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Dual Role for Inositol-Requiring Enzyme 1α in Promoting the Development of Hepatocellular Carcinoma During Diet-Induced Obesity in Mice

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Obesity is associated with both endoplasmic reticulum (ER) stress and chronic metabolic inflammation. ER stress activates the unfolded protein response (UPR) and has been implicated in a variety of cancers, including hepatocellular carcinoma (HCC). It is unclear whether individual UPR pathways are mechanistically linked to HCC development, however. Here we report a dual role for inositol-requiring enzyme 1α (IRE1α), the ER-localized UPR signal transducer, in obesity-promoted HCC development. We found that genetic ablation of IRE1α in hepatocytes not only markedly reduced the occurrence of diethylnitrosamine (DEN)-induced HCC in liver-specific *IRE1α* knockout (LKO) mice when fed a normal chow (NC) diet, but also protected against the acceleration of HCC progression during high-fat diet (HFD) feeding. Irrespective of their adiposity states, LKO mice showed decreased hepatocyte proliferation and signal transducer and activator of transcription 3 (STAT3) activation, even in the face of increased hepatic apoptosis. Furthermore, IRE1α abrogation blunted obesity-associated activation of hepatic inhibitor of nuclear factor kappa B kinase subunit beta (IKKβ)-nuclear factor kappa B (NF-κB) pathway, leading to reduced production of the tumor-promoting inflammatory cytokines tumor necrosis factor (TNF) and interleukin 6 (IL-6). Importantly, higher IRE1α expression along with elevated STAT3 phosphorylation was also observed in the tumor tissues from human HCC patients, correlating with their poorer survival rate. *Conclusion*: IRE1α acts in a feed-forward loop during obesity-induced metabolic inflammation to promote HCC development through STAT3-mediated hepatocyte proliferation. (HEPATOLOGY 2018; 68:533-546).

Hepatocellular carcinoma (HCC) is the major form of primary liver cancer, and is the second leading cause of cancer deaths, owing to its poor 5-year survival rate.[1] The initiation and malignant progression of HCC rely upon the complex interactions between genetic, environmental, and lifestyle factors. Human epidemiological studies have established overweight and obesity as profound carcinogenic risk factors,[2] and an up to 4.5-fold increase in relative HCC risk has been documented in male

Abbreviations: ANOVA, analysis of variance; Bloc1s1, biogenesis of lysosomal organelles complex-1, subunit 1; CHOP, C/EBP homologous protein; DEN, diethylnitrosamine; DR5, death receptor 5; eIF2α, eukaryotic translation initiation factor 2 alpha; ER, endoplasmic reticulum; ERN1, endoplasmic reticulum to nucleus signaling 1; HCC, hepatocellular carcinoma; HFD, high-fat diet; IHC, immunohistochemistry; IKKβ, inhibitor of nuclear factor kappa B kinase subunit beta; IL-6, interleukin 6; IRE1, inositl-requiring enzyme 1; IRE1α, inositol-requiring enzyme 1α; LKO, liver specific IRE1α *knockout; NASH, nonalcoholic steatohepatitis; NF-κB, nuclear factor kappa B; STAT3, signal transducer and activator of transcription 3; TMA, tissue microarray; TNF, tumor necrosis factor; TRAF2, TNF-receptor-associated factor 2; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; UPR, unfolded protein response; PCNA, proliferating cell nuclear antigen; PERK, PKR-like endoplasmic reticulum kinase; XBP1, X-box binding protein 1.*

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subjects with obesity.[3] Given that the global prevalence of obesity is reaching an alarming epidemic proportion with estimated 300 million obese individuals worldwide,[4,5] such an increase in HCC risk will pose a daunting public health problem. Thus, it is of utmost importance to understand obesity-associated tumor-promoting mechanisms underlying the development of HCC.

A great majority of HCC develops in the context of chronic liver damage arising from exposure to carcinogens, hepatitis viral infections, and nonalcoholic steatohepatitis (NASH).[6] NASH is a severe stage of nonalcoholic fatty-liver disease (NAFLD) that is highly associated with obesity.[7] Recent studies have revealed several potential mechanisms for the HCC–promoting impact of obesity and NAFLD, including inflammation, endoplasmic reticulum (ER) stress, and oxidative stress.[8-13] Proinflammatory cytokines, particularly tumor necrosis factor (TNF) and interleukin 6 (IL-6), whose circulating levels are typically elevated in obesity,[14] have been shown to play pivotal roles in obesity and NASH promotion of carcinogen-induced HCC development.[9] Moreover, both ER and oxidative stresses are known to induce hepatocyte death, resulting in liver damage that stimulates the compensatory proliferation of differentiated hepatocytes.[10,11] During this process, activation of nuclear factor kappa β kinase subunit beta (IKKb)-nuclear factor kappa β (NF-κB) as well as signal transducer and activator of transcription 3 (STAT3) in initiated hepatocytes is thought to drive the malignant progression of HCC.[8,9] Notably, in chemical carcinogen diethylnitrosamine (DEN)-induced HCC mouse models, TNF signaling was shown to be critical in the promotion by obesity and ER stress of HCC development under the condition of overnutrition[9,10]; however, loss of hepatocyte inhibitor of nuclear factor kappa β kinase subunit beta (IKKβ) or NF-κB, the downstream component of the canonical TNF pathway, was reported to enhance chemical hepatocarcinogenesis in the absence of obesity.[11,12,15] Thus, it remains largely obscure whether there exist common mechanisms linking obesity, ER stress, and hepatic inflammatory microenvironment to the malignant progression of HCC.

Perturbations of the ER function as a result of excessive accumulation of unfolded/misfolded proteins or alterations in ER lipid compositions lead to ER stress and activation of the adaptive unfolded protein response (UPR).[16,17] Three ER-localized transmembrane signal transducers, inositol-requiring enzyme 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6), function to govern the cellular UPR program.[16] Persistent activation of these ER stress sensors is thought to confer a greater tumorigenic capacity upon malignant cells.[18,19] Although both metabolic ER stress and chronic inflammation have been found in the state of obesity,[20-22] it has yet to be dissected if individual UPR branches are directly involved in promoting liver damage, inflammation, and compensatory proliferation during HCC development in the face of obesity. In particular, IRE1 is the most conserved ER stress sensor that possesses both Ser/Thr kinase and endoribonuclease activities.[23] Upon ER stress, IRE1 is activated through autophosphorylation and dimerization/oligomerization, and regulates the non-conventional splicing of X-box binding protein 1 (*Xbp1*) mRNA or degrades a select subset of mRNAs via a process termed regulated IRE1-dependent decay (RIDD).[23] Our previous studies in mouse models have revealed that hepatic inositol-requiring enzyme 1α (IRE1α) is hyperactivated in the state of obesity,[24,25] and it has an important role during reparative liver regeneration.[26] Here we investigated whether IRE1α is able to connect metabolic ER stress to promotion of hepatocyte proliferation and HCC

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development during overnutrition-induced obesity. We found that IRE1α expression correlated with human HCC, and through its impact upon both the IKKβ-NF-κB and STAT3 pathways, IRE1α could exert dual actions in obesity acceleration of carcinogen-induced HCC development.

Materials and Methods

ANIMALS

Liver-specific IRE1α knockout (LKO) mice on the C57BL/6 background were generated by intercrossing the endoplasmic reticulum to nucleus signaling 1 (*Ern1*) floxed (*flox/flox*) mice, in which the exon 2 of the *Ern1* allele was flanked by *loxP* sites, with the Alb-*cre* mice as described.[26,27] Mice were maintained at 23±3°C with a humidity of 35±5% under a 12/12-hour dark/light cycle (lights on at 6:30 AM), with free access to water and food (Shanghai Laboratory Animal Co. Ltd, Shanghai, China). HCC was induced by intraperitoneal injection with one dose of DEN (#N0725, Sigma Aldrich, St. Louis, MO) at 25 mg/kg in 2-week-old male mice. After 4 weeks, mice were either fed the NC or an high-fat diet (HFD) (60% fat in calories; Research Diets, #D12492, New Brunswick, NJ) for the desired periods of time until being sacrificed. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

HISTOLOGIC, TUNEL AND IMMUNOHISTOCHEMICAL ANALYSES

Sectioned liver tissues were fixed in 10% formalin or embedded in Tissue-Tek O.C.T. Compound (#SA62550-01, Sakura Finetek, Torrance, CA) for paraffin and frozen block preparation, respectively. Paraffin-embedded liver sections were subjected to hematoxylin and eosin staining, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) analysis, and immunohistochemistry (IHC). Cellular apoptosis was analyzed using the Dead End Fluorometric TUNEL System (Promega Corp., Madison, WI) according to the manufacturer's instructions. Apoptotic signals in liver sections were visualized by fluorescence microscopy. IHC analyses of Ki-67, proliferating cell nuclear antigen (PCNA) and K19 were performed. Briefly, liver slices were permeabilized with blocking buffer (5% BSA/0.25% TX-100 in phosphate-buffered saline [PBS]) and incubated with the Ki-67 (#550609, BD Bioscience, San Jose, CA), PCNA (#13110, Cell Signaling Technology, Danvers, MA) or K19 (sc-376126, Santa Cruz, Dallas, Texas) antibody overnight at 4 °C. After washing with PBS, samples were incubated with HRP-conjugated secondary antibody (Invitrogen, Carlsbad, CA) before analysis by microscopy (PerkinElmer, Waltham, MA). Ki67-, PCNA- and TUNEL-positive cells were quantified using Image J software.

All other materials and methods are described in Supplemental Information.

STATISTICAL ANALYSIS

Data are presented as the mean ± standard errors of the mean (s.e.m.). Statistical analysis was conducted using unpaired two-tailed t-test, one-way or two-way analysis of variance (ANOVA), followed by Bonferroni's post hoc tests with GraphPad Prism 5.0. $P<0.05$ was considered statistically significant.

Results

ABLATION OF HEPATOCYTE IRE1α PROTECTS AGAINST OBESITY-PROMOTED HCC DEVELOPMENT

Overnutrition has been reported to promote DEN-induced or ER stress-evoked hepatic tumorigenesis in mice maintained on an HFD.[9,10] In accordance with these studies, we also observed markedly increased incidence of HCC in DEN-treated male mice following 24 weeks of HFD feeding when compared to those fed a normal chow (NC) diet (Fig. S1A-C). Moreover, immunoblot analysis revealed significantly higher phosphorylation levels of hepatic IRE1α, PERK and eukaryotic translation initiation factor 2 alpha (eIF2α), and elevated expression levels of BiP, in parallel with prominently increased phosphorylation activation of the oncogenic transcription factor STAT3, in livers of HFD-fed DEN-treated mice (Fig. S1D). Phos-tag gel analysis of IRE1α (Fig. S1E) and RT-PCR assessment of *Xbp1* mRNA splicing (Fig. S1F) further affirmed higher activation of liver IRE1α in HFD-fed animals. Thus, during obesity promotion of DEN-induced tumorigenesis, HFD feeding results in hyperactivation of hepatic IRE1

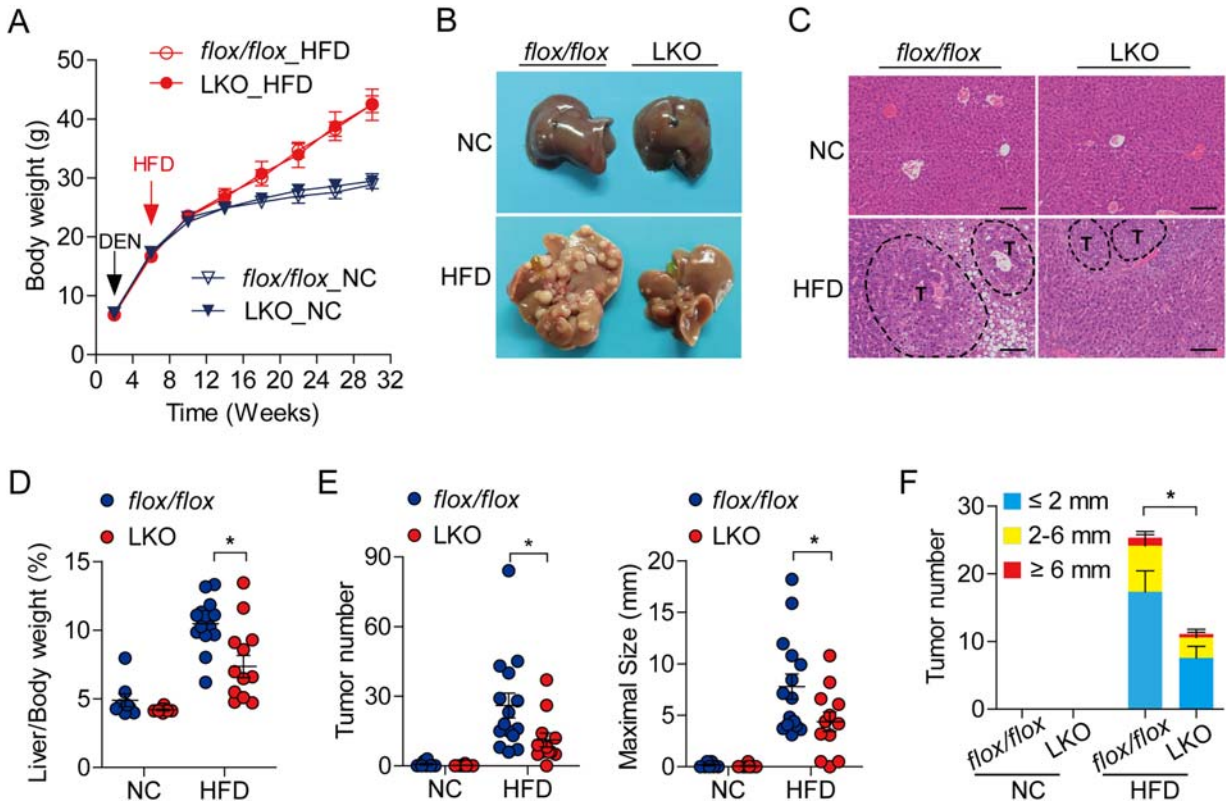

FIG. 1. LKO mice are protected against obesity-promoted HCC development. Two-week-old male LKO mice and their *flox/flox* littermates were i.p. injected with DEN (50 mg/kg body weight) and were maintained on an NC (n=8 per genotype) or fed an HFD beginning at 6 weeks of age (n=12 for LKO, n=15 for *flox/flox*). Mice were sacrificed at 30 weeks of age for HCC analysis. (A) Body weight. (B) Representative images of mouse livers from the indicated group. (C) H&E staining of livers. Tumor (T) regions were indicated by circles. Scale bars, 100 μm. (D) Ratio of liver weight to body weight. (E) Number and maximal size by diameter of liver tumors. (F) Number of liver tumors of various sizes. Data in (D-F) are shown as the mean ± s.e.m., $^*P < 0.05$ by two-tailed unpaired Student's *t*-test or two-way ANOVA.

and PERK pathways of the UPR that accompanied increased STAT3 activation. This indicates a potential link between sustained activation of the ER stress sensors and acceleration of HCC progression.

Given that IRE1α activation was increased in livers of HFD-fed HCC-bearing mice, we employed the LKO mouse model[26,27] to test if hepatocyte IRE1α contributed to the promotion of HCC progression during overnutrition. Intriguingly, following a short-term HFD feeding of 16 weeks, we observed lower liver weight (an indicator of tumor burden) and reduced DEN-induced HCC incidence in male LKO mice than in *flox/flox* control mice (Fig. S2A-C). After 24 weeks of feeding, while no HCC tumors were detectable in NC-fed DEN-treated mice, loss of IRE1α markedly blunted HFD-induced development of HCC in LKO mice without affecting their body weight gain as compared to their *flox/flox* counterparts (Fig. 1A-C). Quantitative analyses revealed that HFD-fed LKO mice had significantly decreased liver weight (by ~40% [Fig. 1D]) and markedly reduced total number (by ~67%) and maximal size (by ~50%) of visible HCC tumors (Fig. 1E). Further distribution assessment showed that the number of various tumor sizes was significantly decreased in LKO mice (Fig. 1F). These results suggest that abrogation of IRE1α in hepatocytes hinders the acceleration of DEN-induced HCC development in the state of dietary obesity.

ABROGATION OF HEPATOCYTE IRE1α SUPPRESSES CHEMICAL TUMORIGENESIS IN LEAN MICE

Next, we asked if IRE1α is directly involved in DEN-induced tumorigenesis in the absence of overnutrition-induced obesity. When maintained on an NC diet and examined at 40 weeks after DEN

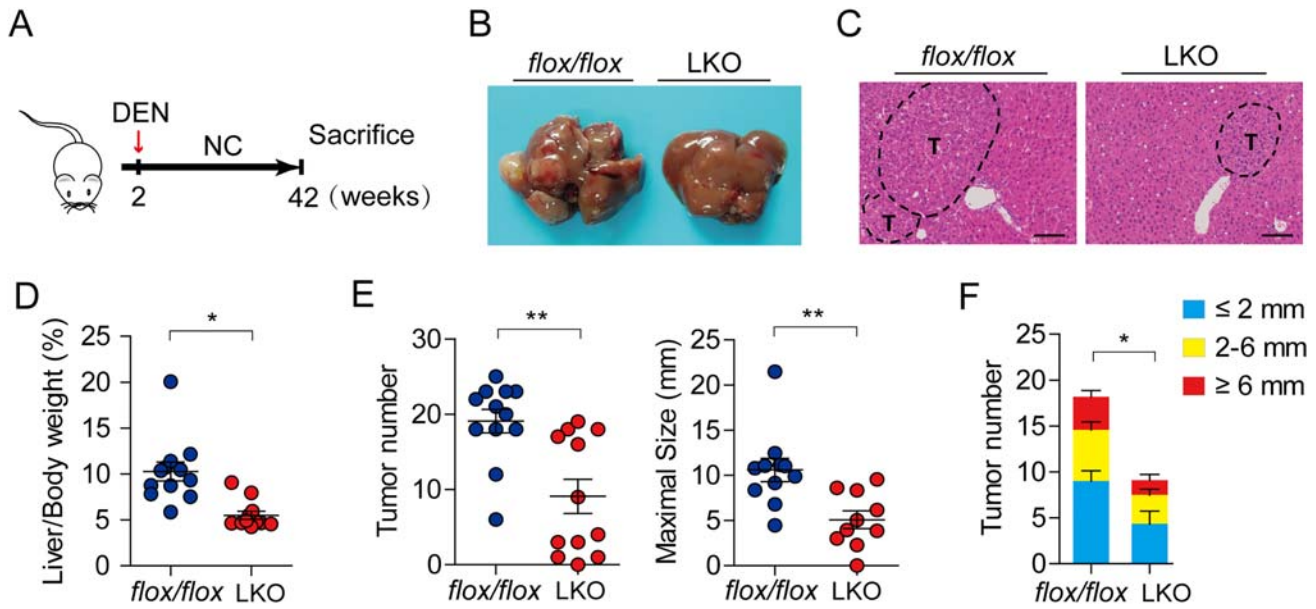

FIG. 2. Hepatocyte IRE1α deletion suppresses HCC development in NC-fed lean LKO mice. Two-week-old LKO mice and *flox/flox* littermates were i.p. injected with DEN (50 mg/kg body weight) and maintained on an NC diet (n=12 per genotype). Mice were sacrificed at 42 weeks of age for HCC analysis. (A) Schematic of the experimental design. (B) Representative images of mouse livers from the indicated genotype. (C) H&E staining of livers. Tumor (T) regions were indicated by circles. Scale bars, 100 μm. (D) Ratio of liver weight to body weight. (E) Number and maximal size of tumors. (F) Number of liver tumors of the indicated sizes. Data in (D-F) are shown as the mean ± s.e.m., $^{*}P<0.05$, $^{**}P<0.01$ by two-tailed unpaired Student's *t*-test or one-way ANOVA.

administration, lower incidence of HCC was detected in LKO mice relative to their *flox/flox* control animals (Fig. 2A-C). Quantitative analysis showed that LKO mice had a ~30% reduction in their liver weight (Fig. 2D) and significant decreases in the number (by ~50%) and maximal size (by ~50%) of HCC tumors (Fig. 2E). Distribution assessment also revealed significantly fewer HCC tumors of various sizes (Fig. 2F). Thus, IRE1α in hepatocytes plays a critical role in carcinogen-induced liver tumorigenesis in addition to mediating the promoting effect of obesity upon HCC progression during overnutrition.

HEPATOCYTE IRE1α ABLATION RESULTS IN ENHANCED HEPATIC APOPTOSIS

To explore the mechanisms by which hepatocyte IRE1α exerts its tumor-promoting effect, we first examined if loss of IRE1α could lead to alterations in hepatic ER stress and cell death that may account for the decreased susceptibility of LKO mice to hepatocarcinogenesis. Short-term DEN administration induced an acute hepatic UPR activation in *flox/flox* control mice, as indicated by elevations in eIF2α phosphorylation, BiP protein expression, *Xbp1* mRNA splicing, as well as the mRNA abundance of C/EBP homologous protein (*Chop*) and its target gene death receptor 5 (*Dr5*),[16,28] and IRE1α deficiency further enhanced these ER stress markers in LKO mice except *Xbp1* mRNA splicing (Fig. S3A-C). At 8 weeks after DEN injection when no HCC tumors were detectable in mice maintained either on NC or HFD, LKO livers showed significantly increased PERK and eIF2α phosphorylation levels despite of decreased PERK protein abundance (Fig. 3A), while exhibiting no significant changes in BiP protein levels. Because hyperactivation of the PERK-eIF2α branch is known to trigger ER stress-associated apoptosis through the activating transcription factor 4 (ATF4)-CHOP cascade,[16,19,28] we measured the mRNA abundance of *Atf4*, *Chop*, and two CHOP target genes, *Dr5* and Tribbles homolog 3 (*Trb3*), as well as DR5 protein level, which was all elevated in LKO livers (Fig. 3A, B). These results indicate that loss of IRE1α could cause an overactivation of the PERK-eIF2α branch in DEN-treated livers, leading to enhanced activation of the apoptotic CHOP pathway.[19] Indeed, TUNEL analyses revealed that

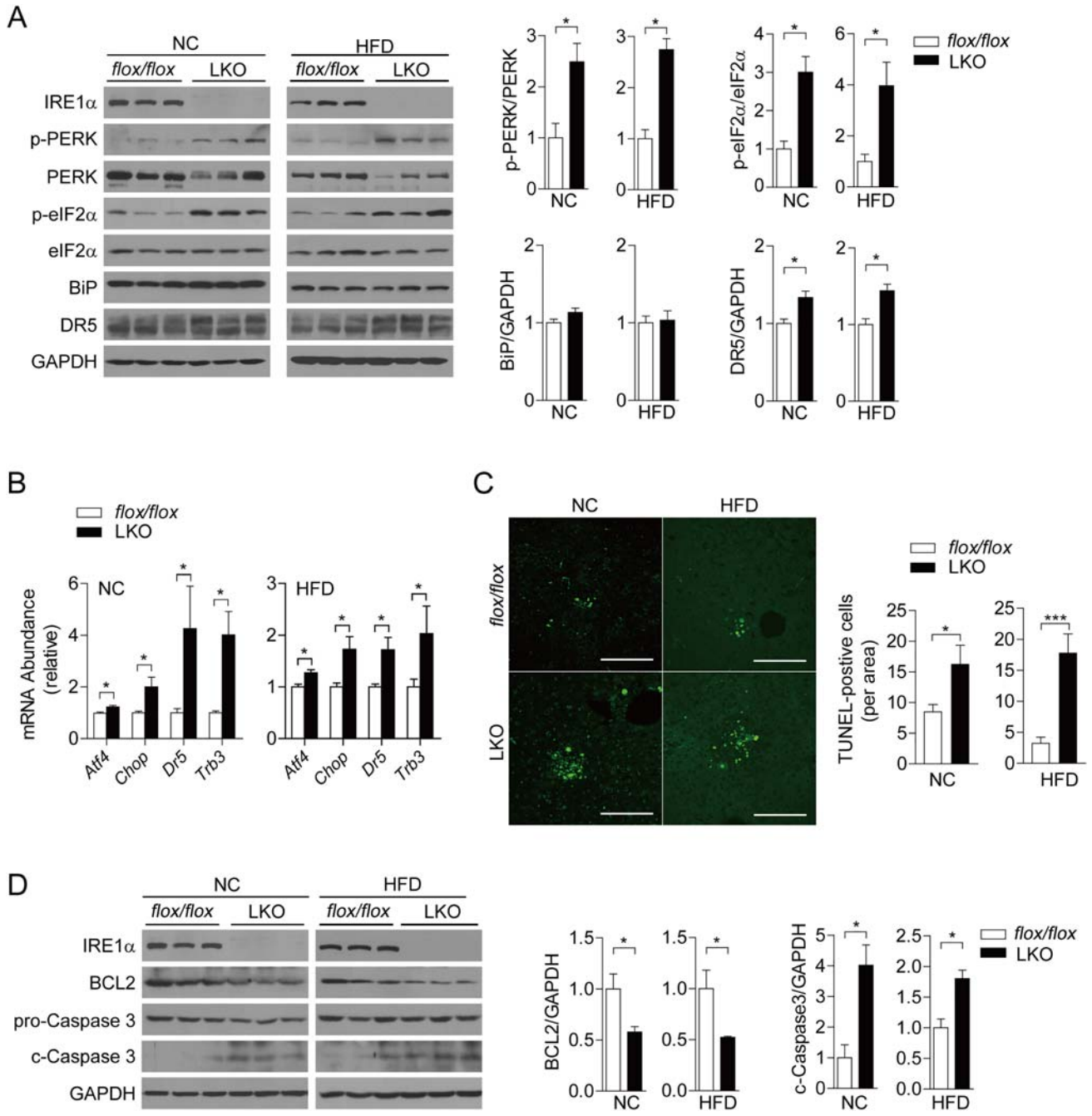

FIG. 3. IRE1α deficiency results in an overactivation of the PERK-eIF2α pathway with increased hepatic apoptosis. Two-week-old male LKO and *flox/flox* mice injected with DEN were either maintained on an NC or an HFD for 8 weeks (n=8 per group). (A) Immunoblot analysis of the phosphorylation of PERK and eIF2α as well as the protein level of BiP and DR5 from liver extracts. Representative immunoblots are shown for three individual mice per group. Shown also are densitometric quantification results after normalization to that of *flox/flox* mice. (B) Quantitative RT-PCR analysis of the indicated genes in livers. (C) Analysis of apoptosis in liver sections. Shown are representative TUNEL labeling images (6 images per liver) and quantifications of TUNEL-positive cells (n=5 per group). Scale bars, 100 μm. (D) Immunoblot analysis of liver BCL2, pro-Caspase-3 and c-Caspase-3 proteins with densitometric quantifications. Data are shown as the mean ± s.e.m, $^{*}P < 0.05$, $^{***}P < 0.001$ by two-tailed unpaired Student's *t*-test.

LKO mice, regardless of NC or HFD feeding after DEN injection, had significantly increased apoptotic cells in their livers relative to their *flox/flox* counterparts (Fig. 3C). Consistently, the anti-apoptotic protein B-cell lymphoma 2 (BCL2) decreased, while the cleaved active form of apoptotic Caspase 3 (c-Caspase 3) increased in LKO livers (Fig. 3D). Moreover, in NC-fed mice at 40 weeks and HFD-fed mice at 24 weeks after DEN injection when HCC tumors were easily detectable (Fig. 1), we detected in HFD-fed animals significantly elevated hepatic *Xbp1* mRNA splicing but an insignificant alteration in the mRNA level of biogenesis of lysosomal organelles complex-1, subunit 1 (*Bloc1s1*) (Fig. S4A), a typical RIDD target gene whose cleavage can be temporally separate from *Xbp1* mRNA splicing.[29,30] Whether fed an NC or an HFD, loss of IRE1α led to markedly reduced *Xbp1* mRNA splicing without a significant change in the mRNA abundance of *Bloc1s1* (Fig. S4A), and resulted in similarly enhanced activation of the PERK-eIF2α branch and increased apoptosis in LKO livers (Fig. S4B,C). Thus, it is possible that the enhanced cell death in LKO livers, if occurring in initiated hepatocytes, might contribute to the protective effect of IRE1α abrogation during DEN-induced tumorigenesis. However, hepatocyte death has been largely documented to stimulate the compensatory proliferation that promotes chemical carcinogenesis in several mouse HCC models.[10-12] Therefore, it is more likely that loss of IRE1α could suppress HCC development through other more predominant mechanisms such as blocking the compensatory proliferation of hepatocytes, even in the face of increased hepatic cell death.

HEPATOCYTE IRE1α ABROGATION IMPEDES HEPATOCYTE PROLIFERATION THROUGH BLUNTING STAT3 ACTIVATION

We then examined the impact of IRE1α deficiency upon hepatocyte proliferation. Indeed, immunostaining analyses revealed markedly reduced Ki-67-, PCNA- and K19-positive cells in LKO livers from DEN-treated mice when maintained on NC for 40 weeks or on HFD for 24 weeks relative to their *flox/flox* counterparts (Fig. 4A-C), suggesting decreased hepatocyte proliferation as a result of IRE1α ablation. Given the critical role of persistent STAT3 activation in driving the proliferation and survival of HCC tumor cells,[9,10,13,31,32] we determined the activation states of STAT3. Immunoblot analysis showed that HFD feeding increased the tyrosine phosphorylation levels of hepatic STAT3 in *flox/flox* mice. By contrast, LKO livers exhibited substantially decreased STAT3 phosphorylation irrespective of NC or HFD feeding (Fig. 4D). Consistently, HFD feeding significantly increased liver mRNA abundance of STAT3 target genes involved in regulating cellular proliferation, myelocytomatosis oncogene (*c-Myc*), FBJ osteosarcoma B-cell lymphoma oncogene (*c-Fos*) and hypoxia inducible factor 1, alpha subunit (*Hif1a*), which were prominently reduced in NC- or HFD-fed LKO mice (Fig. 4E). These results indicate that IRE1α acts to promote hepatocyte proliferation and HCC progression through maintaining the activation of the STAT3 pathway, supporting our previous finding that IRE1α could interact with STAT3 and sustain its activation during reparative liver regeneration.[26] To gain insight into the mechanism for IRE1α regulation of STAT3 activation, we utilized chemical inhibitors of IRE1α. Interestingly, blocking IRE1α's kinase activity by KIRA6 not only reduced the ER stressor tunicamycin-induced IRE1α phosphorylation and *Xbp1* splicing, but also blunted IL-6-stimulated STAT3 phosphorylation in a dose-dependent manner in HepG2 cells (Fig. S5A). By contrast, inhibition of its RNase activity by 4μ8C did not show a discernable effect on STAT3 phosphorylation (Fig. S5B). These data further suggest that IRE1α's kinase, but not RNase, activity may be critical in regulating the activation status of STAT3, in accordance with our reported study showing that IRE1α's kinase but not RNase domain is required for interacting with STAT3.[26]

HEPATIC IRE1α DEFICIENCY ALLEVIATES HFD-INDUCED HEPATOSTEATOSIS AND METABOLIC INFLAMMATION

We further investigated whether hepatocyte IRE1α abrogation prevented obesity-induced acceleration of HCC progression through affecting liver steatosis and metabolic inflammation. HFD feeding for 4 weeks following DEN treatment resulted in higher hepatic lipid and triglyceride levels relative to NC feeding in *flox/flox* mice, and IRE1α ablation significantly reduced this HFD-induced liver steatosis in LKO mice (Fig. 5A,B). HFD feeding in *flox/flox* mice also led to increased serum and hepatic levels of TNFα and IL-6, which were significantly blunted in LKO animals (Fig. 5C,D). Given NF-κB as the classical transcriptional regulator that drives the expression of numerous cytokines and chemokines to maintain the tumor-promoting inflammatory microenvironment,[9] we examined its activation state in the livers of DEN-treated mice. Indeed, IRE1α

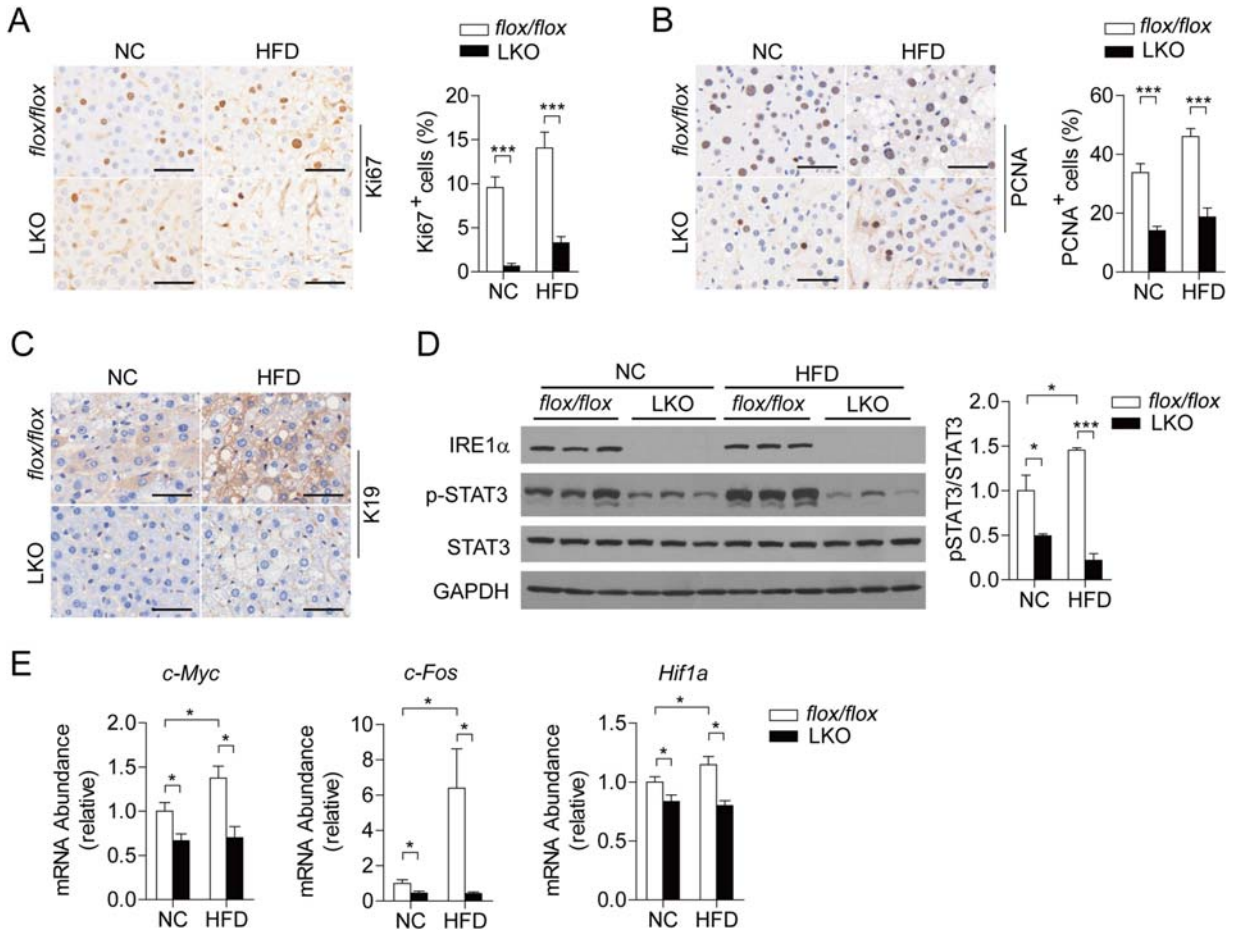

FIG. 4. Loss of IRE1α impairs hepatocyte proliferation and hepatic STAT3 activation. Two-week-old male LKO and *flox/flox* mice were injected with DEN and then maintained on an NC for 40 weeks or fed an HFD for 24 weeks, beginning at 6 weeks of age (n=8 per group). (A, B, C) Representative IHC images of livers stained with anti-Ki67 (A), anti-PCNA (B) or anti-K19 (C) antibody (6 images per liver; n=5 per group). Scale bars, 50 μm. Ki67-positive and PCNA-positive cells were quantified and are shown in percentages, respectively. (D) Immunoblot analysis of liver IRE1α protein and STAT3 tyrosine phosphorylation. Averaged p-STAT3/STAT3 ratios are shown after normalization to that of NC-fed *flox/flox* mice. (E) Quantitative RT-PCR analysis of the indicated STAT3 target genes in the livers. All data are shown as the mean ± s.e.m., $^{*}P < 0.05$, $^{***}P < 0.001$ by two-way ANOVA.

ablation blunted HFD-induced increases in hepatic P65 phosphorylation and *Il-6* mRNA abundance in LKO mice (Fig. 5E,F). These data suggest that hepatocyte IRE1α serves as a crucial promoter of obesity-associated metabolic inflammation to further enhance the IL-6-STAT3 pathway, thus contributing to the acceleration of HCC progression in the state of obesity.

HEPATOCYTE IRE1α MEDIATES HFD-INDUCED ACTIVATION OF THE IKKβ-NF-κB PATHWAY

TNF receptor signaling has been shown to be required for obesity- or ER stress-promoted HCC development.[9,10] Thus, we wondered if IRE1α could link overnutrition-induced obesity to hepatic activation of the canonical TNF signaling cascade, i.e., the IKKβ-NF-κB pathway, in DEN-treated mice. When maintained on an NC diet, hepatocyte IRE1α ablation showed no effects upon the phosphorylation of IKKβ, IκBα and P65, as well as the protein level of IκBα, in livers of DEN-treated LKO mice relative to their *flox/flox* counterparts (Fig. 6A). By contrast, following 4 weeks of HFD feeding, DEN-treated LKO livers exhibited marked reductions in the phosphorylation of IKKβ, IκBα and P65, along with an increase in IκBα protein level (Fig. 6A). Consistently, increased cytoplasmic accumulation and decreased nuclear level of P65

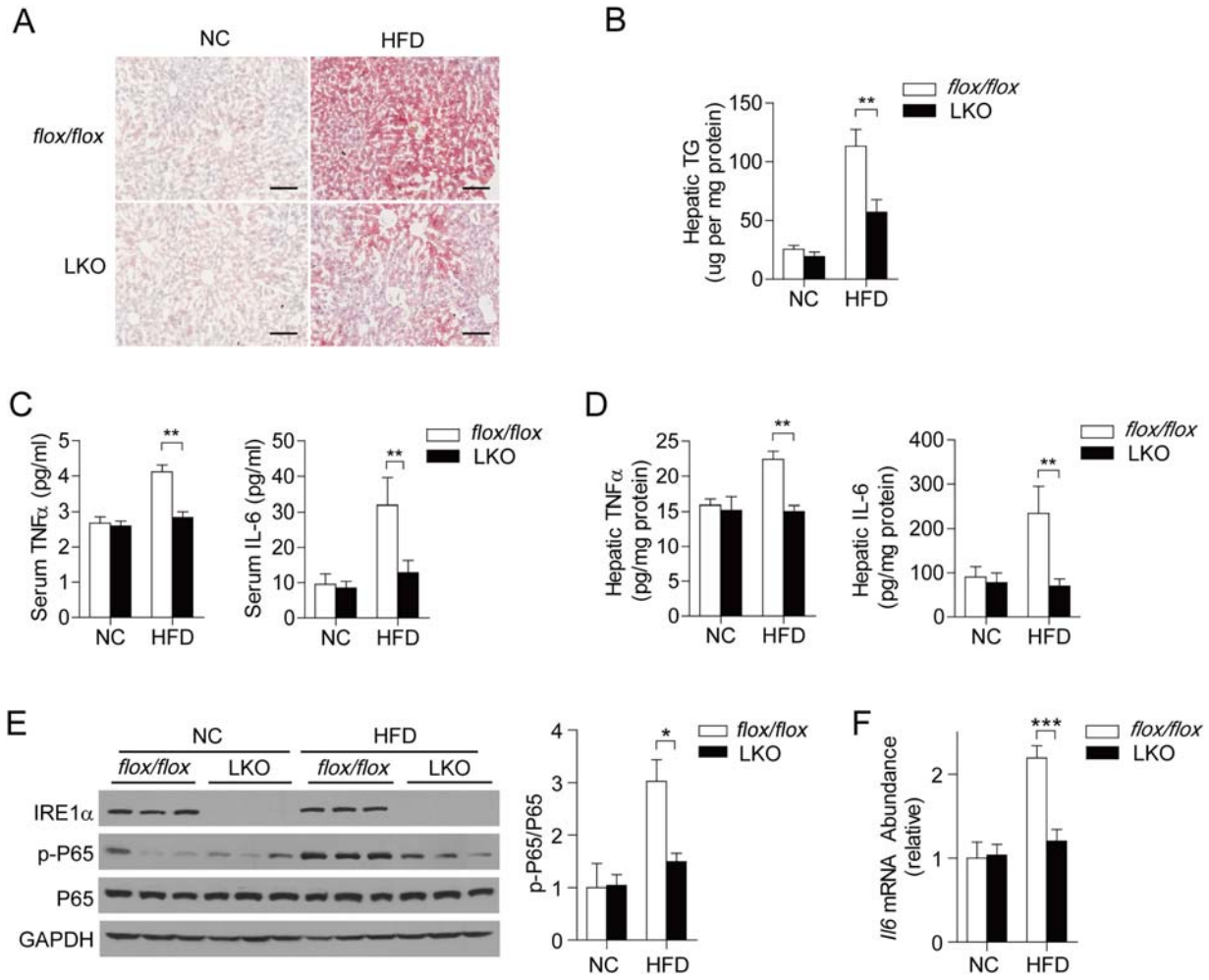

FIG. 5. Loss of hepatic IRE1α attenuates HFD-induced hepatic steatosis and inflammation. Male LKO and *flox/flox* mice injected with DEN were either maintained on an NC or an HFD for 4 weeks (n=8 per group). (A) Representative images of livers stained with Oil-red O (10 images per liver; n=5 per group). Scale bars, 100 μm. (B) Liver triglycerides content. (C) Circulating levels of TNFα and IL-6. (D) Liver levels of TNFα and IL-6. (E) Immunoblot analysis of liver IRE1α protein and phosphorylation of NF-κB P65 subunit. Shown are representative results of three individual mice from each group. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control. Phosphorylation levels of P65 were determined by densitometric quantification of the immunoblots, and averaged p-P65/P65 ratios are shown after normalization to that of NC-fed *flox/flox* mice. (F) Relative *Il6* mRNA abundance in the livers. Results in (B-F) are shown as the mean ± s.e.m., $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by two-way ANOVA.

protein were seen in LKO livers following HFD-feeding (Fig. 6B). These data indicate that hepatocyte IRE1α abrogation leads to suppression of obesity-associated activation of hepatic IKKβ-NF-κB pathway.

Then we examined whether IRE1α was able to mediate TNF activation of the IKKβ-NF-κB pathway in a cell-autonomous fashion. NF-κB-activated luciferase reporter assays showed that siRNA knockdown of the expression of IRE1α substantially diminished the stimulation by recombinant TNFα of NF-κB transcription activity in HepG2 cells (Fig. 6C). Consistently, IRE1α deficiency resulted in marked suppression of TNFα-induced phosphorylation of IKKβ, IκBα and P65, along with elevated protein level of IκBα in HepG2 cells (Fig. 6D). However, siRNA knockdown of XBP1 expression (Fig. S6A) had no effect on TNFα-stimulated transcriptional activation of NF-κB (Fig. S6B), and did not alter the phosphorylation state of IKKβ, IκBα or P65 while causing an appreciable elevation of IRE1α phosphorylation (Fig. S6C). These results demonstrate that IRE1α is coupled to TNF signaling to promote NF-κB activation

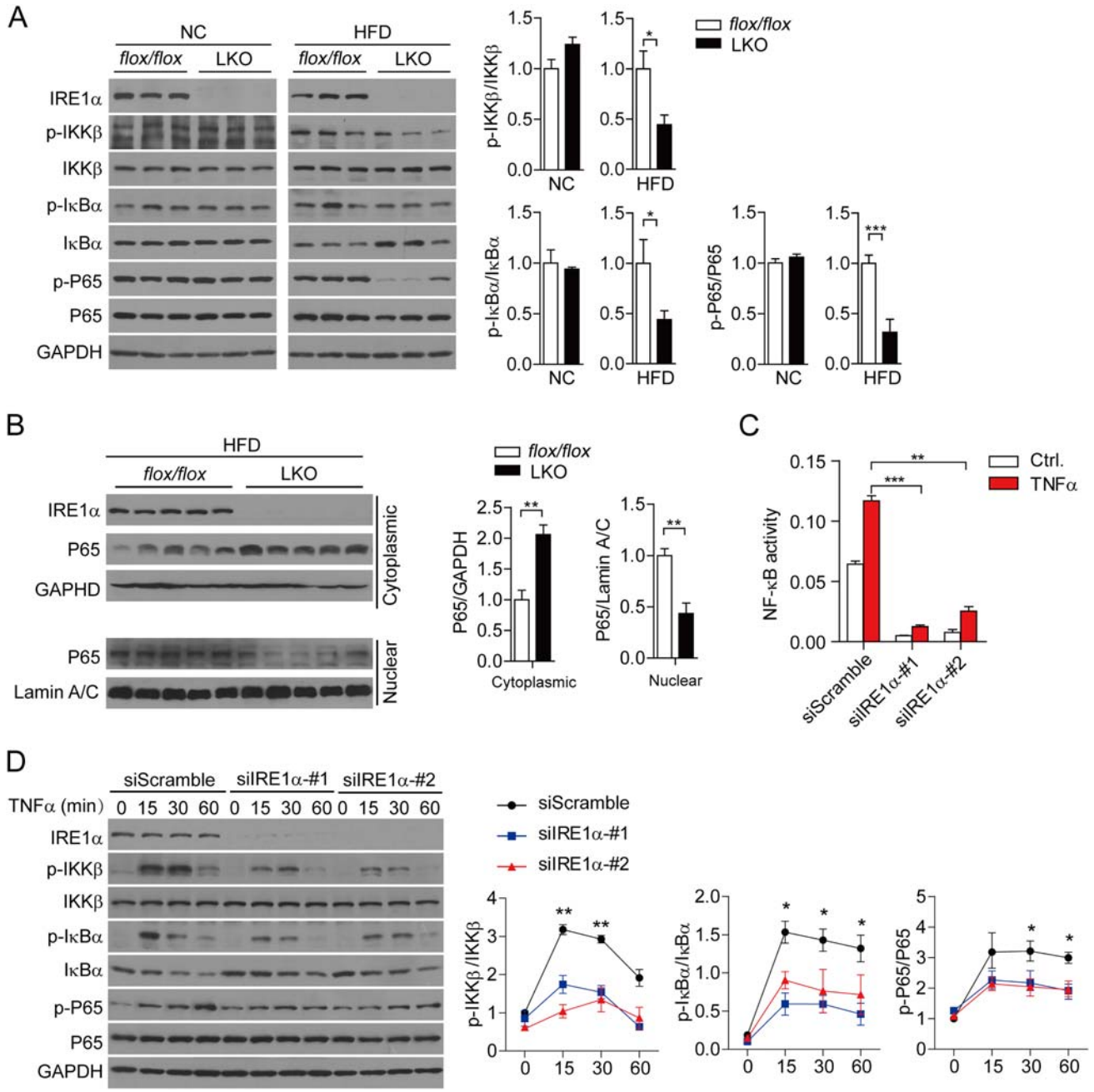

FIG. 6. IRE1α deficiency results in suppression of the inflammatory IKK*β*-NF-*κ*B pathway. (A-B) Hepatocyte IRE1α ablation reduced the activation of hepatic IKK*β* pathway in HFD-fed mice. DEN-treated male LKO and *flox/flox* mice were either maintained on an NC or an HFD for 4 weeks (n=8 per group). (A) Immunoblot analysis of phosphorylation of IKK*β*, IκBα, and P65 in liver extracts. Representative results are shown for three individual mice from each group. Averaged phosphorylation levels were determined by densitometric quantification after normalization to that of *flox/flox* mice. (B) Immunoblot analysis of the cytoplasmic and nuclear P65 protein levels in livers of HFD-fed mice. GAPDH or Lamin A/C was used as the loading control for the cytoplasmic or nuclear fractions. The densitometric quantification results are also shown. (C-D) Knockdown of IRE1α expression attenuated TNFα-activated IKK*β*-NF-*κ*B pathway. (C) HepG2 cells were co-transfected for 48 hours with the NF-*κ*B-luciferase reporter plasmid along with two siRNAs directed against IRE1α or a scramble control. Cells were then treated with TNFα (10 ng/ml) for 24 hours, and NF-*κ*B activity was determined by the luciferase assay. Shown are relative luciferase activities after normalization to Renilla that was used as the internal control. (D) HepG2 cells were likewise transfected with the two IRE1α siRNAs and then treated with TNFα (10 ng/ml) for the indicated time intervals. Phosphorylation of IKK*β*, IκBα and P65 proteins was analyzed and shown after normalization to the value of the untreated control cells (n=4 independent experiments). All data are presented as the mean ± s.e.m., $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by two-tailed unpaired Student's *t*-test or two-way ANOVA.

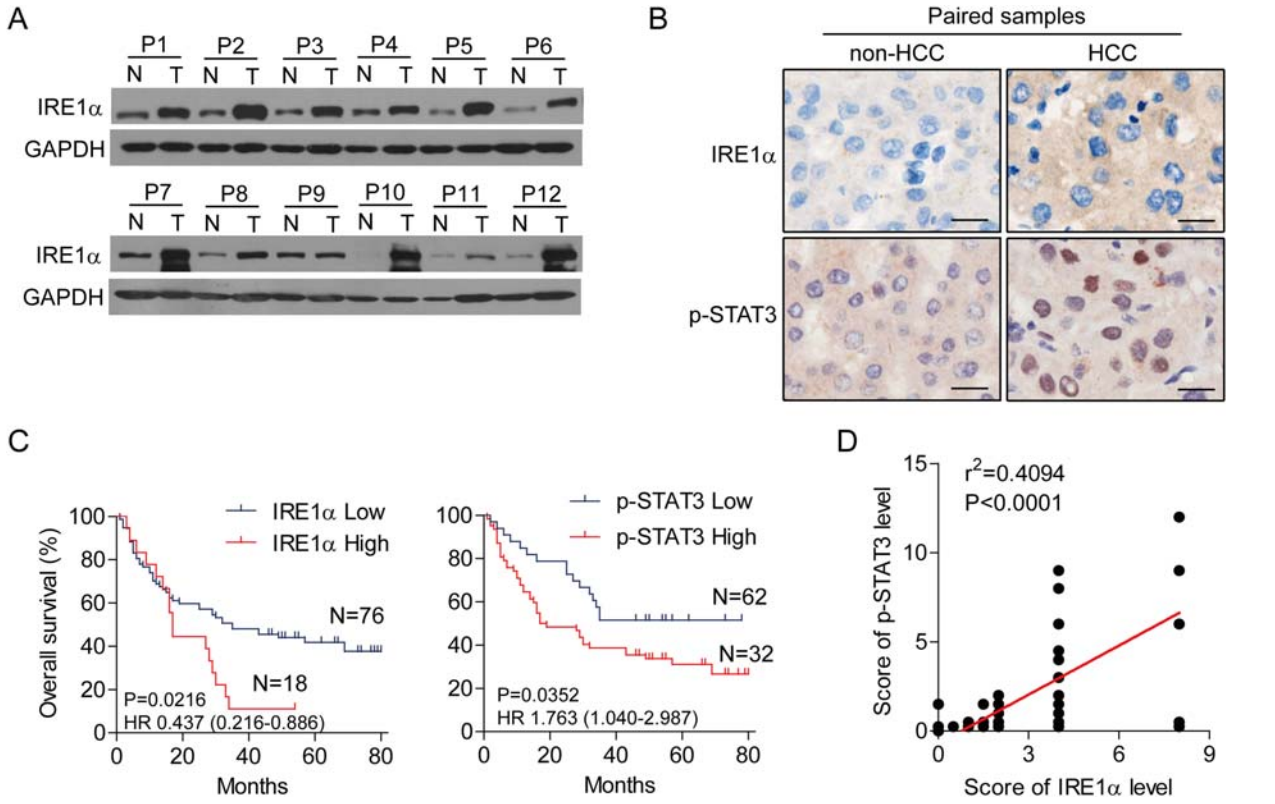

FIG. 7. IRE1α correlates with STAT3 activation and HCC in human patients. (A) Immunoblot analysis of IRE1α protein in extracts of paired tumor (T) versus non-tumor (N) samples from 12 human HCC patients. (B-D) TMA analysis. (B) Representative images of IHC staining of IRE1α protein and phosphorylated STAT3 in paired non-HCC and HCC tissue slides from human patients (n=83 patients). Scale bars, 20 μm. (C) Kaplan-Meier analysis (log-rank test) of the overall survival of human HCC patients in relation with high (n=18) or low (n=76) expression levels of IRE1α protein and with high (n=32) or low (n=62) levels of phosphorylated STAT3. The *P*-value, hazard ratio (HR) and 95% confidence interval (CI, in brackets) are indicated. (D) Linear regression analysis of IRE1α protein expression and STAT3 phosphorylation in HCC tumors. The coefficient of determination (r^2) and *P*-value are indicated.

without requiring XBP1 during metabolic inflammation, which most likely resembles the experimental ER stress state giving rise to IRE1α-directed activation of NF-κB.[33,34]

HIGHER IRE1α EXPRESSION CORRELATES WITH STAT3 ACTIVATION IN HUMAN HCC

To understand the clinical relevance of IRE1α in human HCC, we examined the expression of IRE1α in matched non-tumor and tumor samples from HCC patients. Remarkably, we detected higher IRE1α protein levels in nearly all of the 12 HCC tumor samples as compared to their non-tumor control tissues (Fig. 7A). We then analyzed the relationship between tumor IRE1α expression, STAT3 phosphorylation and the overall survival of HCC patients by tissue microarray (TMA) analysis of 94 HCC patients. TMA assessment also revealed elevated levels of IRE1α protein, as well as higher phosphorylation of STAT3 in HCC tumors relative to their matched non-tumor tissues (Fig. 7B). Moreover, higher extent of elevations in IRE1α protein levels and in STAT3 activation states significantly correlated with decreased overall survival rate in 18 and 32 HCC patients, respectively (Fig. 7C). Linear regression analysis also revealed an appreciable association between IRE1α protein and STAT3 activation (r^2=0.409, $p<0.0001$) in human HCC (Fig. 7D). These results suggest that IRE1α indeed represents an important aspect linking the UPR activation to the malignant development of human HCC through the STAT3 pathway.

Discussion

Persistent activation of the UPR pathways is thought to enable cancer cells to better cope with hostile environments and thus has an important role in promoting tumorigenesis.[18] Chronic ER stress in metabolic organs is a common feature in obesity,[20] but it remains unclear whether the IRE1α branch of the UPR links obesity-associated inflammation to the promotion of chemical carcinogenesis in the liver. Utilizing a mouse HCC model, this study reveals that during HFD-induced obesity, IRE1α in hepatocytes have multifaceted actions in accelerating the malignant progression of HCC. On one hand, hepatocyte IRE1α promotes obesity-associated activation of the IKKβ-NF-κB pathway, which leads to increased hepatic production of typical pro-inflammatory cytokines such as IL-6; on the other hand, hepatocyte IRE1α serves to maintain the activation of STAT3, thus promoting hepatocyte proliferation. Given that the IL-6-STAT3 pathway is essential in hepatocyte proliferation during obesity-promoted hepatocarcinogenesis,[9,10] our results uncover a critical IRE1α-mediated feed-forward loop for accelerating HCC development through STAT3-promoted proliferation in the context of overnutrition-induced obesity and metabolic inflammation.

Our findings highlight the crucial IRE1α-STAT3 axis in promoting the malignant progression of HCC regardless of the adiposity states. Following DEN administration, hepatocyte IRE1α ablation resulted in diminished STAT3 activation along with reduced hepatocyte proliferation, thus protecting against HCC development not only in HFD-fed obese mice, but also in NC-fed lean mice. This is congruent with our previous finding that hepatic IRE1α could directly interact with STAT3 to maintain its activation, thereby promoting reparative liver regeneration after tissue damage.[26] STAT3 is the oncogenic transcription factor that is central in regulating the proliferation and survival of tumor cells,[9,10,13,31] and frequent activation of STAT3 has been documented in human HCCs with poor prognosis.[35] Many studies have also established that STAT3 can be activated in cancer by a myriad of cellular pathways in addition to IL-6 and its family members.[31] Although the precise mechanism by which IRE1α controls STAT3 activation remains to be deciphered, IRE1α's kinase activity appeared to be involved. Targeted disruption of IRE1α-STAT3 association may block the STAT3 pathway and prevent the progression of HCC, even in the absence of obesity and metabolic inflammation.

Both TNFα and IL-6 signaling pathways have been found to be important in mediating the HCC-promoting effects of obesity and ER stress.[9,10] Our results also reveal that hepatocyte IRE1α is coupled to metabolic inflammation through enhancing TNFα activation of the IKKβ-NF-κB cascade. IRE1α was reported to associate with TNF-receptor-associated factor 2 (TRAF2) [36] and form a complex with IKKβ through TRAF2 to mediate NF-κB activation during experimental ER stress.[33,34] Recent reported studies also suggested that IRE1α's kinase activity was involved in maintaining the basal activity of IKKβ.[37,38] It is likely that during obesity-associated metabolic ER stress, IRE1α could enhance activation of the IKKβ-NF-κB cascade in hepatocytes through similar mechanisms, leading to higher hepatic production of IL-6 and presumably other inflammatory mediators as well.[39,40] While IRE1α ablation resulted in reduced hepatic levels of TNFα and IL-6 during dietary obesity, we did not detect apparent changes in macrophages or T cells from HCC tumors (data not shown). However, it has yet to be further investigated whether IRE1α-deficient hepatocytes could affect the infiltrated immune cells with regard to their capacity of cytokine production to maintain the inflammatory microenvironment in the liver throughout the process of HCC progression. Nevertheless, the reduced production of IL-6 in IRE1α-deficient livers could be a key contributor in diminishing its autocrine activation of STAT3 to blunt the acceleration of HCC in the face of dietary obesity.

It is worth noting that hepatocyte IRE1α ablation resulted in alleviation of hepatosteatosis and liver inflammation during HFD feeding but led to increased cell death that could be ascribed to hyperactivation of the PERK-eIF2α pathway and/or deficient STAT3 activation in both obese and lean mice. Whereas enhanced apoptosis in transformed hepatocytes of DEN-treated LKO mice might contribute in hindering the progression of HCC, it is more likely that such increased apoptosis was unable to trigger the compensatory proliferation of hepatocytes[41,42] owing to the impairment of STAT3 activation in IRE1α-deficient hepatocytes. Of interesting note, we also observed a reduction of PERK protein abundance despite of its higher phosphorylation level in IRE1α-deficient hepatocytes. This suggests a possible, as-yet-unrecognized interconnection between these two UPR branches in this particular experimental context. Moreover, given its roles in hepatic lipid metabolism,[27,43] it remains to be fully understood whether the IRE1α-XBP1 branch

contributed to, or the IRE1α-STAT3 axis was also involved in, overnutrition-associated hepatosteatosis during obesity-promoted HCC progression.

Finally, we have found high levels of IRE1α expression in association with human HCC tumors, which appreciably correlated with increased STAT3 activation and poorer survival rate of the examined patients. It remains to be dissected, however, what exact activation features enable IRE1α to promote STAT3 activation in human HCC. Interestingly, human cancer genome studies of HCC have uncovered a number of somatic mutations within *ERN1*, the human gene encoding IRE1α.[44,45] Further functional investigations are warranted to delineate the potential impact of these mutations upon its role in maintaining the persistent activation of STAT3 and NF-κB in human HCC, whether it is associated with higher risks endowed by obesity, NASH, or HBV/HCV infection. In short, our work demonstrates a crucial tumor-promoting activity of IRE1α in HCC development. A variety of human cancer has been documented to have increased ER stress.[19] Given the essential importance of STAT3 and NF-κB in other inflammation-associated cancers,[9,46-50] IRE1α may contribute to the malignant progression of other types of tumor as well. Targeted blocking of the molecular connections between IRE1α and STAT3 or IKKβ may bear promising translational potentials for the development of therapeutics against not only HCC but also many other malignancies.

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Supporting Information

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