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. Blockading 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.
NCOA5 haploinsufficiency results in the onset of glucose intolerance in 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 intra-peritoneal 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.

**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 nucleioli 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 intra-peritoneal 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.
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 perportal 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
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 indicate 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.
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). Since NCOA5 is a coactivator for ERα, 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 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 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+/C0 livers when compared with Ncoa5+/+ livers (Figure 4K), whereas the assembly of ERα on the promoter was not changed in Ncoa5+/C0 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.
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+/− liver derived from 5-month-old male mice (n = 4). Values are mean ± SEM; *p < 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-β1 (Figure S5) 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 ± SD of triplicates from a representative experiment (*p < 0.05).

(H) qRT-PCR of Fasn mRNA levels in 10-month-old WT and Ncoa5+/− male mouse livers (n = 4). Values are mean ± SEM; *p ≤ 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-β1, 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-κB, 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

See also Figure S5.
Ncoa5+/-Il-6+/+

Menendez et al., 2009; Nagasue et al., 1992; Postic and Girard, found to contribute to hepatocarcinogenesis and hepatic insulin resistance. IL-6 relative to wild-type control livers (Figure 5I). FAS was previously shown to play a key role in the development of T2D and HCC in Ncoa5+/+ male mice. We generated mice bearing dual heterozygous deletions of Ncoa5 and Il-6 genes by crossing Ncoa5+/- male mice with Il-6+/- (B6.129S6-Il-6tm1Kopf) mice. The level of Il-6 mRNA in livers of Ncoa5+/+Il-6+/- male mice was decreased by ~50% compared to livers of Ncoa5+/-Il-6+/- male littersmates (Figure 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.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+/+ littermates at the age of 6 weeks (Figures 6B–6D). Improved fasting blood glucose levels and GTTs were also observed in Ncoa5+/-Il-6+/+ male mice (n = 5 per group). Values are mean ± SEM; *p < 0.05; **p < 0.01.

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 Ncoa5+/-Il-6+/- littermates, Ncoa5+/-Il-6+/- male mice exhibited a significant improvement in fasting blood glucose levels, GTTs, and ITTs compared with their Ncoa5+/-Il-6+/+ littermates at the age of 6 weeks (Figures 6B–6D). Improved fasting blood glucose levels and GTTs were also observed in Ncoa5+/-Il-6+/+ male mice (n = 5 per group). Values are mean ± SEM; *p < 0.05. Moreover, heterozygous Il-6 deletion did not block tumor initiation, as Ncoa5+/-Il-6+/- male mice exhibited a significant improvement in fasting blood glucose levels, GTTs, and ITTs compared with their Ncoa5+/-Il-6+/+ littermates at the age of 6 weeks (Figures 6B–6D). Improved fasting blood glucose levels and GTTs were also observed in Ncoa5+/-Il-6+/+ male mice (n = 5 per group). Values are mean ± SEM; *p < 0.05.

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-6tm1Kopf) 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 littersmates (Figure 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.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+/+ littermates at the age of 6 weeks (Figures 6B–6D). Improved fasting blood glucose levels and GTTs were also observed in Ncoa5+/-Il-6+/+ male mice (n = 5 per group). Values are mean ± SEM; *p < 0.05. Moreover, heterozygous Il-6 deletion did not block tumor initiation, as Ncoa5+/-Il-6+/- male mice exhibited a significant improvement in fasting blood glucose levels, GTTs, and ITTs compared with their Ncoa5+/-Il-6+/+ littermates at the age of 6 weeks (Figures 6B–6D). Improved fasting blood glucose levels and GTTs were also observed in Ncoa5+/-Il-6+/+ male mice (n = 5 per group). Values are mean ± SEM; *p < 0.05.
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 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 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 pathogenetic 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 childhood obesity (Naugler et al., 2007), but also point to evidence that Kupffer-cell-derived IL-6 contributes to the development of T2D and HCC (Kaira 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 the transcription factors ZAP3, ILF2, and ILF3 (Ulle-Lermée 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 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 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-κB 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 × C57BL/6 or Balb/c genetic background are provided in Supplemental Experimental Procedures. All mice were housed in microisolator cages at Michigan State University animal facility. B6.129S6-Il-6tm1Kopf/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 × 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 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 χ2 test. Values are expressed as mean ± SEM or SD; p ≤ 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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