c-JUN inhibits mTORC2 and glucose uptake to promote self-renewal and obesity

Highlights
- c-Jun metabolically reprograms cancer cells by disrupting insulin, mTORC2-AKT pathways
- Hepatic c-Jun abundance leads to oncogenesis and insulin resistance in obesity
- Reduced phosphorylation of AKT-S473 leads to insulin resistance
- c-Jun represses the expression of Rictor through c-Jun binding sites of the Rictor promoter
c-JUN inhibits mTORC2 and glucose uptake to promote self-renewal and obesity

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SUMMARY
Metabolic syndrome is associated with obesity, insulin resistance, and the risk of cancer. We tested whether oncogenic transcription factor c-JUN metabolically reprogrammed cells to induce obesity and cancer by reduction of glucose uptake, with promotion of the stemness phenotype leading to malignant transformation. Liquid alcohol, high-cholesterol, fat diet (HCFD), and isocaloric dextrin were fed to wild-type or experimental mice for 12 months to promote hepatocellular carcinoma (HCC). We demonstrated 40% of mice developed liver tumors after chronic HCFD feeding. Disruption of liver-specific c-Jun reduced tumor incidence 4-fold and improved insulin sensitivity. Overexpression of c-JUN downregulated RICTOR transcription, leading to inhibition of the mTORC2/AKT and glycolysis pathways. c-JUN inhibited GLUT1, 2, and 3 transactivation to suppress glucose uptake. Silencing of RICTOR or c-JUN overexpression promoted self-renewal ability. Taken together, c-JUN inhibited mTORC2 via RICTOR downregulation and inhibited glucose uptake via downregulation of glucose intake, leading to self-renewal and obesity.

INTRODUCTION
The proportion of overweight or obese population members has greatly increased over the past few decades due to lifestyle changes that affect diet and/or physical activity. About half of the adult population in developed countries are overweight or obese and face some effects of metabolic disorders (Qatanani and Lazar, 2007). Compelling epidemiological evidence exists for a synergism between HCV infection and diabetes for a 100-fold excess risk of developing HCC in the context of either hepatitis B virus (HBV) or HCV infections (Nordenstedt et al., 2010). Morbid obesity and type 2 diabetes mellitus (T2DM) are associated with premature mortality and many other complications including increased incidence of nonalcoholic fatty liver disease (NAFLD)/Nonalcoholic steatohepatitis (NASH) with higher risk of HCC in HCV patients (Kopelman, 2007). In addition, coexistent diabetes increases the recurrence of HCC after curative therapy (Choi et al., 2017). The linkage of cancer and inflammation is achieved through c-JUN N-terminal kinases (JNKs) (Johnson and Nakamura, 2007).

Our previous HCC studies in mouse models identified the HCV core protein as an independent tumor initiator that induced liver oncogenesis, whereas HCV NSSA promotes Tlr4 expression and enhances tumorigenesis through Tlr4 pathway (Chen et al., 2016). This occurs in a c-Jun-dependent manner through mitochondrial oxidant stress, inflammation, hepatocyte proliferation, and impaired DNA repair. Concomitant Tlr4 upregulation due to increased portal levels of endotoxin is also a crucial event responsible for synergistic induction of inflammation, oxidant stress, and cancer development in diabetes.

The risk of diabetes development is dependent upon changes to the insulin hormonal pathway. Insulin is secreted by pancreatic beta cells in response to elevated levels of nutrients (i.e., glucose) in the circulation. Insulin triggers the uptake of glucose, fatty acids, and amino acids into the liver, adipose tissue, and muscles, promoting the storage of these nutrients in the form of glycogen, lipids, and protein, respectively. The failure to transport and store nutrients is associated with insulin resistance and could result from defects in the insulin signaling pathway. Insulin-mediated activation of AKT in hepatocytes results in the phosphorylation of nuclear FOXO1 transcription factor, leading to its exit into the cytoplasm and degradation. This outcome is reduced by the expression of G6Pase and PEPCK, leading to diminished glucose production...
and elevated synthesis of glycogen in the liver. On the other hand, the absence of AKT-S473 phosphorylation reduces the phosphorylation of FOXO1 (Ni et al., 2007), thus retaining the transcriptional activity of FOXO factors. The mTORC2 phosphorylation of AKT at Ser-473 leads to activation of down-stream pathways (McDonald et al., 2008); however, conditions for mTORC2 regulation, expression, and/or activity are still elusive.

Recent studies have pointed out a link between insulin resistance and cancer (Arcidiacono et al., 2012). Although the mechanisms for this association are unknown, hyperinsulinemia (one hallmark of insulin resistance) and the increase in bioavailable insulin-like growth factor I (IGF-I) reportedly have roles in tumor initiation and progression in insulin-resistant patients (Arcidiacono et al., 2012). Lipid accumulation in obesity triggers cancer-related pathways including c-Jun N-terminal kinase (JNK), NF-κB, and TLR signaling, resulting in oncogenic gene overexpression. The TLR4 receptor rapidly activates not only the NF-κB pathway but also MAPK pathways, including JNK, ERK, and p38. Many of the downstream targets of MAPK pathways are transcription factors, which include c-JUN, ATF2, and ELK-1 (Liu et al., 2009). Transcription factor c-JUN (a component of activating protein-1, AP-1) is a proto-oncogene but has a role in metabolic disorders and controls cellular responses to stimuli that regulate proliferation, differentiation, oncogenic transformation, and apoptosis.

AP-1 is a heterodimer of basic region-leucine zipper (bZIP) proteins that belong to the JUN, FOS, MAF, and ATF sub-families (Chinenov and Kerppola, 2001). Among these, c-JUN is the major component of the AP-1 complex with c-FOS as its best-known binding partner (Zhang et al., 2007). We hypothesized that c-JUN activation is required for synergistic liver tumor development in HCFD-fed NSSA Tg mice because c-Jun is required in HCV NSSA-induced potentiation of chemically induced liver carcinogenesis (Eferl et al., 2003). We previously demonstrated that NSSA induces the expression of Tlr4, which is the co-receptor for endotoxin. Consequently, increased plasma levels of endotoxin due to a high-fat diet may promote the synergism between HCV and obesity in liver disease progression beginning with Tlr4 and its downstream gene c-Jun. Accordingly, we investigated this possibility by using a knockout approach to confirm the role of c-Jun in this association of NSSA with obesity.

We further postulated that c-Jun metabolically reprograms hepatocytes as an underlying condition, leading to the induction of hepatocarcinogenesis; this is based on our previous report demonstrating the requirement of c-Jun in HCV core-gene-induced potentiation of chemically induced liver carcinogenesis (Machida et al., 2010). To determine the downstream effects of c-Jun deficiency, we analyzed insulin-mediated glucose uptake and TIC formation, which would have direct relevance to tumor formation in a c-Jun-dependent manner (Eferl et al., 2003; Machida et al., 2010). In this study, we aimed to determine the effect of c-Jun gene disruption on synergistic tumor incidence and metabolic reprogramming caused by HCFD in the NSSA Tg model.

RESULTS

The c-Jun gene disruption in hepatocytes reduces synergistic tumor incidence and TIC formation induced by NSSA transgene and obesity

We hypothesized that HCV NSSA and obesity synergistically induce liver tumors (Figure S1A) through activation of TIR4 expression and its downstream effector c-Jun to account for the tumor promotion effect (Figures S1B and S1C). We examined the requirement of c-Jun for synergistic liver cancer development in NSSA Tg mice given alcohol or HCFD (Figure 1A, left); for this we crossbred c-Junfl/fl;Alb::Cre mice (Behrens et al., 2002; Stepniak et al., 2006) with NSSA Tg mice. As a control, we used c-Junfl/fl mice without Alb::Cre. The c-Jun deficiency in the hepatocytes of the former attenuated liver cancer incidence (Figure 1A, right). When Alb::Cre; c-Junfl/fl mice were fed HCFD, the final body weights were significantly less than those of c-Junfl/fl mice without Alb::Cre (Figure 1A right Table, 1C top). From this result, the c-Jun gene knockout prevented synergistic liver tumor incidence as observed in NSSA Tg mice given alcohol (Figure 1A, right). These results indicated that NSSA-induced Tlr4 expression led to HCC with HCFD.

Combination between obesity and NSSA induces c-Jun and TIC markers

HCFD acts by altering the LPS-induced redistribution of components of the Tlr4 complex within the membrane lipid raft. This change is related to rearrangement of the actin cytoskeleton, receptor clustering, with effects on subsequent signaling (Szabo et al., 2007). Serum endotoxin levels increased independently of HCFD-fed mice genotypes (Figure 1D), indicating that HCFD feeding consistently increased endotoxin.
**A**

| Genotype          | Dist No. | Tumor (%) | Survival (%) | Body weight | Liver weight | Ratio of intestines |
|-------------------|----------|-----------|--------------|-------------|--------------|---------------------|
| c-Jun+/+          | Control  | 19        | 0            | 93          | 24.5 ± 4.2   | 31.8 ± 2.5           | 1.4 ± 0.5            | 4.6                |
| Alb::Cre;c-Jun+/+ | Control  | 21        | 0            | 92          | 24.1 ± 3.1   | 30.8 ± 2.1           | 1.4 ± 0.4            | 4.1                |
| c-Jun+/+;NS5A     | Control  | 23        | 0            | 89          | 24.8 ± 4.1   | 32.9 ± 4.1           | 1.3 ± 0.7            | 4.2                |
| Alb::Cre;c-Jun+/+ | Control  | 21        | 0            | 90          | 25.9 ± 3.2   | 30.8 ± 3.2           | 1.2 ± 0.3            | 3.9                |
| c-Jun−/−          | HCFD     | 18        | 6            | 88          | 24.5 ± 4.5   | 53.5 ± 5.8           | 2.2 ± 1.5            | 6.2                |
| Alb::Cre;c-Jun−/− | HCFD     | 21        | 0            | 85          | 25.9 ± 3.9   | 34.5 ± 6.4**         | 1.5 ± 1.1**          | 3.6**              |
| c-Jun−/−;NS5A     | HCFD     | 24        | 40           | 78          | 24.6 ± 4.5   | 58.5x ± 8.9          | 2.9 ± 1.8            | 6.8                |
| Alb::Cre;c-Jun−/−;NS5A | HCFD  | 18        | 9            | 82          | 24.0 ± 4.0   | 35.9 ± 4.0**         | 1.7 ± 0.8**          | 4.1**               |

*P < 0.005, **P < 0.001, Student’s T-test was performed to calculate statistical significance in comparison to those of wild type mice fed HCFD.

**B**

| Tumor mass |
|------------|
| HCFD+      |
| HCFD−      |
| Alb::Cre;c-Jun+/+;NS5A |

**C**

**D**

**E**

**F**

**G**

**H**

**I**

**J**

**Notes:**

1. Control diet feeding.
2. High-cholesterol/fat feeding.
3. Glucose Uptake.
4. Insulin Resistance.
5. Glucose Production.
Figure 1. c-Jun disruption in hepatocytes reduces liver tumor development and restores glucose intolerance and insulin resistance phenotypes

(A) (Left) Experimental design to define the role of c-Jun in liver tumors induced by HCV NSSA and alcohol/diabetes. HCV NSSA Tg mice or their control non-Tg littermates were fed HCFD from 8 weeks of age for 12 months. These transgenic mice express Cre recombinase driven by liver-specific albumin-promoter, which induces the recombination oflox sites that flank the c-Jun gene and its deletion (“floxed”). (A) (Right) Mice were euthanized after 12 months of feeding for analysis of tumor incidence. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

(B) Quantitative data are shown for tumor size (ratios of liver tumors weights versus total liver weights) of HCFD-fed Tg mice. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

(C) (Top) Representative images of HCFD-fed NSSA Tg mice. (C, Bottom) H&E-stained liver sections from mice. Note the widespread hemorrhaging in the liver of NSSA Tg mice. Representative H&E-stained sections of mouse organs. Scale bars represent 10 µm.

(D) Serum endotoxin levels were quantified. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

(E) Immunoblot analyses validated that c-JUN knockout in hepatocytes reduced stemness marker Nanog and malignant HCC marker Vimentin expression in mouse tissues.

(F) Tumor-initiating stem-like cells (TICs) are induced in NSSA Tg mice fed HCFD. CD133 + CD49f + cells are considered as TICs. Double staining of liver sections from NSSA Tg mice showed that some of the hepatocytes have both CD133 and CD49f expression, indicating that HCV NSSA increased cancer stem cells in liver. To determine if NSSA expression induces Nanog with higher ligand levels caused by HCFD feeding, liver sections from NSSA Tg mice at the age of 12 months were stained with antibodies directed to Nanog and CD49f. Most of Nanog-expressing hepatocytes have high levels of CD49f, which is a marker of hepatic progenitor cells. Scale bars represent 10 µm.

(G) Glucose tolerance test. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

(H) Insulin resistance test. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

(I) c-Jun overexpression inhibited glucose uptake in the HepG2 cells. FACs analysis using fluorescent d-glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) showed that c-Jun overexpression in HepG2 cells decreased glucose uptake. N = 6. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

(J) c-Jun overexpression in HepG2 cells also showed the increase in the gluconeogenesis/glucose production, which was performed using glucose assay kit (Sigma). N = 5. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

Levels. A poor prognostic subtype of human HCC is derived from hepatic progenitor cells (Lee et al., 2006). Feeding of HCFD induced NSSA and Jun protein levels. Expression of NSSA further increased the Tlr4 and Jun protein levels (Figure 1E, left).

We examined mouse mortality, tumor incidence, tumor size, and liver pathology, combined with immunostaining to determine co-localization of c-Jun and TIC markers (CD133 and CD49f). Western blot analyses of c-Jun knockout mice showed c-Jun, Nanog, and vimentin protein levels were reduced (Figure 1E right). We observed c-Jun disruption reduced the extent of fatty livers in NSSA Tg mice fed HCFD (Figure 1E, left, Table S2). Thus, to determine if c-Jun is responsible for TIC formation, we examined c-Jun and TIC markers (CD133 and CD49f) in the livers of Alb::Cre;c-Junfl/fl;NS5A (c-Jun-snap) mice fed HCFD. In these c-Jun-snap-deficient mice, CD133 or c-Jun induction was partly prevented (Figure 1F). To further test whether CD133 or c-Jun is downstream of Tlr4 signaling, we examined the expression of CD133 or c-Jun in liver tissue sections isolated from NSSA Tg mice in the absence or presence of c-Jun knock out. We confirmed that c-Jun protein induction by NSSA was largely prevented in c-Jun liver-specific knockout mice (Figures 1E and 1F). These results suggested that transcription and expression of stem cell marker Nanog and CD133 were induced through both NSSA and c-Jun signaling.

As the body weights of Alb::Cre; c-Junfl/fl;NSSA Tg mice fed HCFD were significantly lower than those of c-Junfl/fl;NSSA Tg mice without Alb::Cre fed the identical diet (Figure 1A, right), we analyzed the downstream parameters of c-Jun deficiency. We measured glucose tolerance and insulin resistance associated with the severity of the obese phenotype. c-Junfl/fl mice without Alb::Cre were glucose intolerant and insulin resistant, whereas HCFD-fed Alb::Cre;c-Junfl/fl mice showed less abnormal parameters, indicating that disruption of c-Jun improved responses to insulin or glucose increases (Figures 1G and 1H). These results indicated that NSSA-induced c-JUN expression promoted HCC development with diabetes. Glucose tolerance and insulin response were reduced in HCFD-fed NSSA Tg mice. Glucose tolerance and insulin resistance were improved in Alb::Cre;c-Junfl/fl;NSSA Tg mice compared with c-Junfl/fl;NSSA Tg mice without Alb::Cre, indicating further that c-Jun played a key role for induction of the diabetic
Figure 2. c-Jun inhibits Akt phosphorylation via Rictor downregulation and inhibition of Torc2 complex formation

(A) Western blots of signaling molecules showing dysregulation in the AKT pathway by decreased p-Akt-S473 and p-Foxo-1 in c-Jun wild-type mice fed high-fat diet. In contrast, the c-Jun knockout samples showed that p-Akt-S473 and p-Foxo-1 signals increased.

(B, left) Western Blots of signaling molecules showing similar dysregulation in the AKT pathway by decreased p-AKT-S473 and p-FOXO-1 in c-JUN overexpressed HepG2 samples. (B, right) c-JUN downregulates the RICTOR levels in mTORC2 complex. Western blot analysis shows that RICTOR levels were decreased in c-JUN overexpressed HepG2 cells, whereas the other components of mTORC2 (MTOR, SIN-1) complex were the same.

(C) Real-time RT-PCR analysis also showed that RICTOR mRNA expression was significantly reduced in c-JUN overexpressed HepG2 cells, whereas the expression of the components remained the same in all the samples. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

(D, left) Immunoprecipitation analysis revealed that there was no complex formation between SIN-1 and RICTOR. (D, right) Reverse IP-western blot was performed to show if c-JUN overexpression inhibited RICTOR-mTLR1-SIN1 complex formation. c-JUN expression inhibited the interactions between mTOR1 and SIN1.

(E) (left) Immunohistochemistry of c-Jun wild-type and c-Jun knockout HCFD-fed mice showed increased Rictor expression in the knockout samples. These liver tissue sections were collected from HCFD-fed Alb-Cre;Junfl/fl;NS5A Tg mice. (Right) Immunoreactivity score was calculated based on...
**Figure 2.** Continued

Both staining frequency and intensity. Scale bars represent 10 μm. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. (F) Dysregulation of AKT pathway disrupts downstream pathways. Activation of FOXO-1 activates the gluconeogenic genes. Real-time RT-PCR analysis revealed that PEPCK and G6Pase are increased in c-Jun overexpressed HepG2 cells. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. (F, the 3rd to the left) Real-time RT-PCR analysis also showed that activation of glucokinases decreased in c-Jun overexpressed HepG2 cells. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

**c-Jun overexpression reduces insulin-mediated glucose uptake**

To examine if c-JUN overexpression causes insulin resistance, we overexpressed c-JUN in human HepG2 hepatocytes. The c-JUN overexpression inhibited insulin-mediated glucose uptake and activated gluconeogenesis when compared with the HepG2 with vector control (Figure 1I). Thus, the overexpression of c-JUN enhanced gluconeogenesis exemplified by increased glucose production in response to increased c-JUN expression with glucose levels in normal HepG2 (Figure 1J).

**Overexpression of c-Jun inhibits phosphorylation of AKT at serine 473**

In c-JUN wild-type liver samples, insulin failed to show phosphorylation of AKT at Ser473, whereas Thr308 phosphorylation was induced (Figure 2A). The disruption of c-Jun in a liver-specific manner promoted phosphorylation of AKT at Ser473 (Figure 2A). Similar results were obtained when c-JUN was overexpressed in HepG2 cells. Phosphorylation of AKT at Ser473 was reduced in HepG2 cells upon c-JUN overexpression compared with the parental HepG2 or empty vector transfected cells (Figure 2B). FOXO-1, a substrate of AKT, is responsible for activation of gluconeogenic genes. The former was significantly hyperphosphorylated in c-Jun knockout liver samples (Figure 2A), whereas phospho-FOXO1 levels were reduced in HepG2 cells with c-Jun overexpression (Figure 2B).

**c-JUN downregulates the RICTOR levels in mTORC2 complex and mTORC2 complex formation**

The mTORC2 complex partially activates AKT via phosphorylation at Ser473 (Sarbassov et al., 2005). Further, PDK1-mediated AKT phosphorylation at Thr308 leads to full AKT activation (Finlay and Cantrell, 2011). The mTORC2 complex comprises mTOR, rapamycin-insensitive companion of mTOR (RICTOR), GβL, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1) (Laplante and Sabatini, 2012). Overexpression of c-JUN inhibited the expression of RICTOR at both protein and mRNA levels in HepG2 cells compared with control HepG2 cells where levels of other components, SIN1 and mTORC2, were unaltered (Figure 2C). Reduction of RICTOR protein inhibited the complex formation between RICTOR and SIN1 shown by immunoprecipitation-immunoblot analysis, indicating that c-Jun inhibited mTORC2 complex formation (Figure 2D, left). Reciprocal IP-western blot analyses further confirmed that c-JUN overexpression inhibited the interactions between Sin1 and mTor proteins (Figure 2D, right). Immunohistochemical analysis also showed higher Rictor expression in Alb:Cre; c-Junfl/fl;NS5A Tg mice fed HCFD, whereas in c-Jun wild type liver specimen control, the expression of Rictor was reduced (Figure 2E).

**Inhibition of AKT phosphorylation disrupts the downstream gluconeogenic pathway**

Activation of FOXO1 leads to production of glucose via induction of gluconeogenic genes, including G6Pase and PEPCK. The expression of both gluconeogenic genes increased in HepG2 cells with c-JUN overexpression as measured by qRT-PCR (Figure 2F, left). Hepatic insulin resistance also affects the synthesis of glycogen, which is synthesized from UDP-glucose by the enzyme glycogen synthase. To test if c-JUN overexpression also affects glycogen synthesis, we measured the expression of glycogen synthase enzyme, as the control of glycogen synthase is a key step in regulating glycogen metabolism and glucose storage. AKT-induced glycogen synthase kinase3 (GSK-3b) inhibits glycogen synthase activity by phosphorylation (Fang et al., 2000). Overexpression of c-JUN induced phosphorylation of glycogen synthase, which is the
from the mouse and showed that c-JUN expressing cells inversely have less RICTOR staining in human HCC specimens as expected (Figures 3E and 3F). Immunohistochemistry analyses of HCC and noncancerous adjacent tissues demonstrated fluorescence staining analysis validated that silencing promoter activity increased significantly, indicating that c-JUN repressed Rictor promoter activity. Immunofluorescence staining analysis showed that after mutating the site between bases −622 and −529, the Rictor expression increased in TICs. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

Using reporter constructs containing serial deletions, we identified that both c-Jun binding sites are important in the phosphorylation of AKT expressed samples (Figure 2F, right). These results demonstrated that overexpression of c-JUN inhibited phosphorylation of AKT at Ser473 (Figure 2F, far right).

Jun in TICs is higher compared to HepG2 cells. Silencing c-JUN inhibited phosphorylation of AKT at Ser473 (Figure 2F, right). Glycogen synthase kinase (GSK-3β) was hypophosphorylated in c-JUN overexpressed samples (Figure 2F, right). These results demonstrated that overexpression of c-JUN inhibited phosphorylation of AKT and glucokinase protein levels in comparison to control HepG2, indicating that c-JUN inhibited phosphorylation of AKT at Ser473 (Figure 2F, far right).

Silencing of c-JUN restores RICTOR expression

To further confirm if c-Jun downregulates Rictor expression, c-Jun was silenced in mouse TICs, as the level of c-Jun in TICs is higher compared to HepG2 cells. Silencing c-Jun increased the expression of P-Akt and Rictor (Figure 3A). To determine if c-Jun inhibited Rictor at the transcriptional level through c-Jun binding sites on the Rictor promoter, ChIP-qPCR of c-Jun was performed. We observed that c-Jun is enriched in binding sites on two fragments of the Rictor promoter between bases −751 and −529 as detected by ChIP-qPCR with c-Jun antibody (Figure 3B). Ectopic expression of c-Jun sh-RNA in mouse TICs activated the Rictor promoter region between bases −852 and −512, which includes both promoter elements where c-Jun is enriched (Figure 3C). Using reporter constructs containing serial deletions, we identified that both c-Jun binding sites are important for Rictor repression. By mutating both c-Jun binding sites individually, we determined that the Rictor promoter activity increased when the second site, i.e., bases −622 to −529, was mutated (Figure 3D). This mutant Rictor promoter activity increased significantly, indicating that c-JUN repressed Rictor promoter activity. Immunofluorescence staining analysis validated that silencing c-JUN increased the expression of RICTOR in HepG2 cells (Figures 3E and 3F). Immunohistochemistry analyses of HCC and noncancerous adjacent tissues demonstrated that c-JUN-positive cells have reduced RICTOR expression (Figure 3G). Double staining of RICTOR and c-JUN showed that c-JUN expressing cells inversely have less RICTOR staining in human HCC specimens as expected from the mouse and in vitro cell culture experiments (Figure 3G).

Activation of c-Jun and induction of TLR4 are evident in HCV patient livers

We hypothesized that TLR4 is induced, and c-JUN is fully activated in HCV-infected diabetic livers. From the gene expression analysis of these samples, this hypothesis was validated by the general observation that c-JUN activation and TLR4 induction as observed in the mouse models also were observed in human HCV liver specimens. Meta-analysis of publicly available Oncomine data analyses demonstrated that c-JUN mRNAs are upregulated in HCC patients, whereas AKT1 and GSK3b are downregulated in HCC patient samples (Figures 4A and 4B).

We examined the patient samples for c-Jun/AP-1 involvement in HCV carcinogenesis in livers from hepatitis C patients for further corroboration of this hypothesis. Liver sections from HCV patients exhibiting different stages of HCV infection were used for immunostaining. These patients were only HCV infected or had co-morbidities of obesity or NASH (Table S1). Hepatitis was also included, as our hypothesis incorporates an enhanced inflammatory response through the TLR4-c-Jun axis. Immunostaining was performed
HCC patients with altered TLR4 expression have poor prognosis.

TCGA data analysis by use of cBioPortal showed that the HCC patients who have higher mRNA levels of TLR4 and/or c-JUN have poorest survival rate for 12 months.

The mRNA expression levels of TLR4 and/or c-JUN are elevated in one-third of HCC patients (32%) from TCGA data analysis by use of cBioPortal.

The silencing effects of c-JUN by shRNA transduction were validated by immunoblot analyses (Figure 6A, Figure S2A, and S2B). These results indicated that TICs have reduced glucose utilization and are deficient in glucose supply.

TICs have reduced glucose utilization and deficient glucose supply

Metabolomics analyses showed TICs have restricted glucose metabolism pathways as evident from levels of glucose-6-phosphate, 2-phosphoglycerate, 3-phosphoglycerate, and glycerol (Figure S3A). mTORC-related genes were downregulated in HCV transgenic mice fed high-cholesterol high-fat diet or alcohol diet for 12 months (Figures S2A and S2B). These results indicated that TICs have reduced glucose utilization and are deficient in glucose supply.

Glucose 6-phosphate and mannose 6-phosphate displayed the greatest differences between TICs and primary hepatocytes

Glucose 6-phosphate, 2-phosphoglycerate, and mannose 6-phosphate were significantly reduced in TICs, whereas fructose, lactate, and γ-aminobutyryl acid (GABA) were significantly elevated in TICs in comparison to those levels in primary hepatocytes (Figures S3A and S3B). Neither glucose nor mannose were measurable in TICs, which may indicate rapid phosphorylation of limiting quantities of these sugars upon import into the cells (Figure S3B). Despite the increased levels of glucose-6-P and mannose-6-P (Figure S3), downstream metabolites of the glycolysis pathway, e.g., pyruvate, were not dramatically increased when compared with either sh-NANOG-transduced TIC or scrambled shRNA-transduced TIC (0.45-fold change; p = 0.24) (Figures 5A and 5B). By contrast, glucose 6-phosphate and mannose 6-phosphate displayed the greatest differences in levels between sh-NANOG-transduced TIC and scrambled shRNA-transduced TICs (Figure S3B).

c-Jun silencing increases extracellular acidification rate (ECAR: glycolysis activity)

To test if c-Jun silencing increased glycolysis, Seahorse metabolic assays were performed in the presence or absence of c-Jun silencing in cells. c-Jun silencing increased ECAR (glycolysis activity) (Figure 5C). We examined if c-Jun suppressed glucose uptake and glucose usage by this method. The knockdown of c-Jun increased glycolysis activity, indicating that c-Jun suppression elevated glucose uptake (Figures 5C and 5D). Glucose assays and gene expression analyses of Nanog and c-Jun were performed in the presence or absence of silencing of c-Jun or Nanog (Figure 5E). Silencing of Nanog or c-Jun increased glucose uptake (Figure 5E, Top). Silencing effects of shRNA transduction against c-Jun were validated by qRT-PCR analyses. Silencing of c-Jun significantly reduced expression of pluripotency transcription factor Nanog (Figure 5E, Bottom). Similarly, silencing of Nanog significantly reduced c-Jun expression (Figure 5E, Bottom), indicating that c-Jun and Nanog were co-dependently expressed. Immunofluorescence analyses showed higher levels of c-JUN and lower levels of GLUT2 in HCC areas in comparison to those of noncancerous counterparts (Figure 5F).

c-JUN inhibits GLUT2 promoter activity through c-JUN binding sites and reduces GLUT1 and GLUT3 protein levels

The silencing effects of c-JUN by shRNA transduction were validated by immunoblot analyses (Figure 6A, left). Immunofluorescence staining showed restoration of GLUT2 expression in c-JUN-silenced cells.
inhibited GLUT3 protein expression (Figure 6J). This indicated that c-JUN and/or phosphorylated c-JUN, but not nonphosphorylatable mutant of c-JUN, downregulated GLUT2 mRNA levels (Figures 6B and 6C). Similarly, mutagenesis of c-JUN/AP-1 binding sites abrogated the c-JUN-mediated repression of GLUT2 promoter activity (Figure 6D). The GLUT2 promoter mutant (−1291) showed an induction of GLUT2 promoter activity, indicating that the API1 binding site within the −1200 area suppressed promoter activity. Similarly, removal of AP1-binding sites similarly allowed unregulated GLUT2 promoter activities (Figure 6E). The large increases in promoter activity observed with constructs containing shorter promoter regions (Figures 6B and 6C) suggested the possibility that AP-1 sites proximal to the TSS might have greater influence on promoter strength than distal AP-1 sites.

Overexpression of nonphosphorylatable c-JUN reduces GLUT2 mRNA levels in HepG2

To study the effect of overexpression and activation of c-JUN has on regulating GLUT2 expression, HepG2 cells were transfected with three different expression vectors containing His6-tagged c-JUN WT (A63/73S) MT35, His6-tagged c-JUN (A63/73A) MT111, and His6-tagged c-JUN (D63/73D) MT112. RT-qPCR results showed a decrease in GLUT2 mRNA when WT (MT35) and phosphomimetic c-JUN (MT112) were overexpressed. In contrast, overexpression of nonphosphorylatable c-JUN (A63/73A) decreased GLUT2 mRNA levels (Figure 6G). Overexpression of phosphomimetic mutant of c-JUN produced a greater increase in GLUT2 protein levels (Figure 6F). Overexpression of nonphosphorylatable c-JUN mutant promoted GLUT1 and GLUT3 expression (Figure 6F, the far-right lane). These results suggested that c-JUN phosphorylation downregulated GLUT1 gene expression.

TLR4 signal activation results in phosphorylation, downregulated c-JUN to promote nuclear translocation to transactivate or suppress the AP-1 target genes. LPS stimulation followed by c-JUN phosphorylation was tested to examine the effects on GLUT2 protein levels. LPS stimulation further increased the GLUT2 promoter levels and protein levels (Figures 6H and 6I). Furthermore, immunofluorescence staining confirmed that c-JUN and phosphomimetic mutant of c-JUN, but not nonphosphorylatable mutant of c-JUN, inhibited GLUT3 protein expression (Figure 6J). This indicated that c-JUN and/or phosphorylated c-JUN inhibited GLUT3, but nonphosphorylated c-JUN did not inhibit GLUT3, suggesting nonphosphorylated c-JUN lost inhibitory effects on GLUT3 protein suppression.

Silencing c-JUN reduces self-renewal, tumor-initiation property, and RICTOR expression in TICs

To determine if c-Jun inhibition reduces oncogenicity, we performed colony formation assays, xenograft tumor growth in mouse models, and immunoblots using a loss-of-function approach by lentivirus shRNA targeting of c-Jun. We observed silencing c-Jun significantly reduced colony numbers in soft agar assays.
Figure 6. Glucose metabolism and GLUT2 gene regulatory mechanism

(A) (Left) Silencing effects of shRNA transduction on c-JUN were validated by immunoblot analyses. (Middle) Immunofluorescence staining analyses showed restoration of GLUT2 expression in c-JUN-silenced HepG2 cells. Scale bars represent 10 μm. (Right) Silencing c-JUN induced GLUT1 and GLUT3 protein levels. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

(B) Effects of cellular c-JUN expression on various regions of the GLUT2 promoter. Silencing of c-JUN increased GLUT2 promoter activity compared with the control in Huh7 cells transfected with all GLUT2 promoter-luciferase constructs compared with the control. No significant difference in luciferase activity was observed.

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AC133 promoter reporter constructs, pGL3-P1-P5 (Figure 7J), were transfected in Huh7 cells. To test whether c-JUN increases stem cell marker expression in HepG2 cells. HepG2 cells were seeded into T-25 flasks and transfected with 500 ng of expression vectors containing WT S63/73S His6-tagged c-JUN (MT35), phosphomimetic D63/73D His6-tagged c-JUN (MT112), or empty vector control (PBSK). Overexpression of MT35 c-JUN produced a mild increase in GLUT2 promoter luciferase reporter activities (p < 0.05) and protein. Overexpression of MT112 c-JUN produced a pronounced increase in GLUT2 mRNA (p < 0.001) and protein. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance.

AC133 promoter activity (Figure 7K). Truncation of AC133 promoter and AC133 promoter activity, several truncation mutants of AC133 promoter were used to test the AC133 promoter luciferase constructs. No significant difference in luciferase activity was observed when −1100 AP-1 binding region was truncated. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

Effects of cellular c-JUN expression on various regions of the GLUT2 promoter. Overexpression of c-JUN produced no significant changes in luciferase expression in the series of GLUT2 promoter-luciferase constructs. No significant difference in luciferase activity was observed when −1100 AP-1 binding region was truncated. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

In vitro mutagenesis of AP-1 consensus sequences in the GLUT2 promoter. The (−206/+308) GLUT2 promoter-luciferase constructs were used to produce mutated AP-1 sites at −144 or −123. (Bottom) Mutation at the −144 AP-1 site resulted in significantly greater luciferase activity. Overexpression of c-JUN reduced luciferase expression in the unmutated condition. In contrast, overexpression continued to produce a significant increase in luciferase activity in the mutant condition. Mutation of one c-JUN/AP1 binding site abrogated the c-JUN-mediated repression of GLUT2 promoter activity. N = 6. Asterisk represents statistical significance (p < 0.05). Constructs containing the full-length (−1291) GLUT2 promoters were mutated at the −1100 AP-1 site. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001.

Mutagenesis of the −1100 AP-1 site resulted in decreased luciferase activity, whereas the overexpression of c-JUN produced a greater luciferase expression compared with the empty vector control. Activation of c-JUN at serine 63/73 increased GLUT2 promoter luciferase reporter activities and protein expression in HepG2 cells. HepG2 cells were seeded into T-25 flasks and transfected with 500 ng of expression vectors containing WT S63/73S His6-tagged c-JUN (MT35), phosphomimetic D63/73D His6-tagged c-JUN (MT112), or empty vector control (PBSK). Overexpression of MT35 c-JUN produced a mild increase in GLUT2 promoter luciferase reporter activities (p < 0.05) and protein. Overexpression of MT112 c-JUN produced a pronounced increase in GLUT2 mRNA (p < 0.001) and protein. Data are represented as mean ± SD. Student’s t test was used to calculate statistical significance.

Expression of c-JUN induced CD133 protein levels (Figure 7E). Immunoblotting of CD133+/CD49F+ cells have higher CD49F, c-JUN, NANOG, and OCT4, whereas CD133(−)/CD49F(−) cells have less c-JUN protein levels, indicating that c-JUN is associated with pluripotency transcription factors. Furthermore, c-JUN silencing reduced Nanog expression, but induced Rictor expression (Figure 7G, left). Xenograft tumors with shRNA targeting of c-JUN showed significantly decreased tumor size, indicating that c-JUN had a key role for self-renewal and the tumor-initiation property (Figure 7G, Right). We performed xenograft c-JUN overexpression in the context of HCFD/NSSA. Xenograft tumors with c-JUN overexpression in the TIC population (CD133+/CD49F+) showed significantly larger tumor sizes with HCFD feeding. Overexpression of c-JUN further promoted tumor growth (Figure 7H). Xenograft tumors with c-JUN overexpression in non-TIC population (CD133−/CD49F−) showed significantly larger tumor sizes with HCFD feeding, indicating that c-JUN and HCFD feeding effects played key roles for self-renewal and the tumor-initiation property. These results indicated that c-JUN overexpression promoted the self-renewal tumor-initiation property of the non-TIC population induced by HCFD feeding (Figure 7I).

To test whether c-JUN increases stem cell marker AC133 promoter activity, several truncation mutants of AC133 promoter reporter constructs, pGL3-P1-P5 (Figure 7J), were transfected in Huh7 cells. AC133 P1 promoter had the highest level of transactivation ability among five promoter regions tested (Figure 7K). CD133+/CD49F+ cells had higher P1 AC133 promoter activity (Figure 7K). Truncation of AC133 promoter and analyses of resulting activity showed that P1 promoter sequence between −341 and +50 was required for the c-JUN-dependent activation of AC133 promoter (Figure 7L). In vitro mutagenesis of the AC133 promoter
More than 1200 CD133(+)/CD49f(+) cells have elevated levels of CD133, CD49f, c-Jun, Nanog, and Oct4 proteins. Overexpression of c-JUN promoted CD133 protein levels. (Top) Colony formation assay. To determine if c-Jun inhibition reduced oncogenicity, colony formation was employed using loss-of-function approach by use of lentivirus expressing shRNA targeting c-Jun. Silencing c-Jun significantly reduced colony numbers in soft agar assays. (Bottom) c-Jun silencing reduced colony numbers. N = 3. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean +/− SD.

Validation of RICTOR silencing in HepG2 cells. (Top) Colony formation assay. To determine if c-Jun inhibition reduced oncogenicity, colony formation was employed using loss-of-function approach by use of lentivirus expressing shRNA targeting RICTOR. Silencing RICTOR significantly reduced colony numbers in soft agar assays. (Bottom) Colony formation assay. To determine if c-Jun inhibition reduced oncogenicity, colony formation was employed using loss-of-function approach by use of lentivirus expressing shRNA targeting RICTOR. Silencing RICTOR significantly reduced colony numbers in soft agar assays. N = 3. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean +/− SD.

Overexpression of c-Jun promoted CD133 protein levels. (A) Colony forming assay. (B) Colony forming assay. (C) Colony forming assay. (D) Colony forming assay. (E) Colony forming assay.

Figure 7. c-Jun silencing reduces self-renewal, tumor-initiation property whereas RICTOR silencing promotes self-renewal ability

(A) (Top) Colony formation assay. To determine if c-Jun inhibition reduced oncogenicity, colony formation was employed using loss-of-function approach by use of lentivirus expressing shRNA targeting c-Jun. Silencing c-Jun significantly reduced colony numbers in soft agar assays. (Bottom) c-Jun silencing reduced colony numbers. N = 3. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean +/− SD.

(B) CD133+ TICs had higher levels of c-Jun expression than those of non-TIC population. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean +/− SD.

(C) Validation of RICTOR silencing in HepG2 cells. (D) (Top) Representative images of spheroids of Huh7 cells transduced with lentivirus expressing shRNA targeting c-JUN, GLUT2 or scrambled shRNAs. (Bottom) RICTOR silencing promoted spheroid formation in Huh7 cells especially in Huh7 cells that are cultured in low-glucose media. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean +/− SD.

(E) Overexpression of c-Jun promoted CD133 protein levels.

(F) CD133+/CD49f(+) cells have elevated levels of CD133, CD49f, c-Jun, Nanog, and Oct4 proteins.
component. The nuclear import of c-Jun showed significantly smaller tumor sizes, indicating that c-JUN played a key role for self-renewal and tumor-initiation property. N = 8 mice per group. Student’s T-Test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean ± SD.

Overexpression of c-JUN further promoted tumor growth. N = 6 mice per groups. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean ± SD.

Xenograft tumors with c-JUN overexpression in non-TIC population (CD133+/CD49F–) showed significantly larger tumor sizes with HCFD feeding, indicating that c-JUN and HCFD feeding effects played key roles for self-renewal and tumor-initiation property. These results indicated that c-Jun overexpression promoted the self-renewal tumor-initiation property of non-TIC population induced by HCFD feeding. N = 7 mice per groups. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean ± SD.

Figure 7. Continued
(G) (Left) c-Jun silencing reduced Nanog expression, but induced Rictor expression. The c-Jun silencing promoted Rictor expression in TICs, indicating that c-Jun was required to maintain stemness and oncogenicity of TICs and partly depended on Rictor suppression. (Right) Xenograft tumors with shRNA targeting c-JUN showed significantly smaller tumor sizes, indicating that c-JUN played a key role for self-renewal and tumor-initiation property. N = 8 mice per groups. Student’s T-Test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean ± SD.

(H) Xenograft tumors with c-JUN overexpression in TIC population (CD133+/CD49F+) showed significantly larger tumor sizes with HCFD feeding. Overexpression of c-JUN further promoted tumor growth. N = 6 mice per groups. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean ± SD.

(I) Xenograft tumors with c-JUN overexpression in non-TIC population (CD133+/CD49F–) showed significantly larger tumor sizes with HCFD feeding, indicating that c-JUN and HCFD feeding effects played key roles for self-renewal and tumor-initiation property. These results indicated that c-Jun overexpression promoted the self-renewal tumor-initiation property of non-TIC population induced by HCFD feeding. N = 7 mice per groups. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean ± SD.

(J) AC133 promoter has five major promoter regions (P1–PS).

(K) The P1 promoter region of AC133 promoter had the highest levels of transactivation activity in Huh7 HCC cell line. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean ± SD.

(L) Truncation promoter analyses showed that the region of AC133 promoter between −341 and +50 had the highest levels of transactivation ability. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean ± SD.

(M) To test if c-Jun/AP1 and/or NANOG binding transactivate the AC133 promoter, in vitro mutagenesis abrogated the AP1 binding sites and NANOG promoter binding sites. These AP1 and/or NANOG binding sites reduced AC133 promoter activity, indicating that AP1 and NANOG proteins are required to transactivate AC133 promoter. Student’s t test was used to calculate statistical significance. Asterisk (*) indicates statistical significance. *p < 0.05, **p < 0.01, ***p < 0.001. Data are represented as mean ± SD.

identified AP1 and/or NANOG binding sites. Mutations of these AP1 or NANOG-binding sites inhibited c-JUN-mediated transactivation of the AC133 promoter (Figure 7M), indicating that c-JUN and/or NANOG transactivates AC133 promoter through the AP1 and/or NANOG transcription-factor-binding sites.

DISCUSSION
In this study, we showed that hepatic c-Jun abundance impaired the insulin response, primarily due to reduced phosphorylation of AKT-Ser473 and further activation of FOXO1 and GSK-3β leading to insulin resistance. Reduction of RICTOR by overexpression of c-Jun impaired the phosphorylation of AKT at Ser473. This absence of AKT-Ser473 phosphorylation reduced the expression of glycogen synthase, glucose kinase, and phosphorylation of FOXO1, which further increased the expression of gluconeogenic genes. Our data demonstrate that c-Jun regulates the expression of Rictor, a component of the mTorc2 complex, by interacting with c-Jun binding sites on the Rictor promoter. The sites where c-Jun binds to the Rictor promoter also includes the SIRT-1 binding site, which is a positive regulator of Rictor expression (Johnson and Nakamura, 2007). Therefore, c-Jun might interfere with the binding of SIRT-1 to the promoter and antagonize increased RICTOR expression. This relationship further illustrates the importance of fine-tuning metabolic pathways needed to maintain insulin responsiveness.

Components of the AP-1 transcription factor complex including c-Jun and c-FOS are important regulators of tumor development. A genome-wide expression analysis of human HCCs revealed that c-JUN is at the center of an oncogenic signaling network characterized with poor disease prognosis (Lopez-Bergami et al., 2010). Using a diethyl nitrosamine (DEN)-induced mouse liver cancer model, c-Jun was found to promote tumorigenesis by suppressing the important cell death regulator p53 (Eferl et al., 2003). In addition, c-Jun was required for mouse liver tumorigenesis during early stages, but dispensable in advanced liver tumors. It is also shown that c-Jun up-regulation correlates with human hepatocyte survival during HCV infection and with early events during cirrhosis-associated human HCC development. Taking advantage of the c-Jun-dependent mouse model, a study showed that c-Jun promotes cell survival during cancer initiation by regulating c-Fos- and Sirt6-dependent expression of Survivin, rather than antagonizing p53 (Min et al., 2012).

In this study, we show the role of c-Jun not only in oncogenesis but also in metabolic reprogramming of cancer cells by dysregulation of the mTorc2/Akt pathway. Saturated FFAs and lipid accumulation activate this proinflammatory transcription factor in hepatocytes, and enhanced AP-1 activation has been demonstrated in obese patients (Dorn et al., 2014). AP-1 is a homo or heterodimer consisting of proteins belonging to the c-Jun, c-FOS, ATF, and JDP families, with c-Jun being the best-characterized AP-1 component. The nuclear import of c-Jun is mediated by multiple mechanisms, and nuclear c-Jun levels
correlate with AP-1 target gene activity. These further increases c-Jun protein abundance, as the Jun proto-oncogene itself is activated by AP-1 in the manner of a positive autogenous regulatory loop. The activity of c-Jun/AP-1 is markedly enhanced by phosphorylation of the transcriptional activation domain by JNKs (Schattenberg et al., 2006). Sustained activation of the JNK pathway mediates the development and progression of experimental-diet-induced NAFLD (Schattenberg et al., 2006; Singh et al., 2009).

Apart from boosting c-JUN/AP-1 activity, JNK activation can also phosphorylate insulin receptor substrates (IRS)-1 and -2, which may lead to insulin resistance by blocking insulin receptor signal transduction (Hirosumi et al., 2002). Furthermore ROS (reactive oxygen species), which are associated with NAFLD progression, activate JNK. c-JUN promotes the nuclear accumulation of JNK, further increasing AP-1 transcriptional activity in a manner of a positive amplification circuit.

Although c-Jun positively regulates gene expression and increases the phosphorylation of Akt, c-Jun also negatively regulates gene expression and inhibits phosphorylation of Akt in the example of NAFLD. Increased hepatic c-Jun expression is observed in the murine NASH model (high fat diet); this was confirmed in NAFLD patients where increased c-JUN abundance was detected even in steatosis. Higher c-JUN levels facilitate NASH development and progression. Nonalcoholic steatohepatitis (NASH) is a common cause of chronic liver disease associated with insulin resistance and obesity, which could lead to diabetes and other metabolic disorders. Decreased hepatic Akt phosphorylation in high-fat-diet-fed mice compared to control mice indicated an impaired insulin response, a key pathological factor for NAFLD development and progression also observed in patients (Dorn et al., 2014).

Abnormal regulation of hepatic glucose production is one of the causes for several human health problems, such as insulin resistance, type 2 diabetes, nonalcoholic fatty liver disease, and liver cirrhosis. The lower PFK-1 activity may prevent full utilization of available glucose 6-P and the shunting of excess fructose 6-P into mannose 6-P as demonstrated by comparison between sh-NANOG-transduced TIC versus scrambled shRNA-transduced TIC. In our study, we showed that c-Jun overexpression caused an increased level of glucose production. We also observed the same phenomenon in vivo using different mouse models. When we tested NSSA Tg mice in which c-Jun was knocked-out in a liver-specific manner and fed a high cholesterol fat diet, we observed that c-Jun wild-type mice developed insulin resistance, whereas c-Jun knockout mice had improved conditions. Hepatic insulin resistance is observed in mice, which is accompanied by increased levels of ROS in their insulin-responsive organs (Wang et al., 2011).

Subtle differences are displayed in metabolites associated with the TCA cycle between TICs and primary hepatocytes (Figure 5D). An examination of metabolites of the TCA cycle revealed some small differences between NANOG- and scrambled shRNA-transduced cells (Chen et al., 2016). The status of isocitrate dehydrogenase (IDH) is unknown for TIC cells. However, as “normal” CD133(−) cells contain 2-hydroxylutarate levels equivalent to the TIC cells, it seems unlikely that 2-hydroxyglutarate levels are related to mutated IDH. 2-hydroxyglutarate was present in measurable amounts in unspent media and can be converted to α-ketoglutarate through the activity of 2-hydroxyglutarate dehydrogenase.

The TCA cycle represents a nexus for balancing energy metabolism with inputs from glycolysis, fatty acid β-oxidation, and amino acid anaplerosis (Chen et al., 2016). One might speculate that the overall trends are consistent with an increase in carbon input into the cycle and/or decreased demand for citrate to support fatty acid synthesis.

Liver-specific c-Jun disruption may accompany adaptive compensation by other members of the Jun family such as JunB and JunD that may still serve to induce proinflammatory and pro-oncogenic mechanisms. However, a published study using the same knockout mice demonstrated similar hepatic levels of JunB and JunD expression in c-Jun KO and wild-type mice (Eferl et al., 2003). Although not performed in the present studies we intend to analyze expression of the Jun family members in human liver specimens in the future.

Any proposed study of clinical specimens may commonly be influenced by inevitable variables such as co-infection with other hepatitis viruses or HIV, drug addiction, and co-morbidities other than diabetes. In future studies we will expand our meta-analysis to account for the effects of pre-existing HBV or HIV positivity on metabolism and oncogenicity.
In conclusion, we have demonstrated that HCFD and HCV induce liver tumors in hepatocytes by activating c-Jun. Data from this in vivo model support the model that activation of c-Jun not only plays a critical role in cancer development but also causes a metabolic shift from basal levels. The potential linkage of c-Jun induction to metabolic abnormalities and hepatocellular carcinomas implies that inhibitors of c-Jun could exert possible therapeutic effects in liver cancer.

Limitations of study
Although our study focused primarily on c-Jun, other members of the Jun family such as JunB and JunD were not analyzed in the present study. Comprehensive analysis of these other Jun family members is needed to draw better conclusions. In addition, preexisting HBV and HIV conditions should also be considered in future studies.

STAR METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104325.

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AUTHOR CONTRIBUTIONS
A.R., R.S., C.C., and K.M. conceived the study. A.R., R.S., C.C., J.L. C.N., and K.M. obtained the data. A.R., J.L., and K.M. provided data management. A.R., S.M.T., and K.M. conducted the data analysis and drafted the report. R.R. provided an essential mouse model. All authors interpreted the data and contributed to the final version of this report.

DECLARATION OF INTERESTS
The authors declare no competing interests.

INCLUSION AND DIVERSITY
We worked to ensure sex balance in the selection of nonhuman subjects. One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community. One or more of the authors of this paper received support from a program designed to increase minority representation in science. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list.

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### Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| c-JUN (1:2000 dilution: H-79, Rb pAb) Antibody | Santa Cruz | sc-1694; RRID: AB_631263 |
| p-c-JUN (1:1000 dilution: Ser 63/73, Gt pAb) Antibody | Santa Cruz | sc-16312; RRID: AB_2129883 |
| c-JUN (1:2000 dilution: Rb pAb), ChIP Grade | Abcam | ab31419; RRID: AB_731605 |
| c-JUN (1:2000 dilution: 60A8, Rb mAb) | Cell Signaling | 60A8; RRID: AB_2796752 |
| RICTOR (1:2000 dilution) | Invitrogen, Thermo Fisher Scientific | PAS-18370; RRID: AB_10983978 |
| RICTOR (1:2000 dilution: H-11, Ms mAb) | Santa Cruz | sc-271081; RRID: AB_10611167 |
| NANOG (1:2000 dilution: M-17, Gt pAb) | Santa Cruz | sc-30329; RRID: AB_2150123 |
| GSK3b (1:2000 dilution: 27C10, Rb pAb) | Cell Signaling | Cat #9335S; RRID: AB_490890 |
| P-GSK3b (1:2000 dilution: S9, 5B3, Rb mAb) | Cell Signaling | Cat #9323S; RRID: AB_2115201 |
| Glycogen synthase (1:2000 dilution: 15B1, Rb mAb) | Cell Signaling | Cat #3886S; RRID: AB_2116392 |
| P-Glycogen synthase (1:2000 dilution: S441, Rb Ab) | Cell Signaling | Cat #3891S; RRID: AB_2116390 |
| GLUT2 (1:2000 dilution: C-10, Ms mAb) | Santa Cruz | sc-518022; RRID: AB_2890904 |
| Flag tag Monoclonal Antibody (M1) | Sigma Aldrich | F3040; RRID: AB_439712 |
| β-Actin mouse Monoclonal Antibody (AC-15) | Sigma Aldrich | sc-69787; RRID: AB_1119529 |
| c-Myc mouse monoclonal Antibody (9E10) | Santa Cruz | sc-40; RRID: AB_627268 |

**Chemicals, peptides, and recombinant proteins**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Cycloheximide       | Sigma Aldrich | 239764-100MG |
| Triton X-100        | Sigma Aldrich | T8787-50ML |
| Paraformaldehyde    | Sigma Aldrich | P6148-500G |
| Polybrene           | Sigma Aldrich | TR-1003 |
| Puromycin Dihydrochloride | Thermo Fisher | A1113803 |
| Protease Inhibitor  | Roche | 4693159001 |
| Proteinase K        | Roche | 3115879001 |
| One Shot™ Stbl3™ Chemically Competent E. coli | Thermo Fisher | C737303 |
| Stellar™ Competent Cells | Takara | 636766 |
| Q5® High-Fidelity DNA Polymerase | NEB | M0491L |
| T4 DNA Ligase       | NEB | M0202L |
| Quick Blunting™ Kit | NEB | E1201L |
| GenClone Fetal Bovine Serum, Heat Inactivated | Genesee Scientific | 25-514H |
| Dulbecco's modified Eagle's medium (DMEM, High Glucose, with L-Glutamine, with Sodium Pyruvate) | Genesee Scientific | 25-500 |
| Glutamax            | Thermo Fisher | 35050061 |
| Antibiotic/An antimycotic Solution | Gemini | 400-101 |
| DPBS                | WWR | WWR0117-0500 |
| BioT                | Bioland Scientific | B01-01 |
| 6X Laemmli Sample Buffer | Bioland Scientific | SAB03-01 |
| LB Broth (Miller) Mix | Genesee Scientific | 11-120 |
| LB Agar (Miller) Mix | Genesee Scientific | 11-122 |
| PVDF membrane       | EMD Millipore | ISEQ85R |
| Nylon membrane      | Thermo Fisher | AM10104 |

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| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| X-ray film          | Thermo Fisher | 34091      |
| Trizol Reagent      | Thermo Fisher | 15596026   |
| SuperScript III reverse transcriptase | Thermo Fisher | 18080085   |
| RNasin® Ribonuclease Inhibitors | Promega | N2511       |
| EvaGreen miRNA qPCR Master Mix | Genomics-online | ABIN4219203 |
| Brilliant II SYBR Green qPCR Master Mix | Stratagene | 600828     |
| SYBR Green PCR Master Mix | Thermo Fisher | 4309155     |
| Pierce ECL plus     | Thermo Fisher | 32132      |
| Immobilon Western chemiluminescent HRP substrate | EMD Millipore | WPKLS0500  |
| Protein A/G PLUS Agarose | Santa Cruz | Sc-2003     |

**Critical commercial assays**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| RIP-assay kit       | MBL    | RN1001     |
| RNeasy mini kit     | QIAGEN | 74104      |
| QIAprep Spin Miniprep Kit | QIAGEN | 27106      |
| MinElute Gel Extraction Kit | QIAGEN | 28604      |
| Lenti-X™ GoStix     | Takara | 631280     |
| Quickchange Lightning site-directed mutagenesis kit | Agilent | 210519   |
| Dual-Luciferase Reporter Assay System | Promega | E1960      |
| MAXiScribe™ SP6/T7 Transcription Kit | Thermo Fisher | AM1322 |
| mirPremier microRNA Isolation Kit | Sigma Aldrich | SNC50-1KT  |
| miRNA cDNA Synthesis Kit | ABM | G270       |
| Northern Blot Assay | Signosis | NB-3001    |

**Experimental models: Cell lines**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Human: HEK 293T     | Human: HEK 293T |            |
| Human: Huh7         | Human: Huh7 |            |
| Human: PH5CH        | Human: PH5CH |            |

**Experimental models: Organisms/strains**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse: Alb-CreERT2  | Gift from Dr. Daniel Metzger and Pierre Chambon, IGBM, Illkirch France | N/A        |
| Mouse: NsSaTg       | Gift from Dr. Ratna Ray (Saint Louis Univ.) | N/A        |
| Mouse: NOD.cg-Prkdc<sup><small>cre</small></sup>/J2rg<sup><small>tg83V/M1</small></sup>/Szj | Jackson Laboratory | 005557     |
| Mouse: FRG          | Gift from Dr. Anne Willis (Leicester Univ.) | N/A        |

**Recombinant DNA**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| pPAX2               | Addgene | Plasmid #12260 |
| pMDZ.G              | Addgene | Plasmid #12259 |
| scrambled shRNA     | Addgene | Plasmid #1864 |
| pRL c-Myc 3’UTR     | Addgene | Plasmid #14806 |
| pRetrosuper Myc shRNA | Addgene | Plasmid #15662 |
| MSI2 Lentiviral Vector | AMB | LV712475   |
| MSI2 shRNA Lentiviral Vector | AMB | i014309   |
| pSK-ML              | Gift from Dr. Anne Willis (Leicester Univ.) | N/A        |
| pSK-GAP-L           | Gift from Dr. Anne Willis (Leicester Univ.) | N/A        |
| pRF                 | Gift from Dr. Anne Willis (Leicester Univ.) | N/A        |

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Keigo Machida (keigo.machida@med.usc.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
● All data reported in this paper will be shared by the lead contact upon request.
● This paper does not report original code.
● Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Detailed materials and methods are described in STAR Methods section, Figures 1–3, and supplemental information.

In vivo animal studies
Animal handling followed AALAC and National Institutes of Health guidelines, and experimental procedures were approved by the IACUC. Both female and male were used.

Subject details
Paraffin embedded tissue sections were obtained from both females and males in accordance with the approved Institutional Review Board (IRB). There were two institutions [University of Southern California and University of Minnesota] that gave Institutional Review Board (IRB) approval for the supplied specimens. Specimens from both females and males were obtained from the Liver Tissue Cell Distribution System (LTDCS) at the University of Minnesota according to the following criteria: surgically excised HCC tissues from 8 patients +/- HCV infection, +/- history of alcohol misuse, +/- obesity/diabetes/BMI>30. Twenty specimens were also obtained from the Hepatobiliary and Liver Transplantation Service at the USC Keck School of Medicine.

METHOD DETAILS

Human subjects
We obtained access to a large collection of liver cryosections from HCV patients exhibiting different stages of HCV infection, including early-stage infection, hepatitis, cirrhosis, HCC, or late-stage HCC. These
selected patient materials were HCV-infected only or had co-morbidities of alcoholism or/and diabetes. Inclusion criteria for further analysis were HCV positivity and/or diabetes. Clinicopathological factors are summarized in Table S1. Informed consent was obtained from human subjects after IRB approval.

Genomic PCR to detect c-Jun
To detect c-Jun<sup>Flox/Flox</sup> genotype, Flox sites targeting the c-Jun sequence flanked by loxP sites were tested by genotyping PCR reactions (Palmada et al., 2002).

Mice and feeding
Hepatocyte-specific Cre expression from Albumin promoter (Alb::Cre) was used to generate liver-specific knockout of c-Jun (c-Jun<sup>fl/fl</sup>;Alb::Cre) (Palmada et al., 2002). c-Jun<sup>fl/fl</sup> mice are gifts from Dr. Carter in Vanderbilt University. NSSA Tg mice were crossed with c-Jun<sup>fl/fl</sup>;Alb::Cre or c-Jun<sup>fl/fl</sup> to establish c-Jun<sup>Δhep</sup> and c-Jun<sup>+/+</sup> Na5a Tg mice. (Sarcar et al., 2004; Van Heek et al., 1997). To determine the effect of c-Jun gene disruption on synergistic tumor incidence influenced by high-cholesterol high-fat diet (HCFD) in the NSSA Tg mice, NSSA Tg mice were crossed with Alb::Cre;c-Jun<sup>fl/fl</sup> (c-Jun<sup>Δhep</sup>) mice or wild type mice to produce the double Tg mice in the c-Jun knockout background. These mice were fed HCFD to determine whether synergistic consequences of c-Jun expression on tumor incidence was abrogated by TLR4 deficiency. Twenty-five mice in each treatment group (based on the power analysis) were used for this experiment. These mice were fed HCFD to determine the effect of c-Jun deficiency on synergism of diet on tumor incidence. At the end of the study period (12 months), mice were euthanized for gross observation and digital photography of the excised whole livers for determination of the presence, size, and number of liver tumors. II mice experiments were approved by the Institutional Animal Care and Use Committee.

Glucose production assay
HepG2 cells (5 x 10<sup>5</sup>/well) were washed three times with PBS to remove glucose and then were incubated in a 6 well plate for 16 h in 2 ml of glucose production medium (glucose- and phenol red-free DMEM containing gluconeogenic substrates, 20 mM sodium lactate, and 2 mM sodium pyruvate) and in the presence of 1 nM insulin (Usbio) during the last 3 h. A quantity of 300 μl of medium was sampled for measurement of glucose concentration using a glucose assay kit (Sigma).

Flow cytometry glucose uptake assay
A glucose uptake assay using 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose) (2-NBDG) was performed as previously described with minor modifications. Briefly, HepG2 cells transfected with c-Jun expression vector or vector control were cultured for 24 h, maintained in serum-free DMEM with or without 1 mM insulin with the absence or presence of 10 mM 2-NBDG for 2 h. The fluorescence intensity of 2-NBDG was recorded on the FL1 channel using a Caliber flow cytometer. Data from 10,000 single-cell events were collected. To exclude false-positivity, cells in the absence of 2-NBDG were measured and used as the background. The relative fluorescence intensities minus the background were used for subsequent data analysis.

Colonies formation assay
DMEM/Ham’s F-12 media (Caisson; 50 mL FBS, 1X non-essential amino acids, 1X Penicillin/Streptomycin/ Glutamine, 200 mg/ml mEGF, and 5 mM dextrose for 500 ml media) was warmed to 37°C in a water bath. 5% agar (Apex BioResearch Products) was melted in PBS in the microwave for 2 min. The 5% agar was mixed in a 1:10 ratio with the warmed DMEM/Ham’s F-12 to give 0.5% agar in culture media. In a Falcon 24-well plate 0.5 ml of the 0.5% agar/culture media was added to each well and allowed to solidify. Core and Core sh-cJun treated (sh-RNA from Sigma) cells were trypsinized from their 10 cm culture dishes and counted in an Invitrogen Countess automated cell counter. 12,500 core or core sh-c-Jun cells were added to 9 ml of culture media and incubated in a 37°C water bath. 3% agarose (Apex BioResearch Products) was melted in PBS in the microwave for 3 min. 1 ml of the 3% melted agarose solution was added to the 9 ml of cell suspension and mixed. 0.5 ml this agarose-cell solution was added to wells in triplicate for each cell line. The plates were incubated in a 37°C humidified incubator for 7 days. The plates were then stained with 0.5 ml of 0.005% crystal violet per well at room temperature for 2 hours and left overnight at 4°C. Plates were washed 15 times with PBS for an hour each wash. Each well was photographed, and the cells were counted via CellCounter program (Nghia Ho).
Luciferase reporter assay
To study the role of c-JUN on human RICTOR and GLUT2 promoter activity, a series of RICTOR-Luc and GLUT2-Luc plasmids were transfected into HepG2 cells using a lipid-based transfection reagent (BioT, Bio-land). 7x10⁴ cells/well were seeded onto a 24-well plate and transfected with luciferase constructs and appropriate control vectors (500ng total plasmid DNA). At 48 h after transfection, samples were harvested and assayed for luciferase activity using a dual-luciferase reporter assay system (E1980, Promega). Luciferase activity was measured with a Lumat LB 9501 instrument (Berthold). Firefly luciferase activity was normalized to Renilla luciferase activity for each sample (n = 3).

In-vitro mutagenesis
Mutant primers were created (Integrated DNA Technologies) to mutate Ap-1/c-Jun transcription factor binding sites on the Rictor and Glut2 promoter luciferase constructs. PCR was used to create mutated plasmids. Mutations were confirmed by Sanger sequencing (Genewiz).

Antibodies
c-JUN (1:2000 dilution: H-79, Rb pAb, sc-1694, Santa Cruz), p-c-JUN (1:10000 dilution: Ser 63/73, Gt pAb, sc-16312, Santa Cruz), c-JUN (1:2000 dilution: Rb pAb, ab31419, abcam) c-JUN (1:2000 dilution: 60A8, Rb mAb, Cell Signaling), RICTOR (1:2000 dilution: PA5-18370, Invitrogen), RICTOR (1:2000 dilution: H-11, Ms mAb, sc-271081, Santa Cruz), NANOG (1:2000 dilution: M-17, Gt pAb, sc-30329, Santa Cruz), GSK3b (1:2000 dilution: 27C10, Rb pAb Cat #9315S, Cell Signaling), P-GSK3b (1:2000 dilution: S9, 5B3, Rb mAb, Cat #9323S, Cell Signaling), c-JUN (1:2000 dilution: 60A8, Rb mAb, abcam) c-JUN (1:2000 dilution: 60A8, Rb mAb, Cell Signaling), RICTOR (1:2000 dilution: PA5-18370, Invitrogen), RICTOR (1:2000 dilution: H-11, Ms mAb, sc-271081, Santa Cruz), NANOG (1:2000 dilution: M-17, Gt pAb, sc-30329, Santa Cruz), GSK3b (1:2000 dilution: 27C10, Rb pAb Cat #9315S, Cell Signaling), P-GSK3b (1:2000 dilution: S9, 5B3, Rb mAb, Cat #9323S, Cell Signaling), Glycogen synthase (1:2000 dilution: 15B1, Rb mAb, sc-3886S, Cell Signaling), P-Glycogen synthase (1:2000 dilution: S64I, Rb Ab, Cat #3891S, Cell Signaling), and GLUT2 (1:2000 dilution: C-10, Ms mAb, sc-518022, Santa Cruz).

Immunohistochemistry
To characterize c-JUN and GLUT2 expression in patient HCC tissue, formalin fixed paraffin embedded (FFPE) sections were used. Selection criteria of patient material included the presence of tumor and adjacent non-tumor sections with comorbidities of HCV and/or alcoholic cirrhosis. Only tissue sections that produced quantifiable c-JUN and GLUT2 staining were included for analysis (n=3). Table S2 summarizes the clinical pathological information of the tissue sections used in this study.

IHC staining of liver tumor sections was optimized by altering individual variables. Optimization of paraffin removal was achieved by varying the time the slides baked and time spent in xylene. Rehydration of tissue was optimized by altering the concentration and time spent in each ethanol solution. Antigen retrieval was optimized by comparing four different antigen retrieval solutions at different pH, temperature, and pressure. Titration of antibodies (1:1000 to 1:200) was used to identify the antibody concentration for optimal staining.

FFPE tissue sections were heated at 60°C overnight. Slides were de-paraffinized with xylene and rehydrated using dilutions of ethanol. Antigen retrieval was performed in Citrate ETDA buffer (10 mM Citric Acid, 2 mM EDTA, 0.05% Tween-20, pH 6.2) using a Tender cooker (Nordic Ware, #62104) and conventional microwave for 20 min on High. Slides were blocked overnight using 5% goat serum and 10% BSA in PBS. Slides were incubated with primary antibodies overnight at 4°C and for 2 hours at room temperature with secondary antibodies. Slides were mounted and DNA was stained using anti-fade mounting media with DAPI (H-1200, Vector). Images were captured using confocal microscopy using Leica software. Immuno-Reactivity Score (IRS) was calculated by multiplying the frequency and intensity of staining on five different areas of each tissue section. An average IRS score was calculated for each tumor section.

Metabolomic analysis
Global biochemical profiles were determined in CD133-negative primary hepatocytes and tumor initiating cells, with comparisons between scrambled shRNA-treated (Scrambled) and NANOG shRNA-treated (NANOG) groups. For media samples, unspent media was provided as a baseline control.

c-Jun genotyping STAR Methods
Advantage cDNA polymerase (50x: Clontech (catalogue #639105) was used for genotyping with genomic DNA (150ng) in 50 µl reaction. The primers for c-Jun genotyping are as follows: forward primer:
5’-AGCAACTTTCCTGACCCAGA-3’; reverse primer: 5’-CGTCCCTGCTTCTGTAACAA-3’.

1) 95°C: 5 min 2) 95°C: 30 sec 3) 58°C: 30 sec 4) 72°C: 1 min Repeat steps 2-4 for cycling 35 times 5) 72°C: 10 min

Expected PCR amplicon sizes are 600 bp for flox/flox loci and 400 bp for WT loci.

Cell culture STAR Methods
HepG2, HEK293T and Mouse TIC cells were used in the study. HepG2 is a HCC cell line that was derived from the liver tissue of a 15-year-old Caucasian American male with a well-differentiated HCC. Because of their high degree of morphological and functional differentiation in vitro, HepG2 cells are a suitable model to study the intracellular trafficking (insulin signaling), sinusoidal membrane proteins and lipids in human hepatocytes in vitro. HEK 293T is a variant type of the Human embryonic kidney 293 cells containing the large T-antigen of the SV40 virus. This variant helps achieve episomal replication of transfected plasmids and generally used in retroviral vectors. These cells form the basis for a lot of retroviral packaging cell lines. Human hepatocellular liver carcinoma (HepG2) cells and HEK293T cells were cultured in DMEM supplemented with 20% heat-inactivated fetal bovine serum, antibiotics and non-essential amino acids. Cell Line was cultured at 37°C in a 5% CO2 humidified atmosphere. Mouse TICs were cultured in DMEM HamF12 supplemented with 20% heat-inactivated fetal bovine serum, antibiotics, non-essential amino acids, Nucleosides, mEGF. HepG2 cells were incubated in serum-free medium with the labeled glucose for glucose uptake assay and glucose free media for glucose production assay.

Transfection STAR Methods
HepG2 cells were transiently transfected with c-Jun overexpression vector using Transient BioT transfection method. This type of transfections was carried out with BioT method.

Western blot and immunoprecipitation
Western blot analysis was carried, using antibodies against C-Jun and Rictor (Santa Cruz Biotechnology Inc); FOXO1, FOXO1S256, AKT, AKTS473, AKTT308, mTOR, GSK and P-GSK (Cell Signaling Technology); and Sin1 (Bethyl Laboratories). Complex formation of mTORC2 complex was analyzed by immunoprecipitation. Liver tissues were collected from high fat diet fed mice and high fat diet fed mice in which c-Jun was knocked out in liver specific manner.

qRT-PCR
Total RNA was isolated from HepG2 cells and HepG2 overexpressed with c-Jun using Qiagen RNeasy mini kit (Qiagen, Inc., Venlo, Netherlands) according to the manufacturer’s protocol, and the RNA concentration was measured using Thermo Scientific NanoDrop™ Spectrophotometer. cDNA was synthesized from the RNA templates using Random primers and 10 mM dNTPs under the following conditions- 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. Real-time PCR analysis was performed on ABI 7900 HT qPCR system (Life Technologies, Carlsbad, CA) using SYBR Green qPCR Master Mix (Stratagene) according to the manufacturers’ instructions. β-actin was used as endogenous reference control.

Luciferase activity assay
pGL3B vectors containing different Rictor promoter sequences were transfected into HepG2 cells and HepG2 cells over expressed with c-Jun. After a 24-h incubation, the luciferase activity was assessed with the Dual-Luciferase Reporter Assay Kit (Promega).

ChIP-qPCR
To determine how signaling pathways differentially regulate gene expression, it is necessary to identify the interactions between transcription factors (TFs) and their cognate regulatory DNA elements. ChIP involves crosslinking of the protein–DNA complex within an intact cell using crosslinking agents, such as formaldehyde. The DNA is then sheared to smaller pieces (~500 bp) by sonication or nuclease digestion. The sheared protein-bound DNA is then immunoprecipitated using a highly specific Ab against the protein. An aliquot of the sheared DNA before immunoprecipitation is used as a reference sample. The protein–DNA complexes from reference and ChIP samples are then reverse crosslinked. The DNA is enriched by chromatin immunoprecipitation over the DNA isolated from reference sample were analyzed using quantitative PCR (1). ChIP assay was performed using mouse TICs on Rictor promoter with AP-1/c-JUN antibody (Abcam Inc.).
**Histology STAR Methods**

Gross observation of human liver slices for histological determination was performed as previously described (2). Hepatitis patient samples were included since our hypothesis included enhanced inflammatory response through the TLR4 and c-Jun axis. Immunostaining for TLR4, and phosphorylated c-Jun in blinded liver sections was conducted and the staining was assessed. Morphometric analysis of the staining was performed and correlated with the patients’ background, liver pathology and tumor formation after the coding was unsealed. We assessed the cell type which expressed these proteins (hepatocytes, Kupffer cells, stellate cells, or inflammatory cells) by immunostaining for cell-type specific markers. This study was approved the USC IRB. Gross observation and digital photographs of the excised whole liver were examined for the presence, size, and number of liver tumors. The standardized protocol for collection of liver slices for histological determination was performed as previously described (2).

**Sample accessioning STAR Methods**

Each sample received was recorded into the Metabolon LIMS system and was assigned by the LIMS a unique identifier, which was associated with the original source identifier only. This identifier was used to track all sample handling, tasks, results etc. The samples (and all derived aliquots) were bar-coded for tracking by the LIMS system. All portions of any sample were automatically assigned a new unique identifier by the LIMS whenever a new process was created; the relationship of these samples was also tracked and recorded. All samples were maintained at -80°C until processed.

**Sample preparation STAR Methods**

The sample preparation process was carried out using the automated MicroLab STAR® system (Hamilton Co.). Recovery standards were included prior to the first step in the extraction process for quality control purposes. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into two fractions; one for analysis by UPLC-MS/MS and one for analysis by GC/MS. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS.

**Quality assurance/quality control STAR Methods**

For QA/QC purposes, a number of additional samples were included with each day’s analysis. Furthermore, a selection of QC compounds was added to each sample, including those under test. These compounds are carefully chosen so as not to interfere with the measurement of the endogenous compounds. These QC samples are primarily used to evaluate the process control for each study as well as aiding in the data curation.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Statistics**

The Student’s t test was used to compare differences between samples analyzed. P values of less than 0.05 were considered as statistically significant. *: p<0.05, **: p<0.01, ***: p<0.001. Data are represented as mean ± SD. Data represent the average ± S.D. Statistical details of experiments can be found in the figure legends, figures, results. N= number of samples used to calculate statistical significance.