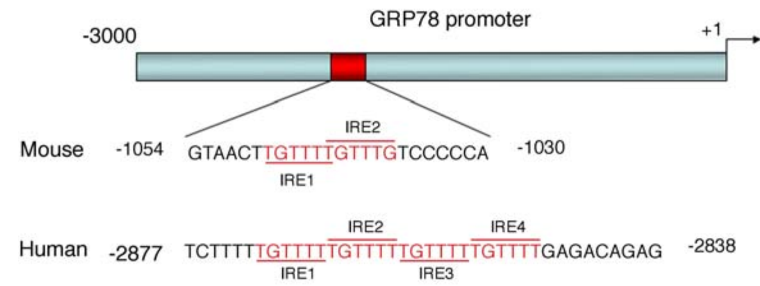
Supplemental Figure 1



Supplemental Figure 2

-1056

IRE

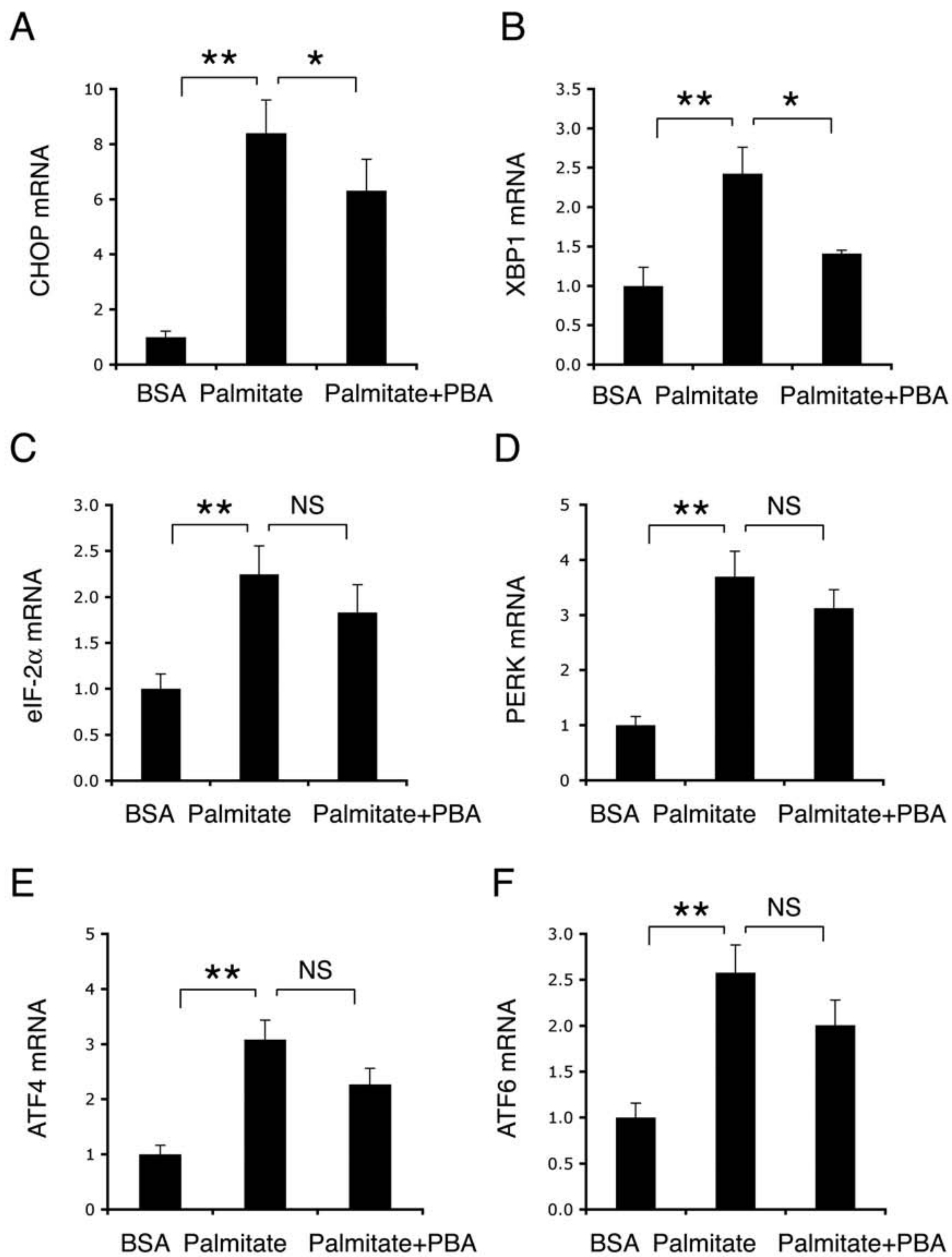
-1023

Wild-type

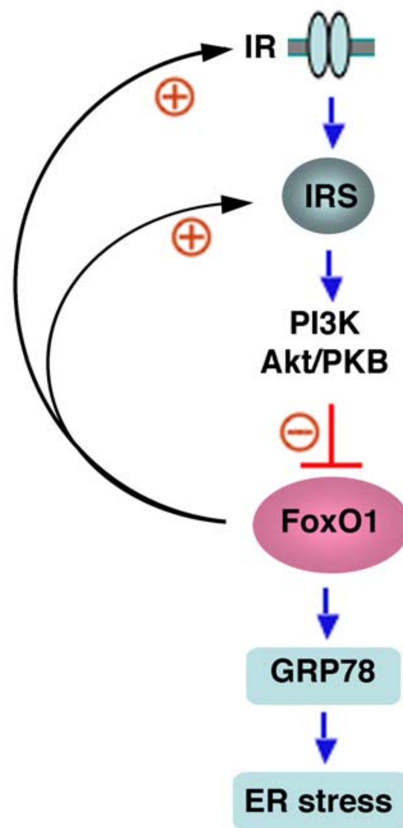
Mutant

GRP78 Promoter

Supplemental Fig. 3



Supplemental Fig 4



Online Support Materials

Materials and Methods

Cell culture and adenovirus transduction: HepG2 cells were purchased from American Type Culture Collection (ATCC, Virginia, USA). Cells were cultured in Minimum Essential Medium Eagle (ATCC) supplemented with 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin in an incubator with 5% CO_2 and 95% air at 37°C. HepG2 cells were transduced with adenoviral vector at a predefined multiplicity of infection (MOI). After 24 hours of incubation, cells were collected for analysis. The adenoviral vectors used were as follows; Adv-CMV-FoxO1 expressing wild type FoxO1 ($1.0x10^{11}$ pfu/ml), Adv-CMV-FoxO1-ADA expressing constitutively active FoxO1-ADA allele ($1.0x10^{11}$ pfu/ml), and the null adenovirus Adv-null ($1.25x10^{11}$ pfu/ml). Adv-FoxO1-RNAi and Adv-scRNAi vectors encoding FoxO1-specific RNAi and scrambled control RNAi have been described (1). All adenoviral vectors were produced in HEK293 cells and purified as described (2). BSA-bound fatty acid solution was prepared as described (3). Palmitate (20 mg) or oleate (20 mg) was dissolved in pre-boiled 0.1 N NaOH, followed by 10-fold dilution in DMEM containing 12% w/v BSA (A6003, Sigma-Aldrich, St. Louis, MO). This yields a molar ratio of 1.5:1.0 between palmitate and BSA, which is equivalent to the corresponding ratio in human serum (4, 5). The solution was adjusted to pH 7.4, followed by the determination of fatty acid concentration using the Wako NEFA assay kit (Wako Chemical USA, Richmond, VA). Palmitate-free BSA (12% w/v) solution was used as control.

RNA isolation and real time RT-PCR: RNA isolation from liver (20 mg) was performed using the RNeasy Mini Kit (QIAGEN, Valencia, CA). Real-time quantitative RT-PCR was used for quantifying mRNA concentrations using the Roche LightCycler-RNA amplification kit (Roche Diagnostics, Indianapolis, IN), as described (1). The primers

were GRP78 forward 5'-GTGGAGATCATAGCCAACGA-3' and reverse 5'-GCTGGTACAGTAACAACTGC-3'; PERK forward 5'-TGCTGCTGCTTCTGTTCCTG-3' and reverse 5'-CGTCCATCTAAAGTGCTGAT-3'; IRE1 forward 5'-CCATCACCATGTATGACACC-3' and reverse 5'-GATACTTCCACTTGGTGATG-3; EDEM1 forward 5'-CGGTGCTCTGGTTGGTCTTC-3' and reverse 5'-CATCAACCAGAGTCAGAGAG-3'; CHOP forward 5'- TTTCACCTTGGAGACGGTGT-3' and reverse 5'-CTTCTCCTTCATGCGTTGCT-3'; GADD34 forward 5'-ATGCTGCTCACCGTGTACTG-3' and reverse 5'- CAAGATCTCCACTGAGCAGA-3'; XBP1 forward 5'-TGCTACTCTTATCTGGCCAG-3' and reverse 5'-CTCTGGAACCTCGTCAGGAT-3'; and ATF4 forward 5'-CTCTTCACGAAATCCAGCAG-3' and reverse 5'-ATCCAACGTGGTCAAGAGCT-3'. Primers for FoxO1 mRNA and 18S rRNA have been described (2, 6, 7). Primers for human hGRP78 are forward 5'-CGTGGAATGACCCGTCTGTG-3' and reverse 5'-CAGCAATAGTTCCAGCGTCT-3'; for hFoxO1 forward 5'-TCAGGTGGTGGAGATCGACC-3' and reverse 5'-CCTGGAAGTCCCCGCACAG-3'; for hCHOP forward 5'-CTCCTTCGGGACACTGTCCA-3' and reverse 5'-CTTTCTCCTTCATGCGCTGC-3'; for hATF4 forward 5'-ACCGAAATGAGCTTCCTGAG-3' and reverse 5'-ACAGGGCATCCAAGTCGAAC-3'; for hATF6 forward 5'-TCACAGGCTGGATGAAGATT-3' and reverse 5'-CATCTGAGAACTAGAAGACA-3'; for hPERK forward 5'-CGATGAGACAGAGTTGCGAC-3' and reverse 5'-TGCTTTCACGGTCTTGGTC-3'; for heIF2α forward 5'-CTCTTGACAGTCCGAGGATC-3' and reverse 5'-GTATCCCAGCTGTGCCATCT-3'. All primers were obtained commercially from Integrated DNA Technologies (Coralville, IA).

Semi-quantitative immunoblot assay: HepG2 cells (~1×10^6 cells) were lysed in 200-µl M-PER (Pierce, Rockford, IL, USA) containing 2-µl Halt Protease Inhibitor Cocktail

(Pierce). Protein extracts were obtained by centrifugation at 13,000 rpm for 10 min. To obtain protein extracts from liver tissue, 20-mg liver tissue were homogenized in 400-μl M-PER supplemented with 4-μl Halt Protease Inhibitor Cocktail (Pierce), followed by centrifugation at 13,000 rpm for 10 min. For the preparation of nuclear fractions, aliquots (40 mg) of liver tissue were homogenized in 400-μl ice-cold CER-I solution (Pierce) supplemented with 4-μl protease inhibitor cocktail (Pierce). Nuclear fractions were separated from cytoplasm and aliquots of 20-μg nuclear or cytoplasmic proteins were resolved on 4–20% SDS-polyacrylamide gels and subjected to immunoblot analysis. Proteins were blotted onto Immun-Blot PVDF Membrane (Bio-Rad, Hercules, CA), and subjected to western blot assay using rabbit anti-GRP78 antibody (Santa Cruz). Polyclonal anti-FoxO1 antibody has been described (6). Rabbit anti-IR β subunit antibody (sc-711) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit ant-IRS1, rabbit anti-IRS2 and mouse monoclonal anti-actin antibodies were purchased from Sigma-Aldrich (Saint Louis, MO). Protein bands were detected by autoradiography and their relative intensities were quantified by densitometry using NIH (National Institutes of Health, Bethesda, MD) image software as described (2).

Determination of hepatic glycogen content: Aliquots (40 mg) of liver tissue were homogenized in 400 μl of 0.03M HCl, followed by centrifugation at 13,000 rpm for 2 min. Aliquots (100 μl) of the supernatant were mixed with 400 μl of 1.25M HCl and incubated at 95 ^{o}C for 1 h for the determination of glycogen and residual glucose concentrations in the liver. In addition, aliquots (100 μl) were mixed with 400 μl of 1.25M HCl and incubated at room temperature for measurement of residual glucose levels. Glucose concentrations were determined using the glucose oxidase reagent (Sigma) and with the rabbit liver glycogen type III as the standard. After subtracting the residual levels of glucose in the liver, hepatic glycogen content was calculated as described (6).

Human primary hepatocytes: Human primary hepatocytes were isolated from donors and were obtained from the Liver Tissue Procurement and Distribution System at University of Pittsburgh Medical Center. Human primary hepatocytes were seeded in 12-well collagen-coated microplates at the density of $1x10^6$ cells/well and cultured in the Hepatocyte Maintenance Medium (HMM, Cat. CC-4192, Lonza, Walkersvile, MD) supplemented with dexamethasone, insulin and GA-1000 according to the manufacturer's instructions (Lonza).

Mouse primary hepatocytes: Mouse primary hepatocytes were isolated from CD1 mice using the protocol, as described (8). The liver was infused in situ through the portal vein first with HBSS supplemented with EGTA (1mM), then with EGTA-free HBSS and finally with HBSS supplemented with collagenase, type V (1.95 mg/ml, Sigma). The liver is harvested, minced and incubated at 37 ^{o}C for 10 min. The cells were dispersed in HBSS, filtered through a 100 mm nylon mesh and washed 3 times with HBSS. Hepatocytes were plated in collagen-coated 6-well plates at 1×10^5 cells/well and cultured in hepatocyte maintenance medium (Lonza) supplemented with dexamethasone, insulin and GA-1000 according to the manufacturer's instructions (Lonza).

ChIP assay: Chromatin immunoprecipitation (ChIP) was used to study the interaction between FoxO1 and GRP78 promoter DNA in cells as described (9). HepG2 cells ($2x10^5$ cells) were transfected with pGRP78 in the presence of FoxO1 vector at an MOI of 100 pfu/cell in triplicate. After 24-h incubation, cells were cross-linked with 1% formaldehyde, followed by sonication in a Microson 100 Watt Ultrasonicator (Structure Probe, West Chester, PA) at 30% of maximum power for 10 consecutives cycles of 10-second pulses. After centrifugation at 18,000xg for 10 min, the supernatant was divided equally

into two portions, one of which was incubated with 8-μg polyclonal rabbit anti-FoxO1 antibody that was generated in our lab (6) and the other was incubated with 8-μg preimmune rabbit IgG, followed by immunoprecipitation using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). The immunoprecipitates were analyzed by immunoblot analysis using goat anti-FoxO1 (FKHR-C20, Santa Cruz Biotechnology, Santa Cruz, CA), and separately by PCR assay to detect co-immunoprecipitated DNA using the GRP78 promoter-specific primers (forward 5'-CACATAGGAGGATCAGTAGT-3', reverse 5'-TATCGAGCGCGCCGTCGCCG-3') flanking the proximal region (-1173/+1 nt) of the mouse GRP78 promotor. As an off-target control, a pair of primers (forward, 5'-AACTAAACAATGACAACAA-3' and reverse 5'- TATGTAAACTATGCAGGATT-3') flanking an upstream region of the mouse GRP78 promoter (-4671/-4652 nt) was used.

GRP78 promoter-directed luciferase reporter system: An 2083-bp DNA fragment containing the mouse GRP78 promoter was amplified from mouse genomic DNA (BioChain Institute Inc, Hayward, CA) by PCR using primers for forward reaction (5'-GCCTCTAACTCAGAGAGATC-3') and reverse reaction (5'-TTACCTCACACACACGCAGA-3'). After verifying its nucleotide sequence, the GRP78 promoter was cloned into the luciferase reporter pGL3-Basic vector (Promega, Madison, WI). To generate promoter variants, DNA fragments covering different lengths of the GRP78 promoter were amplified by PCR using primers corresponding to the nucleotide sequence at which the promoter was truncated and cloned into pGL3. The forward primers were as follows; 5'-CACATAGGAGGATCAGTAGT-3' for pGRP78-A, 5'-GTACATAATAAGCTCAAGTAGTAAC-3' for pGRP78-B, and 5'-GAGGGAAAGAGGAAGGACT-3' for pGRP78-C. To alter the IRE DNA, six nucleotide substitutions were introduced into the core IRE motif in the GRP78 promoter using the site-directed mutagenesis kit (Invitrogen, Carlsbad, CA). The primers for site-directed

mutagenesis were the forward 5'-AAGTAGTAACTTGcccTGcccGTCCCCCAACA-3' and the reverse, 5'-CAAGTTACTACTTGAGCTTATTATGTACTTCA-3'. The resulting mutant GRP78-M promoter was cloned in pGL3 plasmid.

Plasmid transfection and luciferase assay: HepG2 cells in 6-well microplates were transfected with 2-μg plasmid encoding the luciferase reporter gene under the control of wild-type or GRP78 promoter variants using the Lipofectamine 2000 (Invitrogen). As a control, 2-μg pCMV-LacZ was used for normalizing transfection efficiency. Adenoviral vectors encoding wild-type FoxO1 or FoxO1-ADA mutant at a defined MOI were added to culture medium. After 24-h incubation, cells were collected and resuspended in 400-μl lysis buffer (Promega) for preparation of cell lysates. After centrifugation at 18,000x*g* for 3 minutes, aliquots (20 μl) of the supernatant were used to determine luciferase activity using the Luciferase Assay kit, and β-gal activity using a β-gal reporter activity detection kit (Promega). After normalizing to the amount of β-gal activity, the relative luciferase activity for each plasmid was reported.

EMSA*:* Electrophoretic mobility shift assay (EMSA) was used to study FoxO1 binding to DNA. FoxO1 protein was prepared from HepG2 cells ($2x10^5$ cells) that had been pre-transduced with FoxO1 vector at an MOI of 200 pfu/cell. 24-h post transduction, cells were resuspended in 200-μl CER-I solution (Pierce) supplemented with 2-μl protease inhibitor cocktail (Pierce). Nuclear factions were separated from cytoplasm and were used in EMSA. The DNA probe used in EMSA was derived from a 24-bp DNA covering the consensus insulin response element (IRE, -1056/-1023 nt) of the mouse GRP78 promoter (5'-GTAACTTGTTTTGTTTGTCCCCCAAC-3'). A mutant probe was made from the same DNA except for 6 base substitutions within the IRE sequence (5'-GTAACTTGcccTGcccGTCCCCCAAC-3'). Oligo nucleotides were labeled with biotin

using the Biotin 3'-End DNA Labeling kit (Pierce), followed by self-annealing to form double-stranded biotin-labeled DNA. EMSA was performed using the Lightshift Chemiluminescent EMSA kit (Pierce). Aliquots of FoxO1-containing nuclear extract (5 µg) were mixed with 50 fentomoles of biotin-labeled DNA probes in a total volume of 20 µl that contains 1x DNA binding buffer (100 mM Tris-Cl pH 7.5, 500 mM KCl, and 10 mM DTT), 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/µl poly(deoxyinosinic-deoxycytidylic acid), and 0.05% NP40. The mixtures were incubated at room temperature for 20 min and resolved on 6% native polyacrylamide gels, followed by electro-blotting to a nylon membrane. After cross-linking to the membrane for 10 min on a transilluminator equipped with 312 nm bulbs, the biotin-labeled DNA in the membrane was detected using streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate (Lightshift Chemiluminescent EMSA kit, Pierce). For DNA supershift assay, 1-µg anti-FoxO1 antibody (goat anti-FoxO1, FKHR-C20, Santa Cruz) was added to the reaction. DNA protein complexes were analyzed as described above.

Immunohistochemistry: CD1 mice were euthanized by CO_2 inhalation and liver tissue was isolated and embedded with Histoprep tissue embedding media (Fisher Scientific). Frozen sections were cut (8 µm) and subjected to immunohistochemistry using rabbit anti-FoxO1 antibody (dilution at 1:500). The second antibody was Cy3-conjugated goat anti-rabbit IgG (dilution 1:200, Jackson ImmunoResearch Laboratories). The nuclei of hepatocytes were visualized by immunostaining with DAPI (Sigma-Aldrich).

Statistics: Statistical analyses of data were performed by analysis of variance (ANOVA) using StatView software (Abacus Concepts, CA). Using ANOVA post-hoc tests, pair-wise comparisons were performed to study the significance between different conditions.

Data were expressed as the mean ± SEM. *P*-values <0.05 were considered statistically significant.

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