Supplemental Information

Materials and Methods

Animals

Liver-specific *IRE1α* knockout (LKO) and *flox/flox*) mice (1, 2) were subjected to HCC induction (3). For analysis of DEN-induced liver apoptosis and ER stress, 4-week-old mice were injected i.p. with one dose of DEN at 100 mg/kg. Immunohistochemical analyses of Ki-67, PCNA and K19 were performed as described (4, 5). 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.

Oil-red O staining

Frozen liver sections (10 mm) were fixed in formalin and then rinsed with 60% isopropanol. After staining with freshly prepared Oil-red O solution for 10 min, the sections were rinsed again with 60% isopropanol, and nuclei were stained with alum haematoxylin before analysis by microscopy (PerkinElmer, Waltham, MA).

Serum and liver measurements

Serum levels of IL-6 and TNFα were measured using the Interleukin-6 and TNFα ELISA Kit (#KMC0061 and #BMS607HS, ThermoFisher, Waltham, MA), respectively, following the manufacturer's instructions. To determine hepatic levels of IL-6 and TNFα, liver tissues were homogenized by sonication in the homogenization buffer (0.01 N HCl, 1 mM EDTA, 4 mM $Na_2S_2O_5$), and the cellular debris was pelleted by centrifugation at 13,000 rpm for 15 min at 4°C. The homogenates were collected and stored at -80°C prior to measurement by ELISA. Hepatic TGs were analyzed as previously described (2). Briefly, 40-50 mg of liver tissues were homogenized in PBS and mixed with $CHCl_3$-CH_3OH (2:1, v/v). The organic phase was transferred, air-dried overnight, and re-suspended in 1% Triton X-100 in absolute ethanol. The concentration of TGs was determined using the serum triglyceride

determination kit (#TR0100, Sigma-Aldrich, St. Louis, MO). All liver measurements were normalized to the total tissue protein concentrations.

Antibodies, chemicals and immunoblotting analysis

Antibodies against IRE1α (#3294), p-eIF2α (#9721), eIF2α (#9722), p-PERK (#3179), PERK (#3192), BiP (#3183), DR5 (#8074), Caspase 3 (#9662), BCL2 (#3498), p-STAT3 (#8204), STAT3 (#14047), p-P65 (#3303), P65 (#3304), P-IκBα (#5209), p-IKKβ (#2697), IKKβ (#8943) and Lamin A/C (#2032) were all from Cell Signaling Technology (Danvers, MA). Antibody (#NB 100-2323) against phosphorylated IRE1α at Ser724 (p-IRE1α) was from Novus Biologicals (Littleton, CO). IκBα (ab32518) antibody was from Abcam (Cambridge, MA), and GAPDH antibody from Kangcheng Biotechnology (Shanghai, China). GAPDH antibody was used at 1:10,000, and all other antibodies were diluted 1:1000. Tunicamycin (#T7765) was purchased from Sigma Aldrich (St. Louis, MO). KIRA (#6532281), an IRE1α kinase inhibitor (6), was from Calbiochem, Merck KGaA (Darmstadt, Germany), and 4μ8C, an inhibitor of IRE1α RNase activity (7, 8), was synthesized in-house at Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

For immunoblotting, tissue or cell lysates were prepared by RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.4). Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (#78833, Thermo Fisher Scientific, Waltham, MA). Protein extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane filter. After incubation with the desired antibodies, the blots were developed with Thermo Scientific's SuperSignal West Pico Chemiluminescent substrate or Millipore's Immunobilon Western Chemiluminescent HRP substrate.

Phos-tag gel analysis was carried out as described (9). Briefly, a 6% SDS-PAGE gel with 25 mM phos-tag was prepared according to the manufacturer's instructions (Phos-tag acrylamide AAL-107, Wako Pure Chemical Industries). IRE1α antibody (#3294, Cell Signaling Technology, Danvers, MA) was used to detect the phosphorylated and non-phosphorylated forms of the IRE1α protein.

Quantitative RT-PCR analysis

For quantitative RT-PCR analysis, total RNA was isolated from liver tissues or cells by TRIzol reagent (#T9424, Invitrogen, Carlsbad, CA), and cDNA was synthesized using M-MLV reverse transcriptase and random hexamer primers (#28025013, Thermo Fisher Scienctific, Waltham, MA). Real-time PCR was performed using the SYBR Green PCR system (#4309155, Applied Biosystems, Waltham, MA). *Gapdh* was utilized as an internal control for normalization. The oligonucleotide primers used are as follows:

mouse *Atf4*, forward primer 5’-CCTTCGACCAGTCGGGTTTG-3’ and reverse primer 5’-CTGTCCCGGAAAAGGCATCC-3’;

mouse *Xbp1t*, forward primer 5’-TGGCCGGGTCTGCTGAGTCCG-3’ and reverse primer 5’-GTCCATGGGAAGATGTTCTGG-3’;

mouse *Xbp1s*, forward primer 5’-CTGAGTCCGAATCAGGTGCAG-3’ and reverse primer 5’-GTCCATGGGAAGATGTTCTGG-3’;

mouse *Chop*, forward primer 5’-CTGGAAGCCTGGTATGAGGAT-3’ and reverse primer 5’-CAGGGTCAAGAGTAGTGAAGGT-3’;

mouse *Dr5*, forward primer 5’-GCAGAGAGGGTATTGACTACACC-3’ and reverse primer 5’-GCATCGGGTTTCTACGACTTT-3’;

mouse *Trb3*, forward primer 5’-*TGCAGGAAGAAACCGTTGGAG*-3’ and reverse primer 5’-CTCGTTTTAGGACTGGACACTTG-3’;

mouse *Gapdh*, forward primer 5’-GGATTTGGCCGTATTGGG-3’ and reverse primer 5’-GTTGAGGTCAATGAAGGGG-3’;

mouse *Bloc1s1*, forward primer 5’-TCCCGCCTGCTCAAAGAAC -3’ and reverse primer 5’- GAGGTGATCCACCAACGCTT-3’;

mouse *c-Myc*, forward primer 5’-ATGCCCCTCAACGTGAACTTC-3’ and reverse primer 5’-GTCGCAGATGAAATAGGGCTG-3’;

mouse *c-Fos*, forward primer 5’-TTGAGCGATCATCCCGGTC-3’ and reverse primer 5’-GCGTGAGTCCATACTGGCAAG-3’;

mouse *Hif1a*, forward primer 5’-GTCCCAGCTACGAAGTTACAGC-3’ and reverse primer 5’-CAGTGCAGGATACACAAGGTTT-3’.

Luciferase reporter assay

To measure NF-κB activation, the luciferase (Luc) reporter plasmid was generated by inserting into the pGL3-Basic plasmid (Promega Corp., Madison, WI) a basic promoter element (TATA box) joined to a tandem repeat of NF-κB binding elements. HepG2 cells were co-transfected for 48 hours with the Luc reporter construct and pRL-TK renilla plasmid (Promega Corp., Madison, WI), along with siRNAs against IRE1α or XBP1 or a scramble control siRNA. Cells were then treated with TNFα for 24 hours, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega Corp., Madison, WI) according to the manufacturer's instructions. The oligonucleotide siRNAs used are as follows:

siIRE1α-#1: sense 5’-GGAGAGAAGCAGCAGACUUdTdT-3’ and antisense 5’-AAGUCUGCUGCUUCUCUCCdTdT-3’;

siIRE1α-#2: sense 5’-GCGUAAAUUCAGGACCUAUdTdT-3’ and antisense 5’-AUAGGUCCUGAAUUUACGCdTdT-3’);

Scramble control siRNA for siIRE1α: sense 5’-CGUACGCGGAAUACUUCGAdTdT-3’ and antisense 5’-UCGAAGUAUUCCGCGUACGdTdT-3’;

siXBP1-#1: sense 5’-CCAGUCAUGUUCUUCAAAUTT-3’ and antisense 5’-AUUUGAAGAACAUGACUGGTT-3’;

siXBP1-#2: sense 5’-GGAACAGCAAGUGGUAGAUTT-3’ and antisense 5’-AUCUACCACUUGCUGUUCCTT-3’;

Scramble control siRNA for siXBP1: sense 5’-UUCUCCGAACGUGUCACGUTT-3’ and antisense 5’-ACGUGACACGUUCGGAGAATT-3’.

Clinical HCC specimens and tissue microarray analysis

With the written informed consent from the patients, twelve pairs of human HCC tissues and their corresponding adjacent non-tumor tissues that were at least 3 to 4 cm away from the tumor, were obtained from the Eastern Hepatobiliary Surgery Hospital. The fresh specimens were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. The experimental protocol was approved by the Institutional Ethical Review Board at the Institute for Nutritional Sciences, Chinese Academy of Sciences.

HCC tissue microarrays, collected from 94 HCC tissues with matched non-HCC liver tissues close to the tumor, were prepared and immunostained with the anti-IRE1α antibody (#ab96481, Abcam, Cambridge, MA) and anti-phospho-STAT3 antibody (#8204, CST, Danvers, MA) by the Biochip Shanghai National Engineering Research Center. The collection and use of these human clinical samples were approved by the Ethical Committee of Taizhou Hospital, Zhejiang, China. IHC scoring was performed by a clinical pathologist according to the histological scoring system (H-score), which was based on the IHC staining intensity (0, no staining; 1, low; 2, moderate; and 3, high) and the percentage of stained cells (0, 0; 1, 1-25%; 2, 25-50%; 3, 51-75% and 4, 76-100%). Expression levels of IRE1α protein and phosphorylated STAT3 in each HCC tissue was designated as either low (H-score ≤4) or high (H-score >4).

References

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Figures

Fig. S1

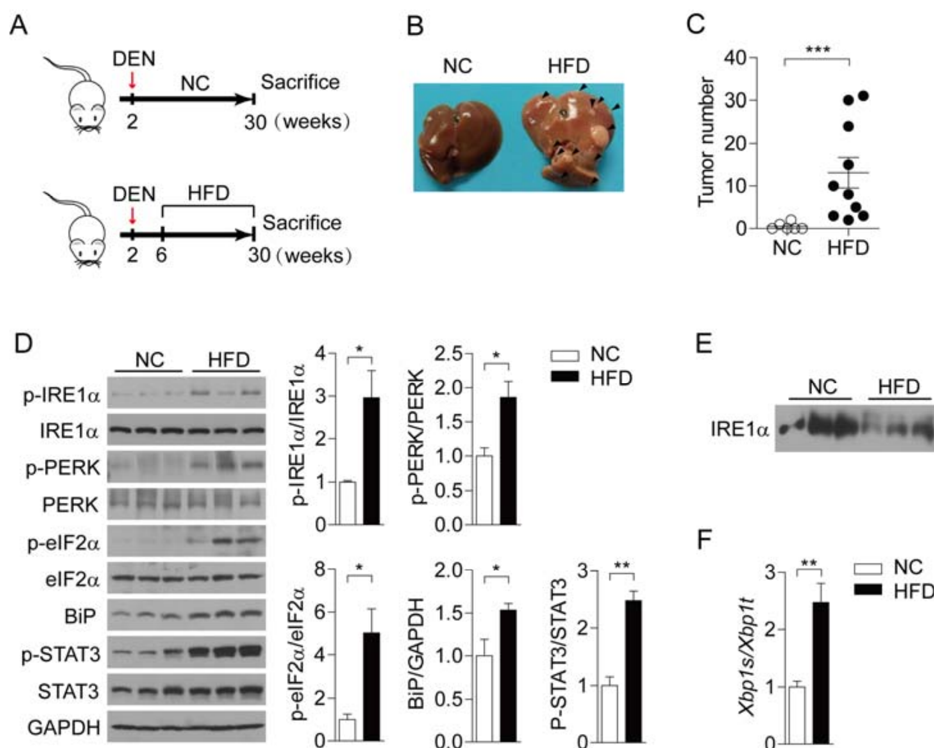

Fig. S1. Promotion by dietary obesity of HCC is accompanied by hepatic ER stress. Two-week-old male C57BL/6 mice were i.p. injected with DEN (50 mg/kg body weight) and were maintained on a normal-chow (NC) diet (n=6), or fed a high-fat diet (HFD) beginning at 6 weeks of age (n=10). Mice were sacrificed at 30 weeks of age. (**A**) Schematic of the experimental design. (**B**) Representative images of mouse livers from the indicated group. Tumors are indicated by arrows. (**C**) The numbers of liver tumors. (**D**) Immunoblot analysis of phosphorylation of IRE1α, PERK, eIF2α and STAT3 as well as BiP protein expression from liver extracts. Representative results are shown for three individual mice per group. GAPDH was used as the loading control. Shown also are results from densitometric quantification of the immunoblots after normalization to that of NC-fed mice. (**E**) Representative phos-tag gel for immunoblot analysis of IRE1α protein. (**F**) Quantitative RT-PCR analysis of *Xbp1* mRNA splicing. Data in (**C**, **D**, **F**) 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.

Fig. S2

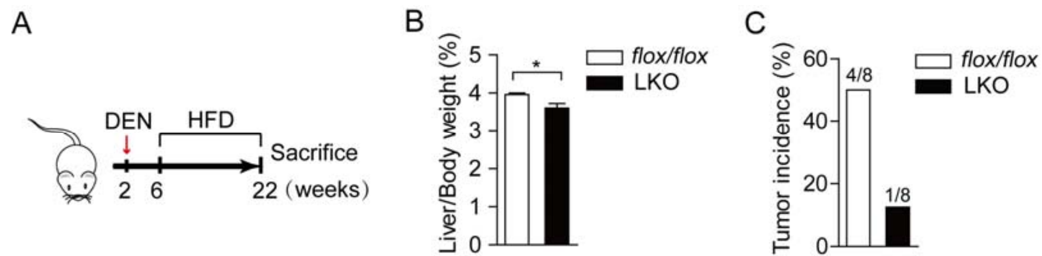

Fig. S2. Hepatocyte IRE1α ablation blunts dietary obesity-accelerated HCC development. Two-week-old male LKO mice and their *flox/flox* littermates were i.p. injected with DEN (50 mg/kg body weight) and were maintained on an HFD beginning at 6 weeks of age (n=8 per genotype). Mice were sacrificed at 22 weeks of age for HCC analysis. (**A**) Schematic of the experimental design. (**B**) Ratios of liver weight to body weight. (**C**) Incidence of HCCs. Data are shown as the mean ± s.e.m., $*P < 0.05$ by two-tailed unpaired Student's *t*-test.

Fig. S3

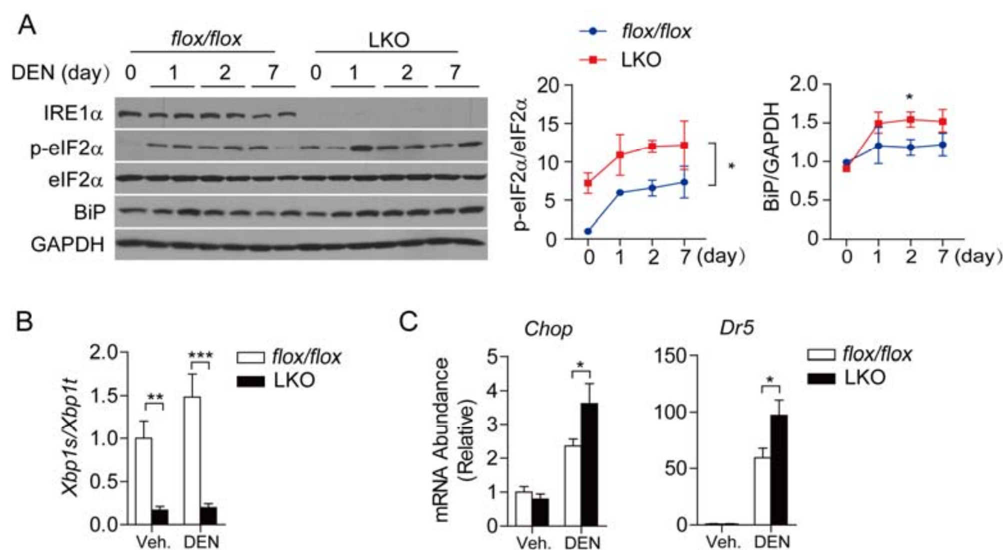

Fig. S3. Abrogation of hepatocyte IRE1α promotes DEN-induced activation of the eIF2α-CHOP branch of ER stress. Four-week-old *flox/flox* and LKO mice were administrated with DEN (100 mg/kg body weight) or saline (vehicle) and were sacrificed at the indicated time after injection (n=5 per group). (**A**) Immunoblot analysis of eIF2α phosphorylation and BiP protein in liver extracts. GAPDH was used as the loading control. Densitometric quantification of eIF2α phosphorylation and BiP protein levels are shown after normalization to the value of untreated *flox/flox* mice. (**B, C**) Quantitative RT-PCR analysis of *Xbp1* mRNA splicing (**B**) and the mRNA abundance of *Chop* and *Dr5* (**C**) in livers of mice at 2 days after DEN administration. Data are shown as the mean ± s.e.m., $*P < 0.05$, $**P<0.01$ by two-tailed unpaired Student's *t*-test or two-way ANOVA.

Fig. S4

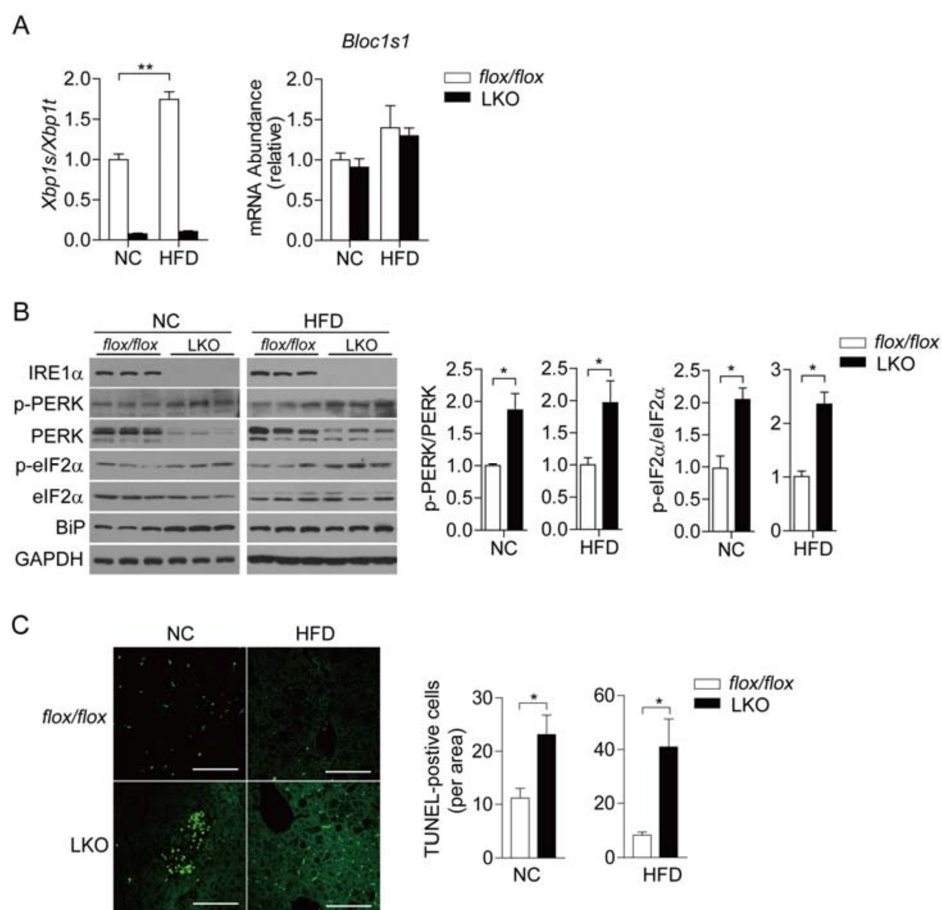

Fig. S4. Hepatocyte IRE1α ablation leads to increased hepatic ER stress and apoptosis in DEN-treated mice. Two-week-old male LKO and *flox/flox* mice were injected with DEN and then maintained on an NC for 40 weeks, or fed an HFD for 24 weeks, starting at 6 weeks of age (n=8 per group). (**A**) Quantitative RT-PCR analysis of *Xbp1* mRNA splicing and the mRNA abundance of *Bloc1s1* in the livers. (**B**) Immunoblot analysis of phosphorylation levels of PERK and eIF2α as well as BiP protein levels from liver extracts. Representative immunoblots are shown for three individual mice per group. Shown also are densitometric quantification results after normalization to those of *flox/flox* mice. (**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. Data are shown as the mean ± s.e.m, *$P < 0.05$ by two-tailed unpaired Student's *t*-test.

Fig. S5

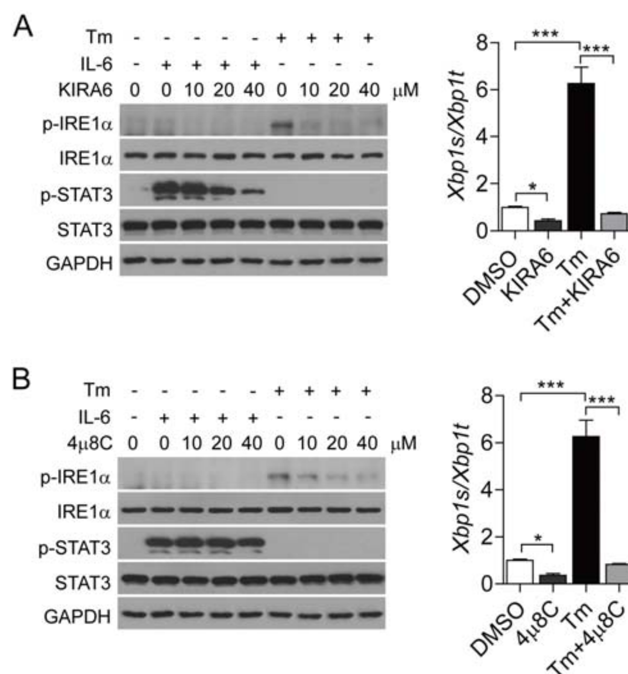

Fig. S5. Blocking IRE1α's kinase but not RNase activity attenuates IL-6-induced STAT3 phosphorylation. HepG2 cells were pretreated with DMSO (-) versus KIRA6 (**A**) or 4μ8C (**B**) for 30 min at the indicated concentrations and then stimulated with IL-6 (10 ng/ml) for another 30 min. Shown are representative immunoblot analyses of IRE1α and STAT3, along with quantitative RT-PCR analysis of *Xbp1* mRNA splicing. Cells likewise treated with tunicamycin (Tm, 10 ng/ml) for another 2 hr were used as the control experiment. Data from 3 independent experiments are presented as the mean ± s.e.m., *$P < 0.05$, ***$P<0.001$ by one-way ANOVA.

Fig. S6

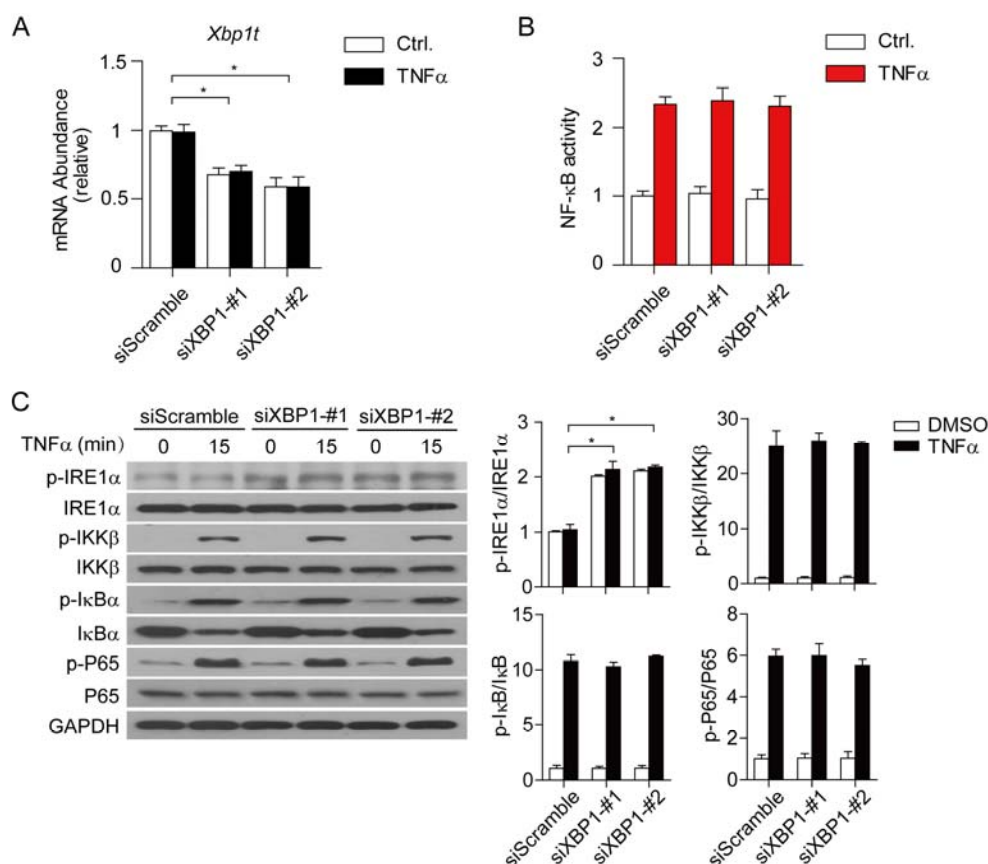

Fig. S6. Knockdown of XBP1 expression does not affect TNFα activation of the IKKβ-NF-κB pathway. (**A**) HepG2 cells were transfected for 48 hours with two siRNAs directed against *Xbp1* or a scramble control. Cells were then treated with TNFα (10 ng/ml) for 24 hours. Quantitative RT-PCR analysis of *Xbp1* mRNA abundance for assessment of knockdown efficiency. (**B**) HepG2 cells were co-transfected for 48 hours with the NF-κB-luciferase reporter plasmid along with *Xbp1* siRNAs or the scramble control. Cells were then treated with TNFα (10 ng/ml) for 24 hours, and NF-κB activity was determined by the luciferase assay. Shown are relative luciferase activities after normalization to renilla that was used as the internal control. (**C**) HepG2 cells were transfected with the scramble or *Xbp1* siRNAs and then treated with TNFα (10 ng/ml) for 15 min. Phosphorylation of IKKβ, IκBα and P65 proteins was analyzed, and data are shown after normalization to the value of the untreated control cells. Results from 4 independent experiments are presented as the mean ± s.e.m., *$P < 0.05$ by two-way ANOVA.