

NCOA5 Haploinsufficiency Results in Glucose Intolerance and Subsequent Hepatocellular Carcinoma

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SUMMARY

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 interleukin-6 (IL-6) expression, early-onset glucose intolerance, and progressive steatosis and dysplasia in livers. Blocking IL-6 overexpression averts glucose intolerance and partially deters HCC development. Moreover, reduced NCOA5 expression is associated with a fraction of human HCCs and HCCs with comorbid T2D. These findings suggest that NCOA5 is a haploinsufficient tumor suppressor and that NCOA5 deficiency increases susceptibility to both glucose intolerance and HCC, partially by increasing IL-6 expression. Thus, our findings open additional avenues for developing therapeutic approaches to combat these diseases.

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 in women. The risk factors for 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, type 2 diabetes (T2D) may become an even more important risk factor for HCC in the future (McGlynn and London, 2011). However, the 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 interleukin-6 (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

Significance

The association between T2D and 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 show that NCOA5 haploinsufficiency activates a pathogenic pathway concomitantly leading to impaired glucose tolerance and HCC development in mice. Reduced NCOA5 expression is observed in a substantial fraction of human HCCs and HCCs with comorbid T2D. These results reveal NCOA5 haploinsufficiency as a genetic link between T2D and HCC. Moreover, our *Ncoa5*^{+/-} mouse model of glucose intolerance with comorbid HCC provides a valuable platform for studying the molecular basis and therapeutic responsiveness of HCC with comorbid T2D.

(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., 2003, 2005; 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., 2003, 2005; 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; Tian et al., 2011). It is well documented that estrogen and estrogen receptor α (ER α) 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. ER α 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 ER α -mediated transcription (Jiang et al., 2004; Sauvé et al., 2001). Recent linkage analysis revealed that NCOA5, along with two nearby genes in the 20q13.1 region, is 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.

RESULTS

NCOA5 Haploinsufficiency 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 S1A and S1B available online). *Ncoa5* expression was detected in all mouse tissues examined, but with variable levels that were lowest in liver (Sauvé et al., 2001). *Ncoa5*^{+/-} mice were found to have a ~50% decrease in NCOA5 expression within the liver (Figures S1C and S1D). *Ncoa5*^{+/-} mice appeared indistinguishable from their wild-type littermates at the age of 8 weeks and had a body weight and liver to body weight ratio similar to the *Ncoa5*^{+/+} male mice (Figures S1E and S1F) at ages 2, 6, and 10 months. However, *Ncoa5*^{+/-} male mice suffered from a severe fertility defect, whereas *Ncoa5*^{+/-} female mice were fertile (S.G., F.C., G. Perez, and H.X., 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 necropsy 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 1A–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 (Figure S1G). Histological analysis revealed that tumors were well to moderately differentiated HCCs, often with a more than two-cell-thick trabecular (Figures 1D–1F) or pseudoglandular pattern (Figure 1G), occasionally with lung metastasis (Figures 1H and 1I) and necrosis (Figure 1J). Tumor cells had morphological resemblance to hepatocytes; however, they displayed nuclear pleomorphism, some with prominent nucleoli and vacuolation (Figures 1F and 1K). Some of the tumor cells were α -fetal protein (AFP) or Ep-CAM positive (Figure S1H). NCOA5 expression was detectable using western blot analysis (Figure S1I) 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 haploinsufficient to suppress HCC development in male mice.

NCOA5 Haploinsufficiency 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 2A–2C). Similar results were obtained in *Ncoa5*^{+/-} mice in a Balb/c genetic background (Figures S2A–S2C). Elevated fasting blood glucose levels and glucose intolerance were continuously present in *Ncoa5*^{+/-} male mice at the age of 20 weeks (Figures S2D–S2F). Interestingly, these differences were not observed between *Ncoa5*^{+/-} and *Ncoa5*^{+/+} female mice (Figure 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 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 and following an intraperitoneal glucose load, was detected between these two groups of mice (Figure 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 2G–2I). Thus, NCOA5 haploinsufficiency 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.

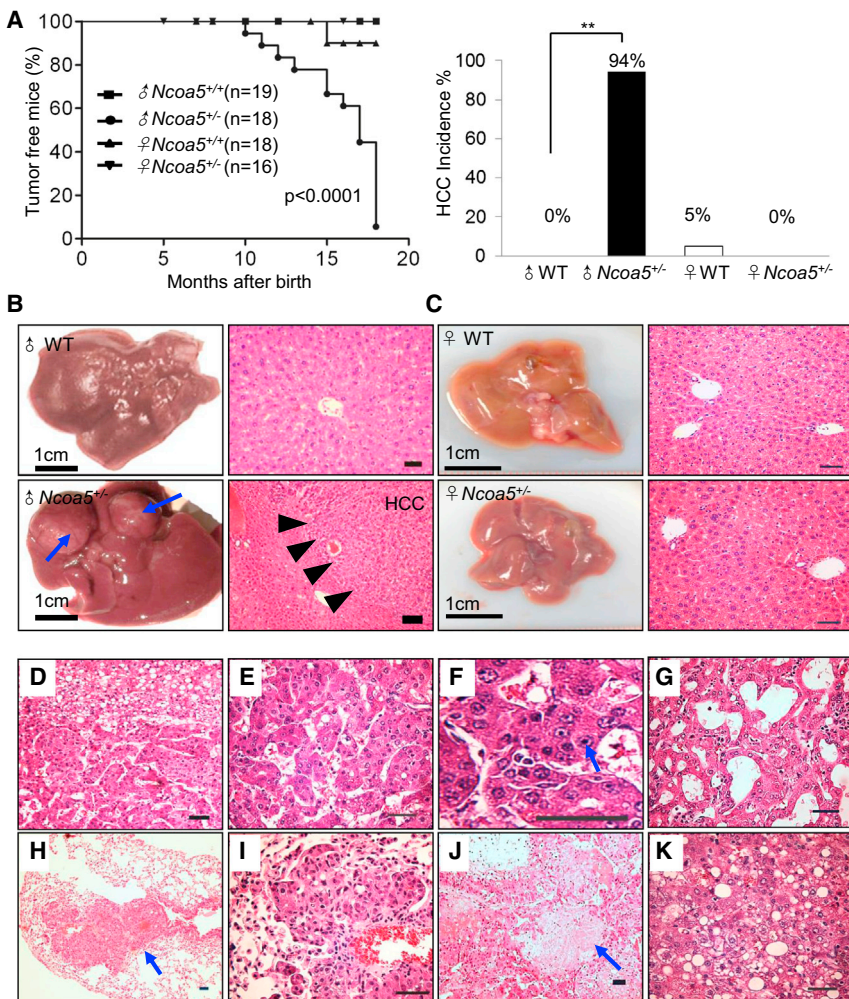


Figure 1. Spontaneous HCC Development in *Ncoa5*^{+/-} Male Mice

(A) Kaplan-Meier curves showing tumor-free survival of wild-type (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 ≤ 0.01).

(B and C) Representative macroscopic appearance of livers and hematoxylin and eosin (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 at 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 at 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.

Scale bars represent 50 μm. See also Figure S1.

***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 the ages of 6 or 10 months, but not the livers of age-matched *Ncoa5*^{+/+} male (Figure 3A) and *Ncoa5*^{+/-} female mice (data not shown), displayed characteristic features of hepatic dysplasia and steatosis such as architectural disorganization, cytological atypia, enlarged nucleus, vacuolated hepatocytes, and increased lipid deposition as revealed by oil red O staining (Figure 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 S3A–S3C). 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 3B). Masson's trichrome staining showed fibrosis with connective tissue fibers in the periportal and periductular areas in livers of 10-month-old *Ncoa5*^{+/-} male mice (Figure 3B). In parallel with these morphologic changes, serum levels of alanine aminotransferase (ALT) and 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 3C and 3D). Moreover, TUNEL assays detected more cell death in the livers of *Ncoa5*^{+/-} male mice (Figure 3E), while PCNA staining revealed more proliferation in the livers and liver tumors of *Ncoa5*^{+/-} male mice (Figure 3F). These results suggest that NCOA5 haploinsufficiency causes development of hepatic inflammation, steatosis, and dysplasia prior to HCC development in male mice.

NCOA5 Deficiency Increased the Transcription of *Il-6* by Enhancing RNA Polymerase II Assembly on the *Il-6* Promoter

Proinflammatory 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 tumor necrosis factor α (TNF- α) in *Ncoa5*^{+/-} and *Ncoa5*^{+/+} mice. The mRNA levels of *Il-6* and *Tnfa* in the livers were significantly increased in *Ncoa5*^{+/-} male mice at the age of 8 and 24 weeks compared to wild-type controls (Figures 4B and S4A), whereas the serum IL-6 levels were not significantly changed (Figure 4A), indicating that NCOA5 exerts its regulation of *Il-6* expression in the liver but does not affect the serum level of IL-6. We next asked which

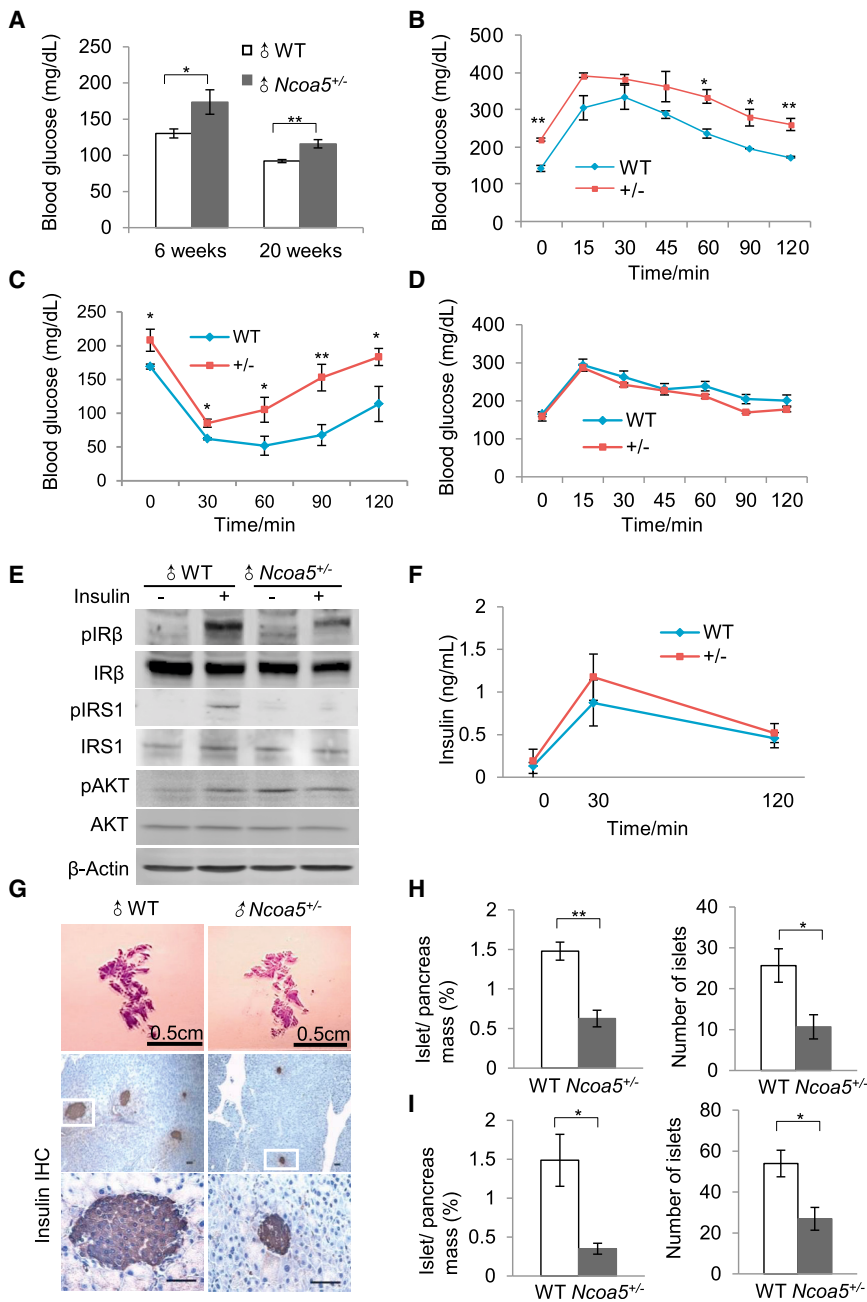


Figure 2. Glucose Sensitivity in *Ncoa5*^{+/-} Male Mice at the Age of 6 Weeks

(A) Blood glucose levels of fasted 6-week-old and 20-week-old male mice with the indicated genotypes (n = 7).

(B) GTT of 6-week-old male mice with the indicated genotypes (n = 3). Blood glucose levels were determined at the indicated times.

(C) ITT of 6-week-old male mice with the indicated genotypes (n = 4). Blood glucose levels of male mice with the indicated genotypes at the indicated times. Note: similar changes in blood glucose levels and GTT were observed in *Ncoa5*^{+/-} male mice in a Balb/c genetic background (Figures S2B–S2D).

(D) GTT of 6-week-old female mice with the indicated genotypes (n = 4; p > 0.05).

(E) 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-phosphotyrosine antibody. Results represent two independent experiments with different pairs of littermates.

(F) Serum insulin concentrations were determined at the 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 at 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 ten 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 < 0.05; **p < 0.01. See also Figure S2.

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 nonparenchymal cells in livers (Figure 4C). The number of positively stained IL-6 cells is significantly increased in *Ncoa5*^{+/-} male livers compared to *Ncoa5*^{+/+} male livers (Figure 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 4E, 4F, and S4B). These results indi-

cate increased activation of Kupffer cells by NCOA5 haploinsufficiency. Strikingly, IL-6 and TNF- α expression in hepatocytes was not apparently changed in *Ncoa5*^{+/-} male mice (Figures 4C and S4B). In agreement with the effects of IL-6 on STAT3 and its canonical target SOCS3 (Senn et al., 2003; Yu et al., 2007), phospho-STAT3 (Tyr705) protein levels and *Socs3* mRNA levels were significantly increased in livers of *Ncoa5*^{+/-} male mice compared with livers of wild-type male mice (Figures 4G and 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 4G). Moreover, we also demonstrated that knockdown of NCOA5 resulted in an increased IL-6 expression in human monocyte/macrophage THP1 cells (Figures S4C and S4D). These results suggest that NCOA5 haploinsufficiency enhances expression of IL-6 and TNF- α in Kupffer cells, which in turn activates STAT3-SOCS3 signaling.

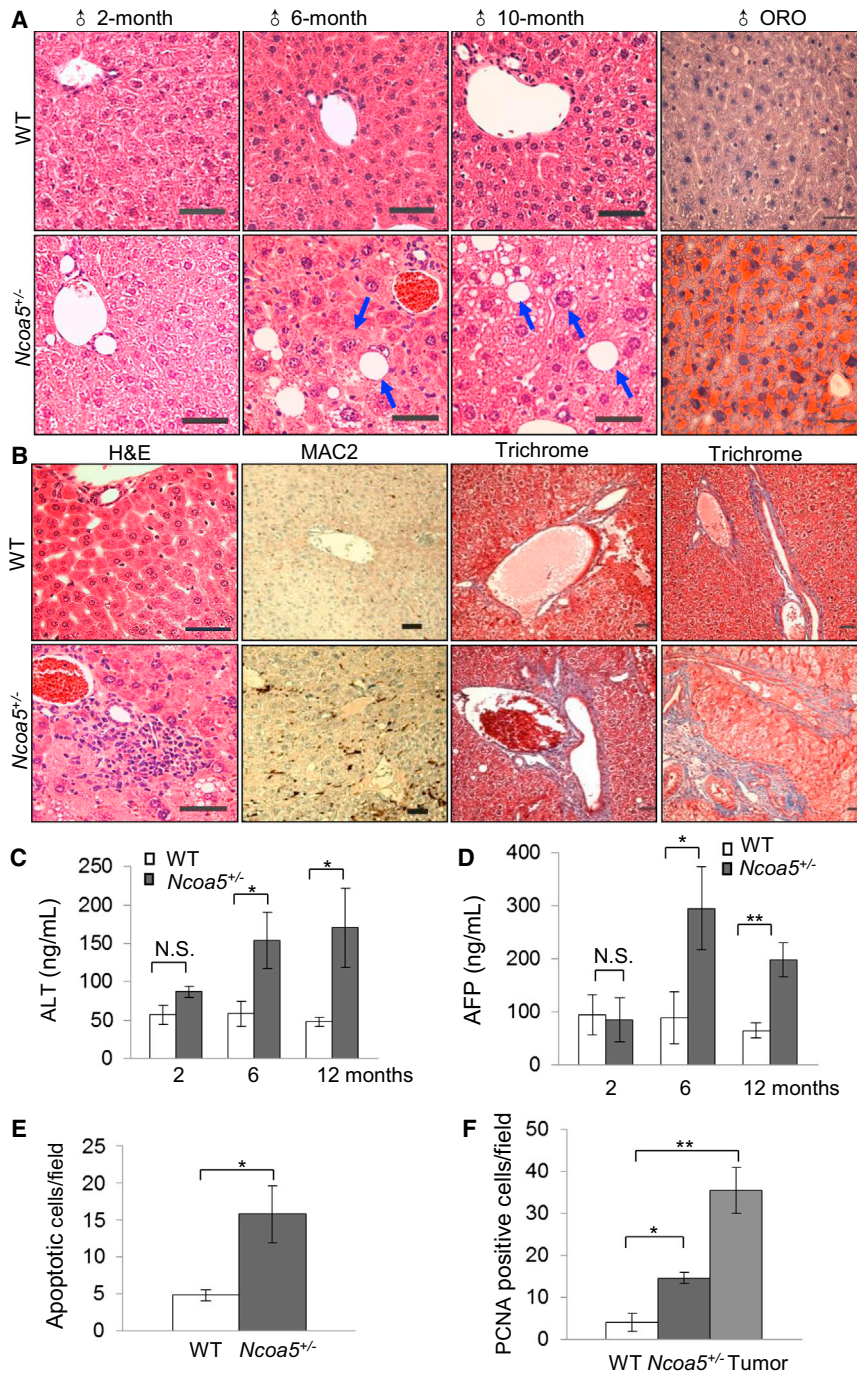


Figure 3. Hepatic Histology and Markers of Liver Function in *Ncoa5*^{+/-} Male Mice Compared to WT Male Mice

(A) Representative H&E-stained liver sections from male mice with the indicated genotypes and ages. Arrows indicate vacuolated or enlarged hepatocytes. A representative oil red O-stained section shows severe accumulation of lipids in the liver of *Ncoa5*^{+/-} male mice at the age of 10 months (bars represent 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 the indicated genotypes (bars represent 50 μm).

(C) Serum levels of ALT of male mice with the indicated ages and genotypes (n = 4–8; *p ≤ 0.05; N.S., no significance). Values are mean ± SEM.

(D) Serum levels of AFP of male mice with the indicated ages and genotypes (n = 4–5; *p ≤ 0.05; **p ≤ 0.01; N.S., no significance). Values are mean ± SEM.

(E) The bar graph shows mean ± SEM of TUNEL-positive cells in the liver sections of 18-month-old WT and *Ncoa5*^{+/-} male mice (n = 3; *p ≤ 0.05).

(F) The bar graph 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 ≤ 0.05). See also Figure S3.

Ligand-bound ER α represses NF- κ B-mediated transcriptional activation of the *IL-6* gene in macrophages through direct interaction with NF- κ B, which binds to the *IL-6* promoter responsive elements (Liebermann and Baltimore, 1990; Naugler et al., 2007; Ray et al., 1994; Stein and Yang, 1995). Since NCOA5 is a coactivator for ER α , we performed quantitative chromatin immunoprecipitation (qChIP) and luciferase reporter assays to examine the molecular mechanism by which NCOA5 regulates *IL-6* expression. qChIP analysis of a cultured mouse macrophage cell line, RAW264.7, indicated that NCOA5 assembly on the

IL-6 promoter (Figure 4I) was increased upon estrogen stimulation, suggesting that NCOA5, along with ER α , 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 assays of the mouse *IL-6* promoter revealed that NCOA5 could repress lipopolysaccharide (LPS)-induced *IL-6* transcription (Figure 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 4K), whereas the assembly of ER α on the promoter was not changed in *Ncoa5*^{+/-} livers. These data indicate that NCOA5 acts as a negative coregulator of *IL-6* transcription in vivo and NCOA5 haploinsufficiency increases *IL-6* expression through enhancing recruitment of RNA Pol II to the *IL-6* promoter.

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

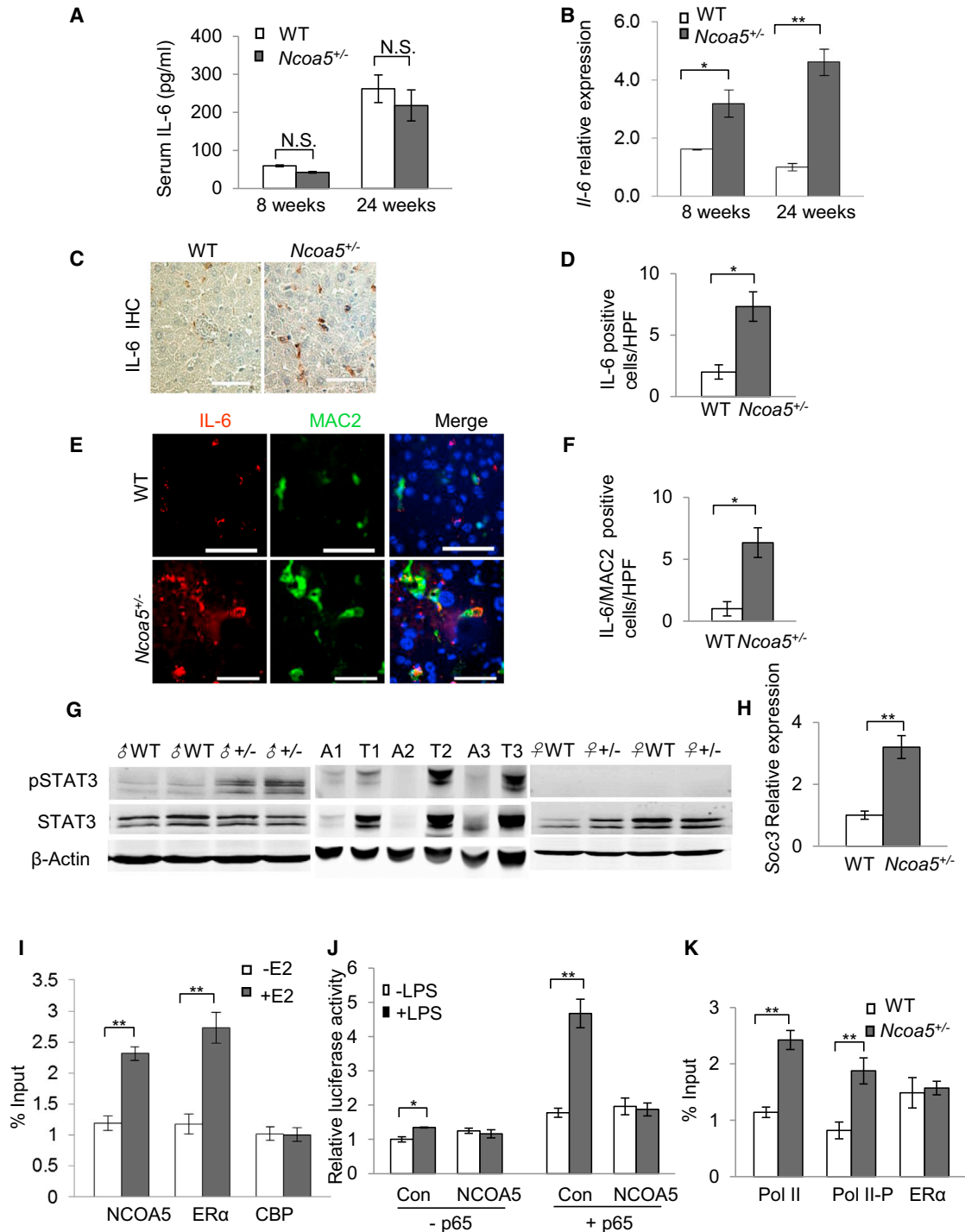


Figure 4. Regulation of *Il-6* Expression in *Ncoa5*^{+/-} Male Mice Compared to WT Male Mice

(A) The bar graph showing serum IL-6 concentration in WT and *Ncoa5*^{+/-} male mice (n = 3) with the indicated ages. Values are mean ± SEM; N.S., no significance. (B) Quantitative RT-PCR (qRT-PCR) of *Il-6* mRNA levels in WT and *Ncoa5*^{+/-} mouse livers with the 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 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, 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.

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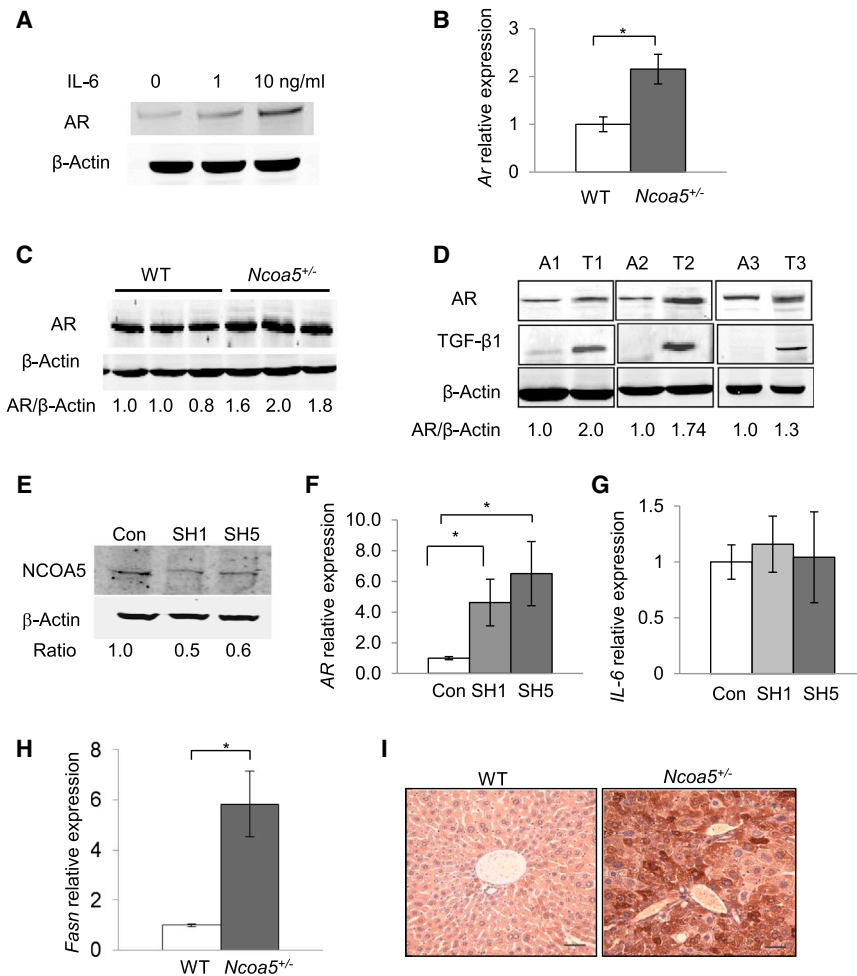


Figure 5. Effects of NCOA5 Deficiency on AR Expression in Mouse Livers and Cultured Human HCC Cell Lines

(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 IL-6, 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 \pm 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) Western blot analysis of AR and TGF- β 1 protein levels in three pairs of liver tumors versus their adjacent nontumorous tissues. The ratios of AR/ β -actin are listed.

(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 against NCOA5 and β -actin (E). The bar graphs show quantitative RT-PCR analysis of AR (F) and IL-6 (G) mRNA levels in indicated cells. Experiments were repeated two times. Data represent mean \pm SD of triplicates from a representative experiment (*p \leq 0.05).

(H) qRT-PCR of *Fasn* mRNA levels in 10-month-old WT and *Ncoa5*^{-/-} male mouse livers (n = 4). Values are mean \pm SEM; *p \leq 0.05.

(I) A representative IHC-stained section of FAS in livers from 10-month-old WT and *Ncoa5*^{-/-} male mice.

See also Figure S5.

androgen-dependent and androgen-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. Initially, we observed that recombinant IL-6 was able to increase AR expression in HCC HepG2 cells in vitro (Figure 5A). Next, we found that levels of *Ar* mRNA and protein were significantly increased in *Ncoa5*^{-/-} livers compared to livers of *Ncoa5*^{+/+} littermates (Figures 5B and 5C). Moreover, the protein levels of AR and an AR downstream target, TGF- β 1, were significantly elevated in HCCs arising in *Ncoa5*^{-/-} mice compared with their adjacent nontumorous liver tissues (Figure 5D). Interestingly, knockdown of

NCOA5 without IL-6 treatment also increased the mRNA level of AR, but not the level of IL-6 mRNA, in cultured human HCC PLC/PRF/5 cells (Figures 5E–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- κ B, androgen, and insulin pathways whose expression might be altered in *Ncoa5*^{-/-} livers (Figure S5). Notably, fatty acid synthase (*Fas/Fasn*) mRNA was about 6-fold higher in *Ncoa5*^{-/-} male mouse livers than in *Ncoa5*^{+/+} control mice (Figure 5H). IHC staining for FAS protein confirmed that FAS expression was increased in *Ncoa5*^{-/-} male mouse livers

(H) qRT-PCR of *Socs3* mRNA of 10-month-old WT and *Ncoa5*^{-/-} male mouse livers (n = 3). Values are mean \pm SEM; **p \leq 0.01.

(I) qChIP assay for NCOA5 and ER α binding on *Il-6* promoter in cultured mouse RAW 264.7 cells in the absence and presence of E2. Antibodies for NCOA5, ER α , and coactivator CBP were used to precipitate DNA-protein complexes. qRT-PCR analysis of precipitated *Il-6* promoter DNA was performed to quantify the recruitment of indicated proteins on the *Il-6* promoter. Fold enrichment of qChIP sample relative to input sample is shown. Experiments were repeated three times. Values are mean \pm SEM; **p \leq 0.01.

(J) The bar graph shows relative luciferase activity of the *Il-6* promoter in RAW 264.7 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 \pm SEM; *p \leq 0.05; **p \leq 0.01.

(K) qChIP analysis of RNA Pol II assembly on the *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 ER α 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 mean \pm SD; **p \leq 0.01. See also Figure S4.

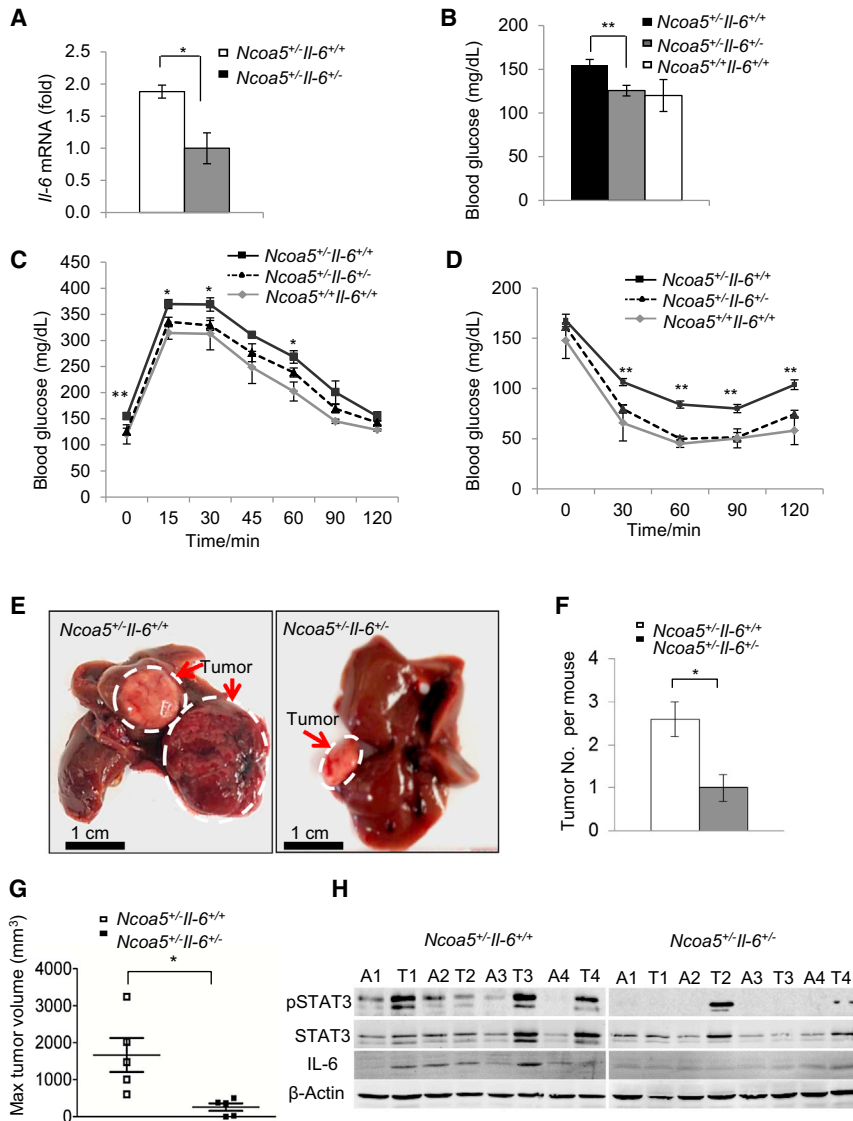


Figure 6. Effects of Heterozygous Deletion of *Il-6* on the Onset of Glucose Intolerance and HCC Development in *Ncoa5*^{+/-} Male Mice

(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-hr-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 and 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 nontumorous liver tissues (A) in 18-month-old *Ncoa5*^{+/-}*Il-6*^{+/-} and *Ncoa5*^{+/-}*Il-6*^{+/-} male mice. β-actin serves as a loading control.

relative to wild-type control livers (Figure 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.

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 ~50% compared to livers of *Ncoa5*^{+/-}*Il-6*^{+/-} male littermates (Figure 6A). Notably, heterozy-

gous *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.G., F.C., G. Perez, and H.X., 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 6B–6D). Improved fasting blood glucose levels and GTTs were also observed in *Ncoa5*^{+/-}*Il-6*^{+/-} Balb/c mice (Figures S2B and S2C). 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 6E–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 nontumorous liver tissues in *Ncoa5*^{+/-}*Il-6*^{+/-} male mice compared with those in *Ncoa5*^{+/-}*Il-6*^{+/-} male mice (Figure 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.

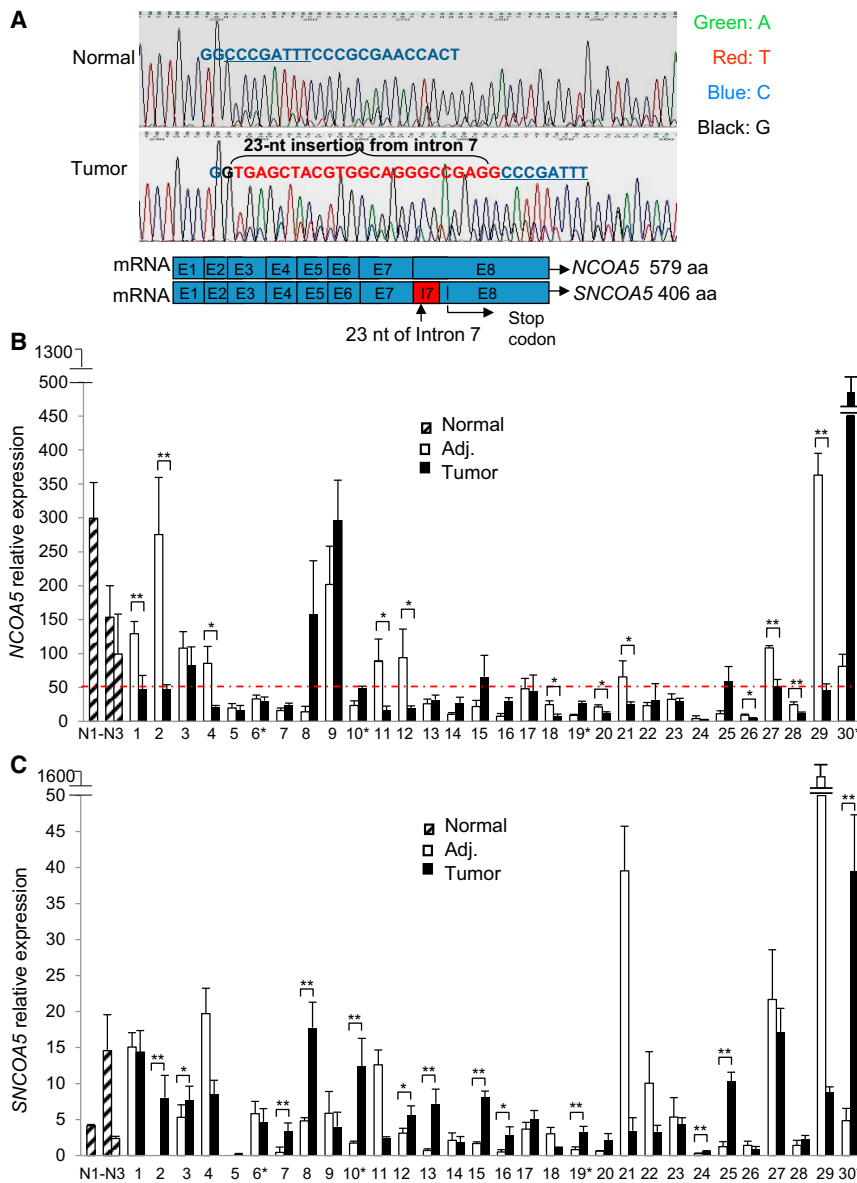


Figure 7. Expression of NCOA5 and SNCOA5 in Human HCC Specimens

(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 adjacent noncancerous 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, and 30 have T2D and are marked with an asterisk (*). Values are mean ± SD; *p ≤ 0.05; **p ≤ 0.01.

See also Figure S6.

mRNA levels of NCOA5 and SNCOA5 in 30 pairs of frozen HCC and adjacent noncancerous tissue specimens (four pairs are from diabetic patients) with quantitative RT-PCR analysis. We detected a statistically significant reduction in NCOA5 expression in 40% (12/30) of HCC specimens when comparing NCOA5 mRNA levels in HCC versus adjacent noncancerous liver (Figure 7B). In contrast, SNCOA5 expression was significantly increased in 43% (13/30) of HCC specimens compared with their adjacent noncancerous tissues (Figure 7C). Western blot analysis confirmed that the protein level of SNCOA5 was significantly increased in two of four tested HCC specimens (Figure S6E). This inverse correlation between low NCOA5 expression and high SNCOA5 expression in human HCC specimens indicates that NCOA5 deficiency may

Decreased Expression of NCOA5 and Overexpression of the Alternatively Spliced Form of NCOA5 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 noncancerous tissue samples from male patients as well as a pooled mRNA sample from five normal male human livers. We identified an alternatively spliced form of NCOA5 mRNA in all of the samples, which encodes a shortened NCOA5 (SNCOA5). It contains 406 amino acids due to a frame-shifting insertion caused by an extended exon 7, containing the first 23 nucleotides of intron 7 (Figure 7A). SNCOA5 is unlikely to have a transcriptional activation function as it lacks the transcriptional activation domain at the carboxyl terminus of NCOA5 and fails to enhance ERα-mediated transcriptional activation in luciferase reporter assays (Figures S6A–S6D). Next, we examined the

contribute to human HCC development. It is noteworthy that at least 63% (19/30) of HCC specimens showed over 50% reduction in the NCOA5 mRNA level in adjacent noncancerous tissues when compared to normal human liver tissue controls (Figure 7B); among them, three of four specimens from diabetic patients showed remarkable reduction in the NCOA5 mRNA level. In addition, we have examined the microarray data of pancreatic islets from seven normal and five T2D patients reported by Kahn and colleagues (Gunton et al., 2005), which is available at the website of the Diabetes Genome Anatomy Project (<http://www.diabetesgenome.org>). We 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). Taken together, our results imply a potential association of reduced NCOA5 expression with human T2D.

DISCUSSION

Here, we describe that NCOA5 plays a critical role in suppressing the development of glucose intolerance, a prediabetic status, and HCC in mice, in part by regulating IL-6 expression in a male-gender-specific fashion. Moreover, we show that reduced NCOA5 expression is associated with a significant portion of human specimens of HCCs and HCCs with concomitant T2D. Taken together, our results suggest that NCOA5 deficiency is 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 a chemical carcinogen (DEN) in 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 ER α that assembles on the *Il-6* promoter through interaction with NF- κ B (Liebermann 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 that NCOA5 is 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). Although increased IL-6 expression was detected predominantly in Kupffer cells in *Ncoa5*^{+/-} male livers, our results do not exclude the possibility that NCOA5 haploinsufficiency also increases autocrine IL-6 production by hepatocellular carcinoma progenitor cells described recently (He et al., 2013). Importantly, reduced NCOA5 expression and increased SNCOA5 were found frequently in the cancerous tissues of human HCC specimens compared to their adjacent noncancerous tissues, indicating that partial loss of NCOA5 function may contribute to human HCC development. The SNCOA5 apparently lacks the coactivation domain of NCOA5 and evidently failed to enhance ER α -mediated transcription in response to estrogen in a luciferase reporter assay, suggesting that SNCOA5 is a good candidate to be examined for its possible promoting role in human HCC development. Thus, our results not only 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 results suggest a critical role of increased hepatic IL-6-STAT3 signaling in hepatocarcinogenesis, which 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 that hepatocyte-specific STAT3-deficient mice treated with DEN developed fewer and smaller HCCs compared to wild-type mice (He et al., 2010). Our results, nevertheless, do 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, we observed that two of three *Ncoa5*^{+/-}*Il-6*^{-/-} male mice did develop three small HCCs (≤ 108 mm³) each at the age of

18 months, 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 haploinsufficiency resulted in aberrant expression of other genes, including *Tnfa*, *Fasn*, 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 ER α -targeted genes via a direct interaction with ER α (Jiang et al., 2004), it is possible that NCOA5 may regulate other ER α -targeting genes in Kupffer cells as well as in hepatocytes. Alternatively, NCOA5 may regulate genes independent of ER α in glucose homeostasis and HCC development, as our current evidence does not prove that the action of NCOA5 is dependent on ER α . Indeed, NCOA5 is able to form a complex with SAM68, hnRNP-G, and the transcription factors ZAP3, ILF2, and ILF3 (Ulke-Lemée 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 coregulator that concomitantly controls the expression of a set of genes in 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 ER α . 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 NCOA5 haploinsufficiency 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- κ B in the liver had elevated serum levels of IL-6 and TNF- α 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, suggest that 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 insulin sensitivity. Notably, neither the serum IL-6 level nor 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 will be a priority, including experiments to measure hepatic pancreatic β cell function and hyperinsulinemic-euglycemic clamp assays to assess hepatic glucose production and insulin sensitivity in adipocytes and muscles in *Ncoa5*^{+/-} and wild-type mice.

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, was 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, three of four human HCC specimens with T2D analyzed in this study had a much lower NCOA5 expression in the adjacent nontumorous 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 with both T2D and HCC.

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. Thus, our findings have tremendous potential impact on the understanding of disease etiology and development of therapeutic strategies for both T2D and HCC

EXPERIMENTAL PROCEDURES

Generation of *Ncoa5*^{+/-} and *Ncoa5*^{+/-}*Il-6*^{+/-} Mice

Details regarding the construction of a *Ncoa5* targeting vector and the generation of a *Ncoa5* knockout mouse on a mixed 129 \times C57BL/6 or Balb/c genetic background are provided in Supplemental Experimental Procedures. 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. To generate *Ncoa5*^{+/-}*Il-6*^{+/-} mice, *Il-6*^{-/-} C57BL/6 or Balb/c male mice were mated with *Ncoa5*^{+/-} female mice of mixed 129 \times 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 \times 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 and conducted in accordance with institutional and national guidelines.

Human Tissue Samples

Total mRNAs from a pooled sample of five normal male human autopsy liver tissues (N1) and a pair of HCC and adjacent tissues from a male patient were purchased from Biochain. Twenty-nine pairs of frozen human HCC and noncancerous adjacent liver tissues from patients (25 males and 4 females) with HCC and two frozen human liver tissues (N2 and N3) from female patients with hepatic hemangioma or gallstones were collected from consenting patients after hepatectomy 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 and the Biomedical and Health Institutional Review Board of Michigan State University.

Statistical Analysis

The differences between groups were analyzed using a Student's two-tailed *t* test. Survival curves were compared using a log-rank (Mantel-Cox) test. Tumor incidences were compared using a χ^2 test. Values are expressed as mean \pm SEM or SD; *p* \leq 0.05 is considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures and six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.ccr.2013.11.005>.

AUTHOR CONTRIBUTIONS

S.G. conducted most of the experiments in this study. A.L., F.L., and S.G. analyzed gene expression in human tissue samples. A.L. and F.C. performed IHC and IF analyses. C.Z., M.W., and Z.K. performed mouse breeding and 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.

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