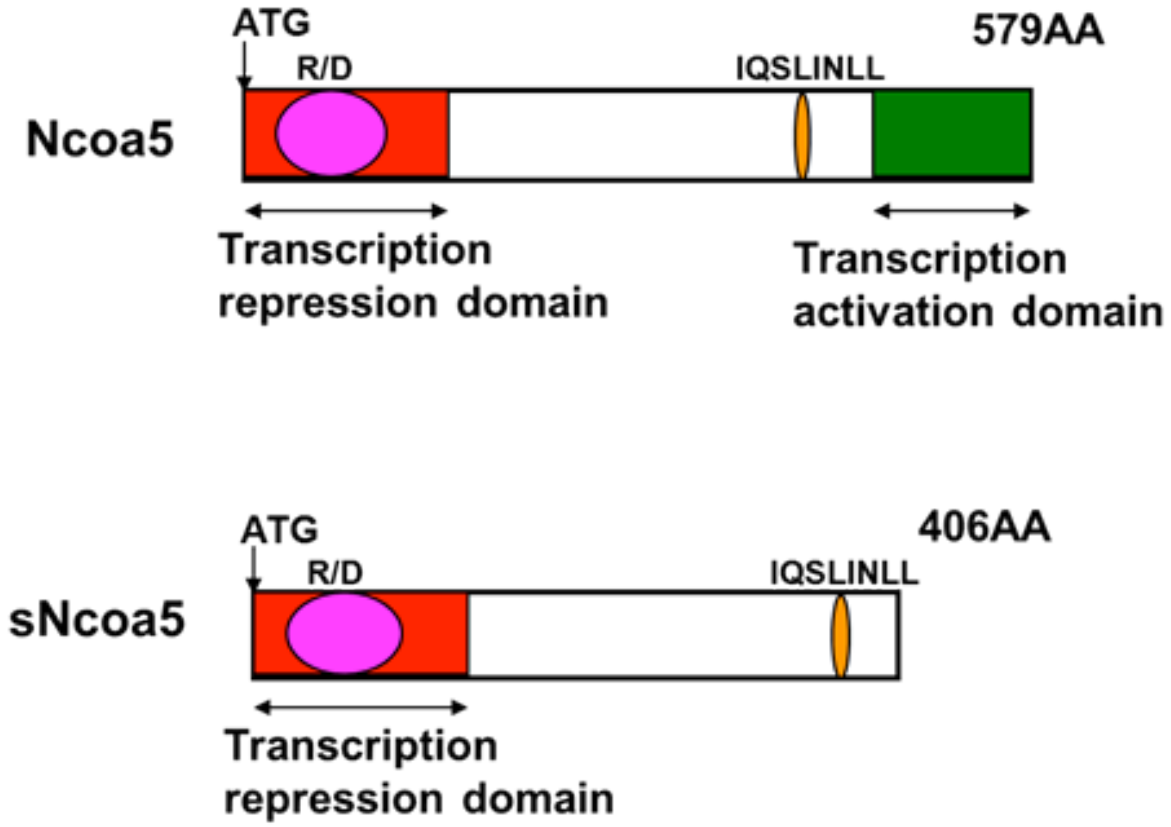


Figure 3.2 Schematic representations of different functional domains of Ncoa5 and sNcoa5

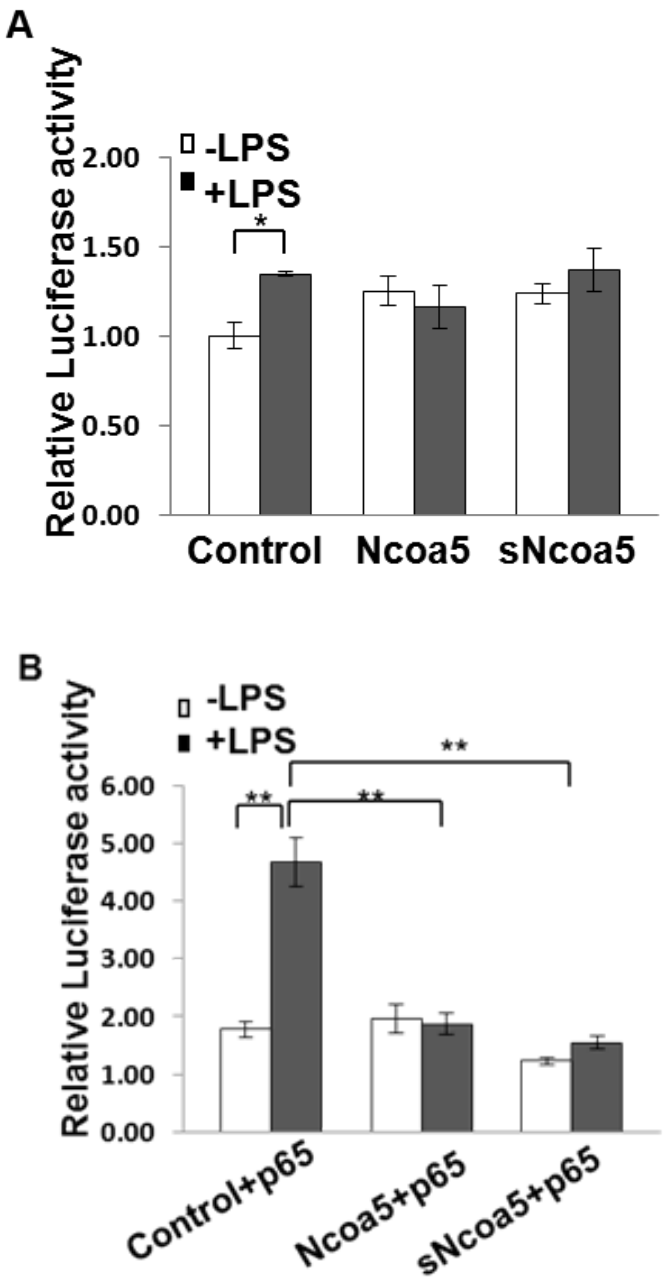
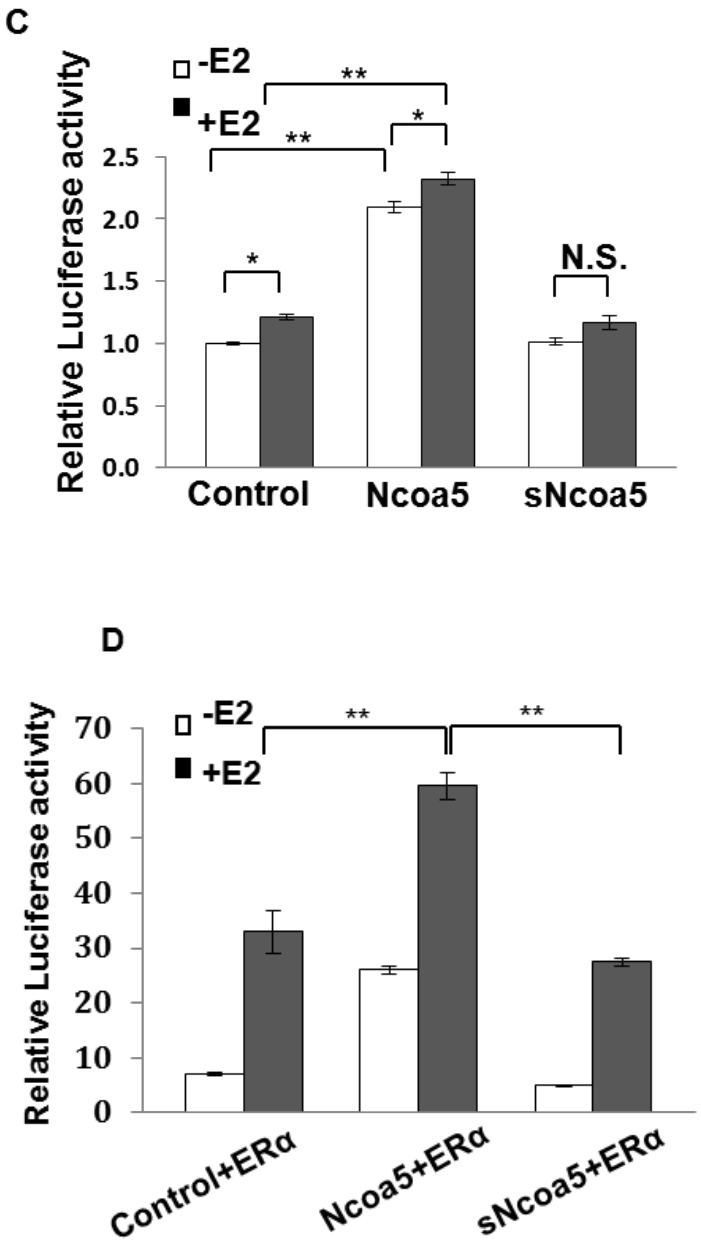


Figure 3.3 Luciferase reporter assay of murine IL-6 promoter using mouse macrophage RAW 264.7 cells and ERE promoter using human liver cancer cell PLC/PRF/5 cell line

Figure 3.3 Cont'd



(A and B) Relative luciferase activity of IL-6 promoter in RAW 264.7 cells transfected with control, Ncoa5 and sNcoa5 plasmids without (A) or with transfected p65 (B) before

and after LPS treatment. Experiments are repeated three times, values are mean ± SEM. (*$P \leq 0.05$, **$P \leq 0.01$)**.** (C and D) Relative luciferase activity of ERE promoter in PP5 cells transfected with control, Ncoa5 and sNcoa5 plasmids (C) or with ERα (D) before and after E2 treatment. Experiments are repeated three times, values are mean ± SEM (*$P \leq 0.05$, **$P \leq 0.01$, N.S. no significance)

3.5 Discussion

In the previous chapters, we have shown that Ncoa5 haplo-insufficiency leads to spontaneous development of early-onset glucose intolerance and late-onset HCC exclusively in male mouse. In this chapter, we extended our findings from the rodent model to human HCC and HCC with comorbid T2D. Through examining the expression levels of Ncoa5 in human HCC specimens, we have identified that reduced Ncoa5 expression was frequently found in the cancerous tissues of human HCC specimens compared to their adjacent non-cancerous tissues and normal human livers, which indicates that partial loss of Ncoa5 function may contribute to human HCC development. Interestingly, we also identified a novel alternatively-spliced Ncoa5 mRNA which encodes a short form of Ncoa5 (sNcoa5) in human HCC specimens. sNcoa5 was found increased in a proportion of HCC cancerous tissues compared to adjacent non-

cancerous livers. Moreover, sNcoa5 lacks the coactivation domain of Ncoa5 and failed to enhance ERα-mediated transcription in response to estrogen in a luciferase reporter assay. These findings suggest sNcoa5 as a good candidate to be examined for its role in human HCC development.

The early onset of glucose intolerance in *Ncoa5*$^{+/-}$ male mice raises a question of whether the Ncoa5 gene is a T2D susceptibility gene in humans. Noteworthy, the chromosomal region 20q13.1, where the Ncoa5 gene locates, has long been known to contain T2D susceptibility genes (Bento et al., 2008). Recently, analysis of candidate genes in this region in two European American case control populations revealed that Ncoa5, along with two other nearby genes were associated with T2D (Bento et al., 2008; Lewis et al., 2010). In addition, we have identified that two out of five patients with T2D displayed a 70-80% reduction of Ncoa5 expression in pancreatic islets relative to normal controls (Gunton et al 2005). Intriguingly, despite the statistical insignificance, 3 of 4 human HCC specimens with T2D analyzed in this study had a much lower Ncoa5 expression in the adjacent non-tumorous tissues compared to the normal liver tissues. Taken together, this implies a potential association of Ncoa5 deficiency with human T2D. Thus, it will be important for further studies to determine whether genetic mutations and/or reduced expression of Ncoa5 in liver and pancreas correlate with patients with T2D or both T2D and HCC.

APPENDIX

Supplemental Figure

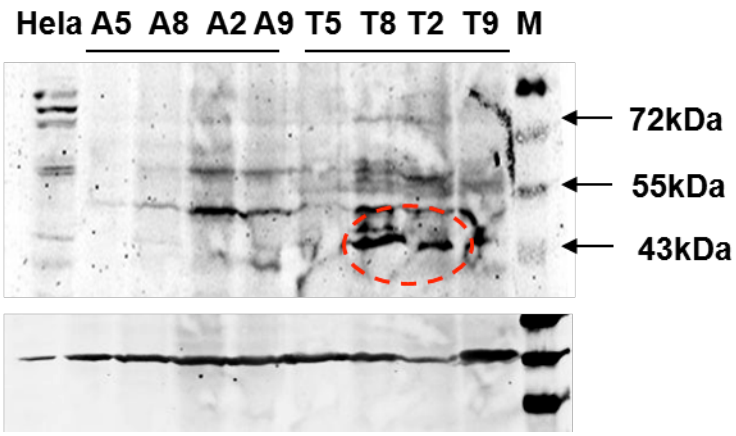


Figure S3.1 Increased sNcoa5 protein level is associated with human HCCs. Relative to Figure 3.1. Lysates made from four pairs of frozen human HCC and adjacent tissues were subjected to Western blotting with antibodies for Ncoa5 and β-actin as indicated. HeLa cell extracts serve as control. The dashed circle indicates sNcoa5 in two of the four HCC samples.

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CHAPTER 4 CONCLUSIONS AND PERSPECTIVES

In the previous chapters, we have shown that Ncoa5 haplo-insufficiency leads to early-onset glucose intolerance and subsequent the development of HCC exclusively in male mice, partly through increased IL-6 expression. We also demonstrated that decreased expression of Ncoa5 and increased expression of sNcoa5 are associated with human HCC development.

On the molecular level, we have demonstrated that Ncoa5 deficiency leads to the transcriptional activation of IL-6 in liver Kupffer cells. Increased IL-6 expression exerts its function on the hepatocyte through IL-6-STAT3 pathway and promotes hepatocytes growth. Loss of Ncoa5 in hepatocytes also results in increased AR and FASN expression, which contribute to the proliferation of hepatocytes. Moreover, IL-6-STAT3-SOCS3 pathway causes hepatic insulin resistance. Meanwhile, Ncoa5 deletion somehow also affects pancreatic β cell expansion and results in glucose intolerance (Figure 4.1).

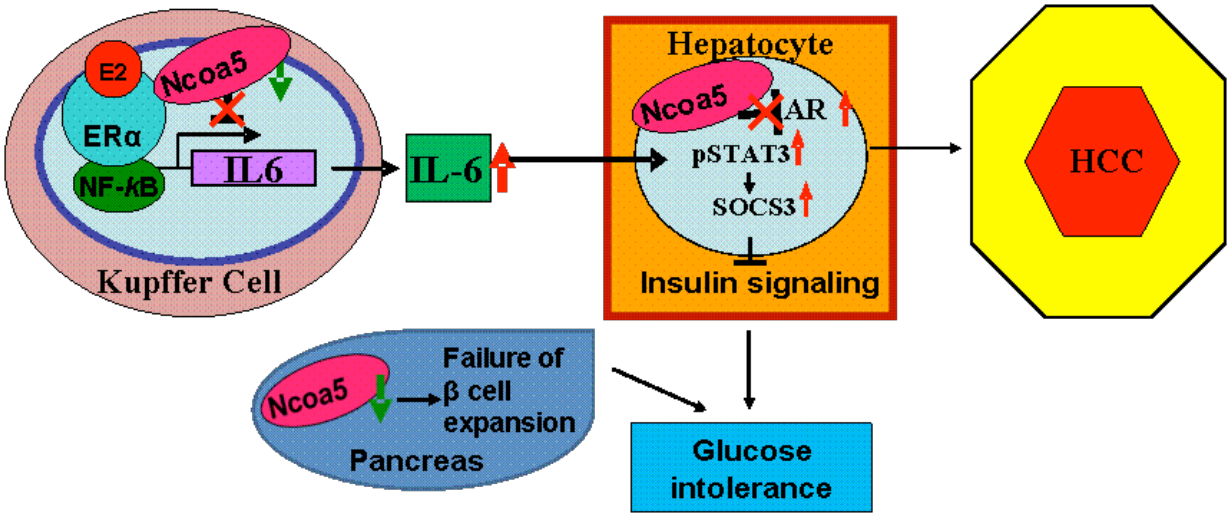

Figure 4.1 Loss of Ncoa5 results in glucose intolerance and HCC

4.1 The uniqueness of our *Ncoa5*$^{+/-}$ mouse model

Firstly, *Ncoa5*$^{+/-}$ mouse spontaneously develops glucose intolerance and HCC without the treatment of high fat diet or chemical carcinogen, which reflects better the development of the diseases. Secondly, *Ncoa5*$^{+/-}$ male mouse develops both early-onset glucose intolerance and subsequent HCC, which is rarely seen in the existing mouse models, provides the research field an ideal platform to study the molecular basis of HCC comorbid with glucose intolerance as the mice do not progress to severe T2D and will not die at younger ages. Thirdly, in regards of the debating about the role of IL-6 in insulin resistance, *Ncoa5*$^{+/-}$ male mouse is not obese and only shows

increased IL-6 in the liver but not in the circulating system, which adds evidence for the previous findings that increased hepatic IL-6 leads to insulin resistance (Johnson et al., 2012; Klover et al., 2005; Klover et al., 2003).

4.2 Ncoa5 haplo-insufficiency leads to early-onset glucose intolerance in male mice

We have shown that *Ncoa5*$^{+/-}$ male mice developed glucose intolerance at the age of 6 weeks with increased fasting glucose, impaired glucose tolerance and insulin tolerance. Insulin treatment experiments revealed that insulin signaling pathway is impaired in *Ncoa5*$^{+/-}$ male mouse liver. The phosphorylation of IR, IRS1 and Akt was reduced after insulin stimulation in *Ncoa5*$^{+/-}$ male mouse liver compared to wild type livers. Future studies aimed at examining the hepatic glucose production and the insulin signaling in peripheral tissues including skeletal muscle and adipose tissue by hyperinsulinemic-euglycemic clamp experiments will be helpful to further understand the global and systemic glucose metabolism in *Ncoa5*$^{+/-}$ mice.

In addition, we did not detect an increased serum insulin level in *Ncoa5*$^{+/-}$ male mice, which suggests a failure of pancreatic β cell compensation. Indeed, we found that the size and the number of β islets were reduced in *Ncoa5*$^{+/-}$ male mice. It would be interesting to know the mechanisms of how Ncoa5 affects β cell expansion and function.

The mechanism might due to the impaired function of ERα in pancreatic β islets. Previous studies have demonstrated the role of ERα in maintaining the normal function of β islets (Tiano et al., 2011). Increased FASN and lipid deposition have been shown to be detrimental to the function of β islets. Since we have detected increased expression of FASN in the liver, it is likely that FASN is also upregulated in the β islets and contributes to the malfunction of β islets. Future studies to examine FASN and lipid levels in β islets of *Ncoa5*$^{+/-}$ mice and wild type mice might be helpful to uncover the mechanisms of how Ncoa5 deficiency leads to the failure of β islets expansion.

4.3 Ncoa5 haplo-insufficiency leads to the spontaneous development of HCC exclusively in male mice

We have shown that 94% of *Ncoa5*$^{+/-}$ male mice in C57BL/6/129SVJ mixed genetic background developed HCC at the age of 10-18 months. Similar results were observed in Balb/C genetic background with an incidence of 74% *Ncoa5*$^{+/-}$ male mice developed HCC. HCC development is preceded by liver inflammation and steatosis. Since we have used Ncoa5 full knockout mice, future studies using conditional knockout of Ncoa5 in specific tissue such as hepatocyte or liver myeloid cells will be beneficial for identifying the distinct functions of Ncoa5 in different tissues.

4.4 Ncoa5 deficiency increased the transcription of IL-6 in Kupffer cells

Elevated hepatic IL-6 expression has been found in *Ncoa5*$^{+/-}$ male mouse liver by qRT-PCR. IHC revealed increased expression of IL-6 in mouse Kupffer cells. Consistently, increased phosphorylation of STAT3, which is the downstream targets of IL-6, has been detected in *Ncoa5*$^{+/-}$ male mouse liver as well as *Ncoa5*$^{+/-}$ male mouse liver tumors compared to wild type livers. Luciferase reporter assays showed Ncoa5 could repress IL-6 transcription in vitro. Moreover, ChIP analysis of IL-6 promoter in mouse macrophage cell line revealed that Ncoa5 was recruited to IL-6 promoter upon E2 treatment. Tissue ChIP analysis found that there were increased RNA polymerase II and phosphor Pol II in IL-6 promoter in *Ncoa5*$^{+/-}$ male mouse liver compared to wild type livers. In addition, knockdown of Ncoa5 in human monocyte/macrophage THP1 cells resulted in increased RNA levels of Ncoa5. Together, these findings suggested that Ncoa5 could regulate IL-6 transcription in macrophages. However, whether the regulation of Ncoa5 is through ERα is still unknown. Monitoring the HCC incidence of ovariectomized female *Ncoa5*$^{+/-}$ mice or *Ncoa5*$^{+/-}$ ERα$^{-/-}$ female mice will provide more information about whether Ncoa5 regulates IL-6 expression through ERα.

4.5 Heterozygous deletion of IL-6 deters HCC development in *Ncoa5*$^{+/-}$ male mice

Our results showed that heterozygous deletion of *IL-6* deters HCC development in *Ncoa5*$^{+/-}$ male mice, paralleled with a reduced Stat3 activation in tumors and adjacent liver tissues. This is consistent with previous reports that deletion of *IL-6* reduced incidence of tumors larger than 0.5 mm in diameter in mice treated with DEN (Naugler et al., 2007) and hepatocyte-specific STAT3-deficient mice treated with DEN developed fewer and smaller HCCs compared to wild type mice (He et al., 2010). These results clearly demonstrate that hepatic IL-6 overexpression per se, but not high serum IL-6 levels, contributes to HCC development. Meanwhile, crossing with IL-6 deficient mouse could not completely block HCC development in *Ncoa5*$^{+/-}$ male mouse, suggesting that in addition to IL-6, there are other factors such as AR, TNFα, FASN, etc. that also contribute to the HCC development in *Ncoa5*$^{+/-}$ male mouse and need further confirmation.

4.6 Decreased expression of Ncoa5 and increased expression of sNcoa5 is frequently associated with human HCC

Our results have shown that the decreased expression of Ncoa5 and increased expression of sNcoa5 is frequently associated with human HCCs, which suggests that Ncoa5 may function as a tumor suppressor in human HCC development. However, currently we have no information about what factors or signaling pathways cause decreased Ncoa5 expression in human HCCs. Methylation of Ncoa5 promoter in human HCCs by its upstream regulatory components might be an explanation. Experiments to

uncover the regulation of Ncoa5 gene expression will be very helpful to further understand how Ncoa5 functions and find therapeutic methods to combat HCC and T2D. It is also urgent to know the function of sNcoa5, whether it is loss of function or gain of function and how the alternative splicing of Ncoa5 is regulated.

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