Supporting Information

Materials and Methods

Isolation of primary hepatocytes and adenovirus infection.

Primary hepatocytes were isolated from male C57BL/6J mice at 8-12 weeks of age according to the procedure described (1). After anesthetization, collagenase perfusion was performed through the portal vein with 50 ml of perfusion buffer (Krebs Ringer buffer with 3.6 mg/ml Glucose, 1 M $CaCl_2$ and 0.66 mg/ml collagenase I (Worthington) at 37°C. The liver was aseptically removed and cut, and hepatocytes were filtrated and washed with cold Hepatocyte Wash Medium (Gibco) for three times and re-suspended in 15 ml of cold HepatoZYME-SFM (Gibco) medium supplemented with 2 mM L-glutamine, 20 units/ml Penicillin and 20 μg/ml Streptomycin. After the viability was determined by Trypan Blue staining, the hepatocytes were plated at 6×10^5 cells/well in 6-well culture dishes pre-coated with collagen. Cells were cultured for 8 hours before further use. Hepatocytes were infected for 48 or 72 hours with adenoviruses at an MOI of 40 and were subsequently treated with the desired reagents prior to protein extraction for Western immunoblot analysis.

Generation of recombinant adenoviruses

Recombinant adenoviruses for the overexpression of EGFP and the wild type or mutant forms of IRE1α were generated as previously described (2) with the AdEasy System (Stratagene) according to the manufacturer's instructions. Briefly, DNA fragments encoding the desired proteins were first subcloned into pShuttle-CMV,

which were then used to produce recombinant adenoviral plasmids through homologous recombination with pAdEasy-1 in Escherichia coli BJ5183 cells. Transfection of HEK 293A cells (Invitrogen) was conducted using the linearized recombinant plasmids to produce the recombinant viruses. The short-hairpin (sh) RNA knockdown adenoviruses targeting IRE1α (Ad-shIRE1α-$^{\#}$1, Ad-shIRE1α-$^{\#}$2) or PKA (Ad-shPKA-$^{\#}$1) or expressing a scrambled control shRNA (Ad-shCON) were generated with the BLOCK-iT Adenoviral RNAi Expression System (Invitrogen) in HEK 293A cells according to the manufacturer's instructions. DNA fragments encoding shRNAs directed against murine IRE1α or PKA, or containing a general scrambled sequence were introduced into pENTR/U6 vector under the control of the human U6 promoter. The two shRNAs designed for the knockdown of IRE1α had the following IRE1α target sequences: Ad-shIRE1α-$^{\#}$1, 5′-GCGAGAAGCAGCAGACTTTGT-3′, and Ad-shIRE1α-$^{\#}$2, 5′-GGAATTACTGGCTTCTCATAG′. The shRNA designed for the knockdown of PKA contained the following PKA target sequence: 5′-GGGTCAATGACATCAAGAACC-3′. The control virus, Ad-shCON, had a core scrambled sequence of 5′-GTTCTCCGAACGTGTCACGTTT-3′. The shRNA for knocking down the expression of XBP1 was as described previously (3). For infection of primary hepatocytes, viruses were used at an MOI (multiplicity of infection) of 40, which was measured according to the manufacturer's instructions. High-titer stocks of amplified recombinant knockdown adenoviruses were purified by two-step ultracentrifugation in cesium chloride gradient. After subsequent dialysis, viral titers

were determined by the tissue culture infectious dose 50 (TCID50) method using 293A cells. Viruses were diluted in PBS and administered through tail vein injection, using approximately $4x10^8$ pfu/mice. Liver function was assessed by measurement of serum levels of alanine transaminase and aspartate transaminase by Alanine Transaminase/Aspartate Transaminase Determination Kit (ShenSuoYouFu).

Glucagon challenge test

After a 15-hour fast period, glucagon was administered through i.p. injection (100 μg/kg for WT mice and 150 μg/kg for *db/db* mice). Tail vein blood was collected at 0, 15, 30, 45, 60 and 75 minutes after glucagon injection. Glucose concentrations were measured by a glucometer (FreeStyle).

Glucose and pyruvate tolerance tests

After a 6-hour fast, *db/db* mice infected with the desired adenoviruses were injected i.p. with 1.5 g/kg glucose or 2 g/kg pyuvate. Tail vein blood was taken for glucose measurement at 0, 30, 60 and 120 minutes after glucose or pyruvate injection.

Glucose production assay

The glucose production assay was conducted according to the protocol described (4). Primary hepatocytes were isolated from mice infected for 5 days with Ad-shCON or Ad-shIRE1α-$^{\#}$2. After attachment, cells were washed three times with PBS and were then incubated for 4 hours at 37 °C with 5% CO_2 in glucose production buffer [DMEM (without glucose, L-glutamine, phenol red, sodium pyruvate or sodium bicarbonate; Sigma) supplemented with 10 mM HEPES (pH 7.4), 0.6% BSA and substrates (5 mM sodium lactate, 5 mM sodium pyruvate)] in the presence of DMSO or 10 μM

forskolin(5). The medium was subsequently collected and glucose concentrations were measured with Glucose (GO) Assay Kit (Sigma).

Chemical reagents, antibodies, plasmids, and Western immunoblotting

Glucagon, thapsigargin, H89, U73122, epinephrine, forskolin, bromo-cAMP and α-tubulin monoclonal antibody were all purchased from Sigma. IRE1α, CREB, pSer^{133}-CREB, eIF2α and pSer^{52}-eIF2α antibodies were from Cell Signaling, and pSer^{724}-IRE1α antibody from Novus Biologicals. Antibody against the catalytic α subunit of PKA was from BD Transduction Laboratories, and BiP monoclonal antibody from Stressgen. For the expression of EGFP-IRE1α fusion protein, mouse IRE1α cDNA was subcloned in-frame into pEGFP-N1 plasmid. For immunoblot analyses, proteins extracted from cells or tissue by RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl) were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane filters (Amersham Biosciences). After incubation with the desired antibodies, the blots were developed with Thermo Scientific's SuperSignal West Pico Chemiluminescent Substrate or Millipore's Immobilon Western Chemiluminescent HRP Substrate. To reduce non-specific protein species detected, pSer^{724}-IRE1α antibody was used after pre-incubation with a PVDF membrane filter that contained transferred mouse cellular proteins.

Expression and purification of recombinant PKA, PKC and cyto-IRE1α proteins

The expression plasmid for the catalytic α subunit of mouse PKA was constructed in pET-15b, which was kindly offered by Susan Taylor (Addgene). Transformation was

performed using BL21 DE3 Codon Plus competent cells, and the recombinant PKA protein was subsequently purified by Ni-NTA Agarose (Qiagen) as described (6).

The cDNA fragment encoding the human full length PKC-ε, a novel isoform of PKC (7), was subcloned into baculovirus expression vector pFastbac-1 (Invitrogen) to produce a GST-PKCε fusion protein. The recombinant virus was generated and amplified according to the manufacturer's instructions. The recombinant PKC-ε protein was expressed in High-Five insect cells and was subsequently purified by GST affinity-chromatography. The enzyme activity of the purified kinase proteins was determined by the Z′-LYTE^{TM} Kinase Assay Kit (Invitrogen) using the peptide substrates for PKA and PKC, according to the manufacturer's instructions.

His-tagged recombinant protein of the cytoplasmic portion of human IRE1α (cyto-IRE1α, spanning amino acid residues 469-977) was bacterially expressed using a PCR-derived cDNA fragment which was subcloned into pET-30a(+). Cyto-IRE1α protein was purified with Ni-NTA Agarose (Qiagen) according to the manufacturer's instructions and subsequently used for phosphorylation assays through incubation with purified PKA or PKC proteins.

Immunoprecipitation of IRE1α for phosphorylation assays

Primary hepatocytes were infected for 48 hours with recombinant adenoviruses expressing Flag-tagged human IRE1α, IRE1α-K599A or IRE1α-S724A proteins. Cells were lysed with the lysis buffer [20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1% Nonidet P-40, 1 mM EDTA and 10% Glycerol containing 1% protease inhibitors cocktail (Sigma) and 1% phosphatase inhibitors cocktail I/II (Sigma)] for 20 minutes

at 4 ^{o}C. After incubation with Flag antibody for 2 hours at 4 ^{o}C with gentle rocking, lysates were mixed with a final concentration of 2.5% protein G Sepharose beads (Amersham Biosciences) and incubated for 2 hours at 4 ^{o}C on a rotator. After washing three times with the washing buffer [20 mM Tris-HCl pH 7.5, 150 mM KCl, 0.5% Nonidet P-40, 1 mM EDTA, and 10% glycerol supplemented with 1% protease inhibitors cocktail (Sigma) and 1% phosphotase inhibitors cocktail I/II (Sigma)], beads were subsequently incubated with purified PKA or PKCε protein at 30℃ for 1 hour in the kinase assay buffer (20 mM TrisHCl, pH 7.5, 5 mM $MgCl_2$, 400 μM ATP, 1 mM DTT, 50 mM NaCl). Reactions were terminated by adding 2×loading buffer and subjected to analysis by immunoblotting.

Microarray analysis

Primary hepatocytes were infected with the desired adenoviruses or treated with glucagon. Total cellular RNA was isolated with TRIzol (Invitrogen) and subjected to analysis by Affymetrix Mouse Genome 430 2.0 Arrays. Three experiments were independently conducted. The original microarray intensities were log2-transformed and quantile-normalized using 'affy' package in Bioconductor (8). Differences in the expression of genes between each two compared samples were determined by RankProd (9), with proportion of false positive (pfp) < 0.1 considered significant. Parametric Analysis of Gene Set Enrichment (PAGE) (10) was conducted based on KEGG pathways downloaded from the KEGG database (http://www.genome.jp/kegg/) on July 5, 2010. Significance of the enrichment was calculated and false discovery rate (FDR) < 0.01 considered significant.

RT-PCR analyses

Total RNA was isolated from cells or mouse livers with TRIzol (Invitrogen). After reverse transcription by M-MLV Reverse Transcriptase (Invitrogen), regular PCR was performed with TaKaRa Taq kits (Takara). Quantitative real-time PCR was done with an ABI Prism 7500 sequence detection system, using Power SYBR Green PCR Master Mix (Applied Biosystems) following the manufacturer's recommendations (Applied Biosystems). Actin was used as an internal control for normalization. The oligonucleotide primers for each target gene examined are listed as follows.

Mouse *Xbp-1*: sense 5'-GAACCAGGAGTTAAGGACACG-3', antisense 5'-GGGGATCTCTAAGACTAGAGGCT-3';

Mouse *G6pase*: sense 5'-CGACTCGCTATCTCCAAGTGA-3', antisense 5'-GTTGAACCAGTCTCCGACCA-3';

Mouse *Pepck*: sense 5'-AAGCATTCAACGCCAGGTTC-3', antisense 5'-GGGCGAGTCTGTCAGTTCAAT-3';

Mouse *Actin*: sense 5'-AGTGTGACGTTGACATCCGTA-3', antisense 5'-GCCAGAGCAGTAATCTCCTTCT-3';

Mouse *Bip*: sense 5'-ACTTGGGGACCACCTATTCCT-3', antisense 5'-ATCGCCAATCAGACGCTCC-3';

Mouse *Chop*: sense 5'-CTGGAAGCCTGGTATGAGGAT-3', antisense 5'-CAGGGTCAAGAGTAGTGAAGGT-3'.

Mouse *Erdj4*: sense 5'-ATAAAAGCCCTGATGCTGAAGC-3', antisense 5'-GCCATTGGTAAAAGCACTGTGT-3'.

Figures and Legends

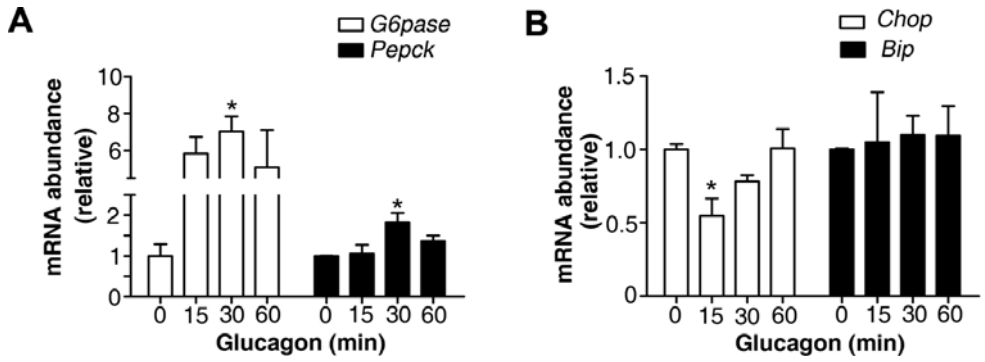

Fig. S1. Glucagon stimulated the expression of gluconeogenic genes but not typical UPR target genes in vivo. Male C57BL/6 mice were treated for the indicated time intervals (n=3/group) by i.p. injection of glucagon (100 μg/kg body weight). The hepatic mRNA abundance of *G6pase*, *Pepck*, *Chop* and *Bip* was analyzed by quantitative real-time RT-PCR, with *actin* used as an internal control. Results for each gene were normalized to the value at 0 min (set as 1) and shown as the mean ± SEM (n=3 independent experiments). *P < 0.05 versus 0 time point by one-way ANOVA.

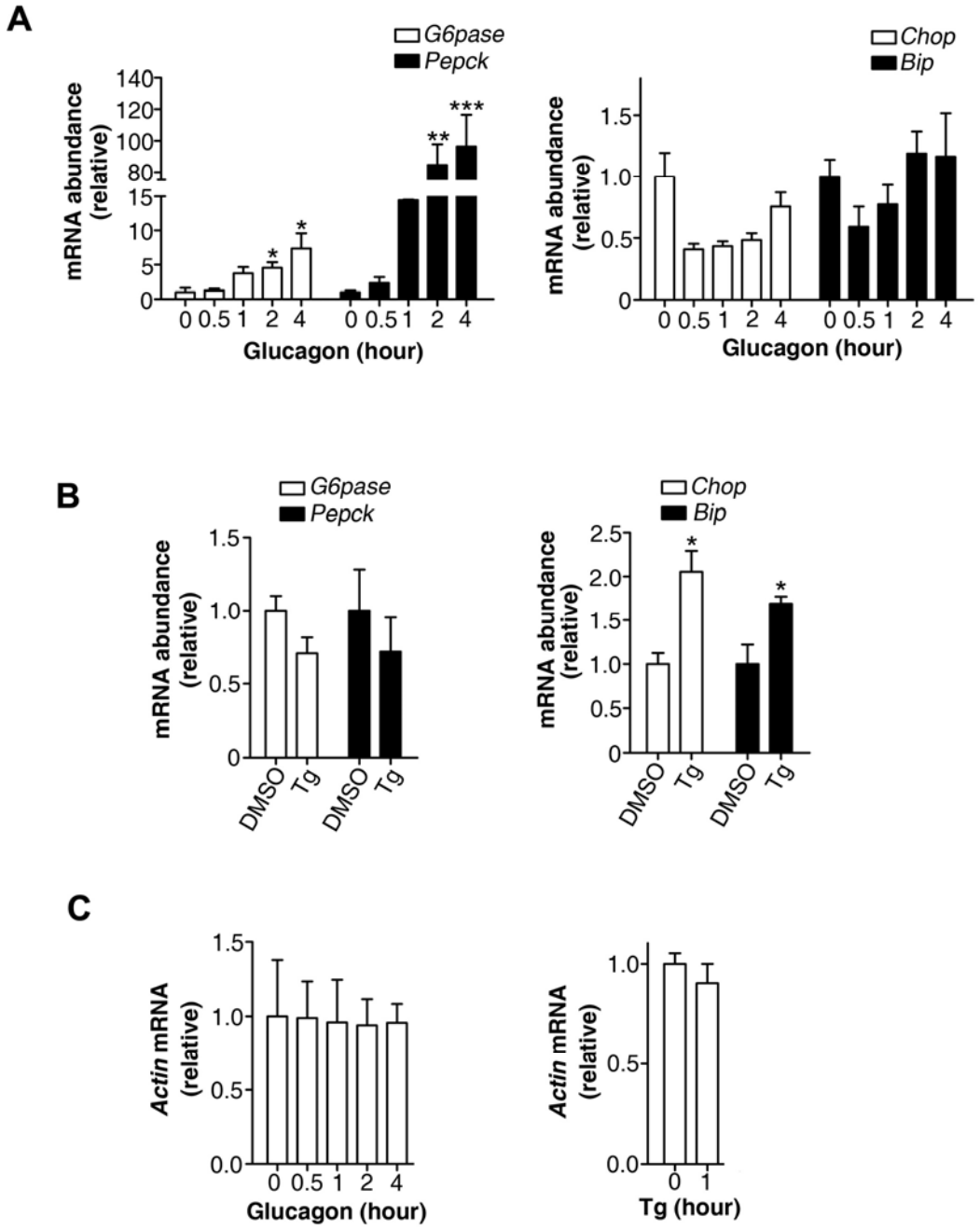

Fig. S2. Glucagon but not thapsigargin stimulated the expression of gluconeogenic genes in primary hepatocytes. Primary hepatocytes were treated (A) with 100 nM glucagon for 0, 0.5, 1, 2 or 4 hours, or (B) with dimethyl sulfoxide (DMSO) or thapsigargin (Tg, 1 μM) for 1 hour. The mRNA abundance of *G6pase*, *Pepck*, *Chop*, and *Bip* was determined by quantitative real-time RT-PCR, using *actin* as an internal control. (C) Glucagon or thapsigargin did not affect the expression of *actin* that was used as the internal control in (A) and (B). The mRNA abundance of *actin* was analyzed by quantitative real-time RT-PCR and shown as relative to 18S rRNA. All results were normalized to the value at 0 min for glucagon treatment or to that of DMSO for Tg treatment. Data are shown as the mean ± SEM (n=3 independent

experiments). *P < 0.05 versus 0 time point or DMSO control, **P < 0.01 and ***P < 0.001 versus 0 time point by one-way ANOVA.

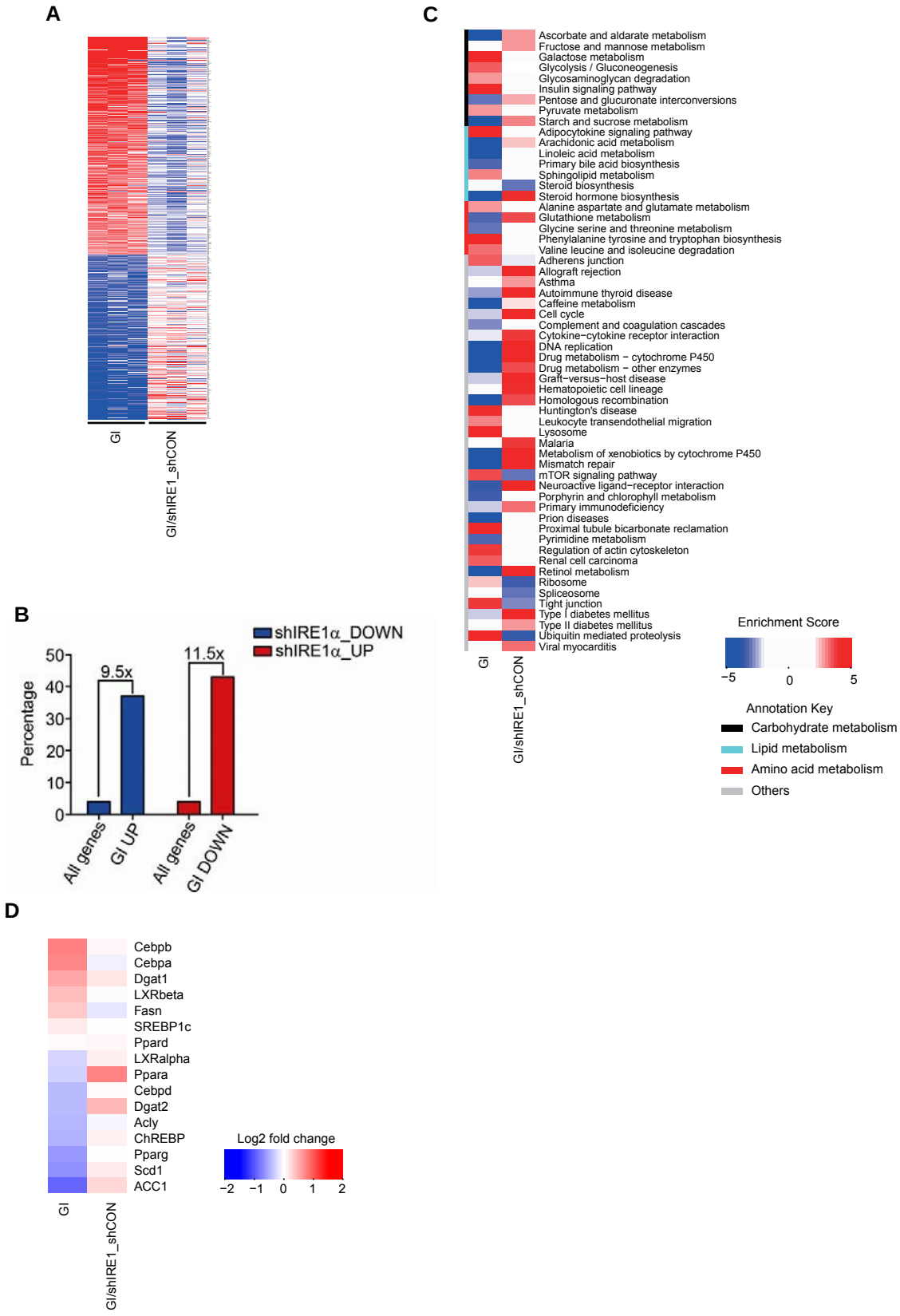
A
GI
GI/shIRE1_shCON
B
shIRE1α_DOWN
shIRE1α_UP
Percentage
50
40
30
20
10
0
9.5x
11.5x
All genes
GI UP
All genes
GI DOWN
C
Ascorbate and aldarate metabolism
Fructose and mannose metabolism
Galactose metabolism
Glycolysis / Gluconeogenesis
Glycosaminoglycan degradation
Insulin signaling pathway
Pentose and glucuronate interconversions
Pyruvate metabolism
Starch and sucrose metabolism
Adipocytokine signaling pathway
Arachidonic acid metabolism
Linoleic acid metabolism
Primary bile acid biosynthesis
Sphingolipid metabolism
Steroid biosynthesis
Steroid hormone biosynthesis
Alanine aspartate and glutamate metabolism
Glutathione metabolism
Glycine serine and threonine metabolism
Phenylalanine tyrosine and tryptophan biosynthesis
Valine leucine and isoleucine degradation
Adherens junction
Allograft rejection
Asthma
Autoimmune thyroid disease
Caffeine metabolism
Cell cycle
Complement and coagulation cascades
Cytokine−cytokine receptor interaction
DNA replication
Drug metabolism − cytochrome P450
Drug metabolism − other enzymes
Graft−versus−host disease
Hematopoietic cell lineage
Homologous recombination
Huntington's disease
Leukocyte transendothelial migration
Lysosome
Malaria
Metabolism of xenobiotics by cytochrome P450
Mismatch repair
mTOR signaling pathway
Neuroactive ligand−receptor interaction
Porphyrin and chlorophyll metabolism
Primary immunodeficiency
Prion diseases
Proximal tubule bicarbonate reclamation
Pyrimidine metabolism
Regulation of actin cytoskeleton
Renal cell carcinoma
Retinol metabolism
Ribosome
Spliceosome
Tight junction
Type I diabetes mellitus
Type II diabetes mellitus
Ubiquitin mediated proteolysis
Viral myocarditis
GI
GI/shIRE1_shCON
Enrichment Score
−5
0
5
Annotation Key
Carbohydrate metabolism
Lipid metabolism
Amino acid metabolism
Others
D
Cebpb
Cebpa
Dgat1
LXRbeta
Fasn
SREBP1c
Ppard
LXRalpha
Ppara
Cebpd
Dgat2
Acly
ChREBP
Pparg
Scd1
ACC1
Log2 fold change
−2
−1
0
1
2
GI
GI/shIRE1_shCON

Fig. S3. Transcriptomic analysis of the impact of IRE1α knockdown on glucagon-regulated gene expression profiles in liver cells. Primary hepatocytes were infected for 48 hours with Ad-shCON or Ad-shIRE1α-$^{\#}$2 and then treated with 100 nM glucagon for 1 hour. Hepatocytes infected for 48 hours with EGFP-expressing adenovirus were left untreated. Total cellular RNA was subjected to whole-genome microarray analysis using Affymetrix Mouse Genome 430 2.0 Arrays. (A) Individual heatmaps from three independent experiments showing differentially expressed genes upon glucagon induction (GI), which were aligned with changes of these genes caused by IRE1α knockdown in the presence of glucagon stimulation. Included are genes exhibiting >1.25-fold changes or trends of significant changes as determined by RankProd with proportion of false positive (pfp) < 0.1. Gene labels are also indicated. (B) Percentages of all array-probed genes (All genes) that were down- or up-regulated by shIRE1α as compared with percentages of glucagon-upregulated (GI UP) or -downregulated (GI DOWN) genes that were down- or up-regulated by shIRE1α relative to shCON control. (C) Heatmaps showing glucagon-regulated cellular pathways or biological processes that were affected by IRE1α knockdown. Parametric Analysis of Gene Set Enrichment (PAGE) was performed using the KEGG database (http://www.genome.jp/kegg/), with false discovery rate (FDR) < 0.01. Pathways related to metabolism of carbohydrates, lipids and amino acids are indicated. (D) Heatmaps showing expression changes of regulatory genes in lipid metabolism and enzymes in triglyceride synthesis as result of IRE1α knockdown in the presence of glucagon stimulation.

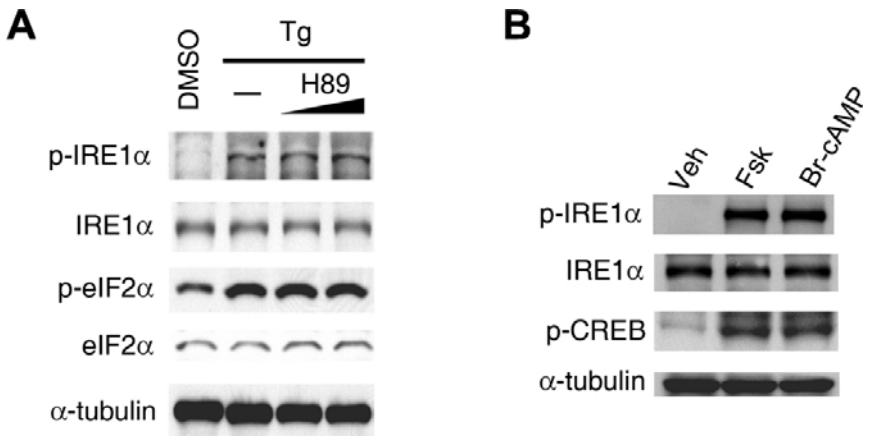

Fig. S4. Effects of PKA inhibitor on IRE1α phosphorylation during ER stress and stimulation of IRE1α phosphorylation by activation of the cAMP pathway. (A) H89 had no effect on ER stress-induced phosphorylation of IRE1α. Primary hepatocytes pre-cultured for 30 minutes with DMSO or H89 (at 5 or 10 μM) were treated with 1 μM Tg for 1 hour. (B) cAMP pathway activators stimulated IRE1α phosphorylation. Primary hepatocytes were treated with DMSO/PBS (Veh), 10 μM forskolin (adenylate cyclase activator), or 100 μM Br-cAMP (cAMP analog) for 1 hour. Immunoblotting was performed using the indicated antibodies. Results shown are representative of three independent experiments.

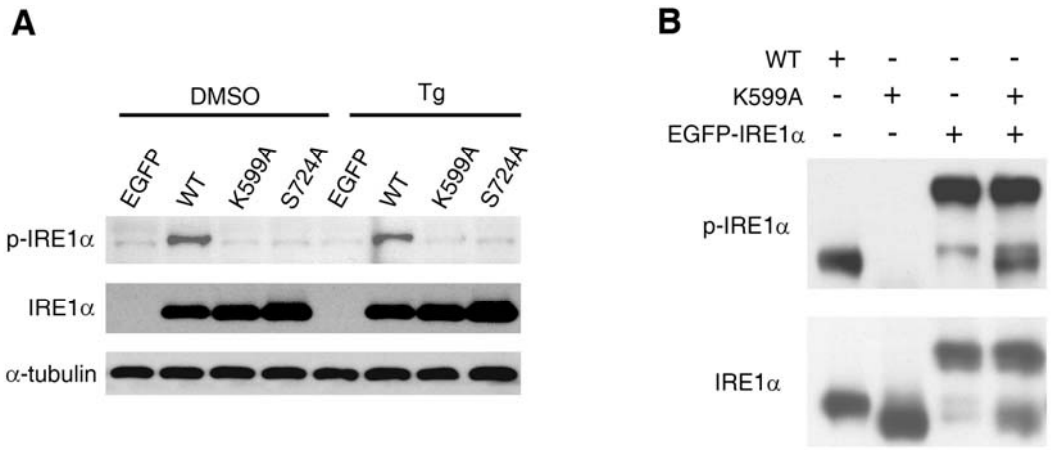

Fig. S5. Thapsigargin did not induce but overexpressed EGFP-IRE1α caused Ser^{724} phosphorylation of the autophosphorylation-defective IRE1α-K599A mutant. (A) Primary hepatocytes were infected for 48 hours with recombinant adenoviruses expressing EGFP, Flag-tagged human wild-type (WT) IRE1α, or IRE1α-K599A and IRE1α-S724A mutants. Cells were then treated with DMSO or 1 μM Tg for 1 hour. (B) HEK293 cells were transfected for 48 hours with plasmids expressing wild-type (WT) IRE1α, IRE1α-K599A or EGFP-IRE1α, or co-transfected with EGFP-IRE1α and IRE1α-K599A. Phosphorylation of IRE1α was analyzed by immunoblotting with IRE1α or phospho-IRE1α antibodies. Results shown are representative of three independent experiments.

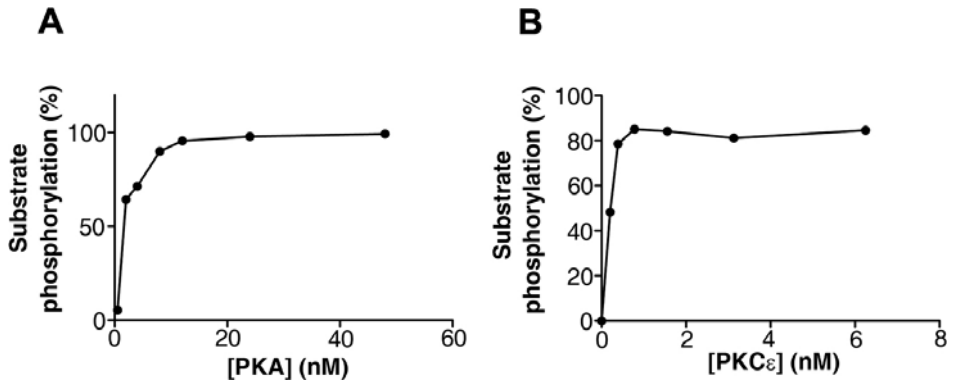

Fig. S6. Enzyme activities of purified PKA and PKC proteins. Recombinant mouse PKA and human PKCε proteins were expressed and purified. Kinase activities were measured with synthetic substrate peptides for PKA (A) and PKC (B) using Z`-LYTE™ Kinase Assay Kit.

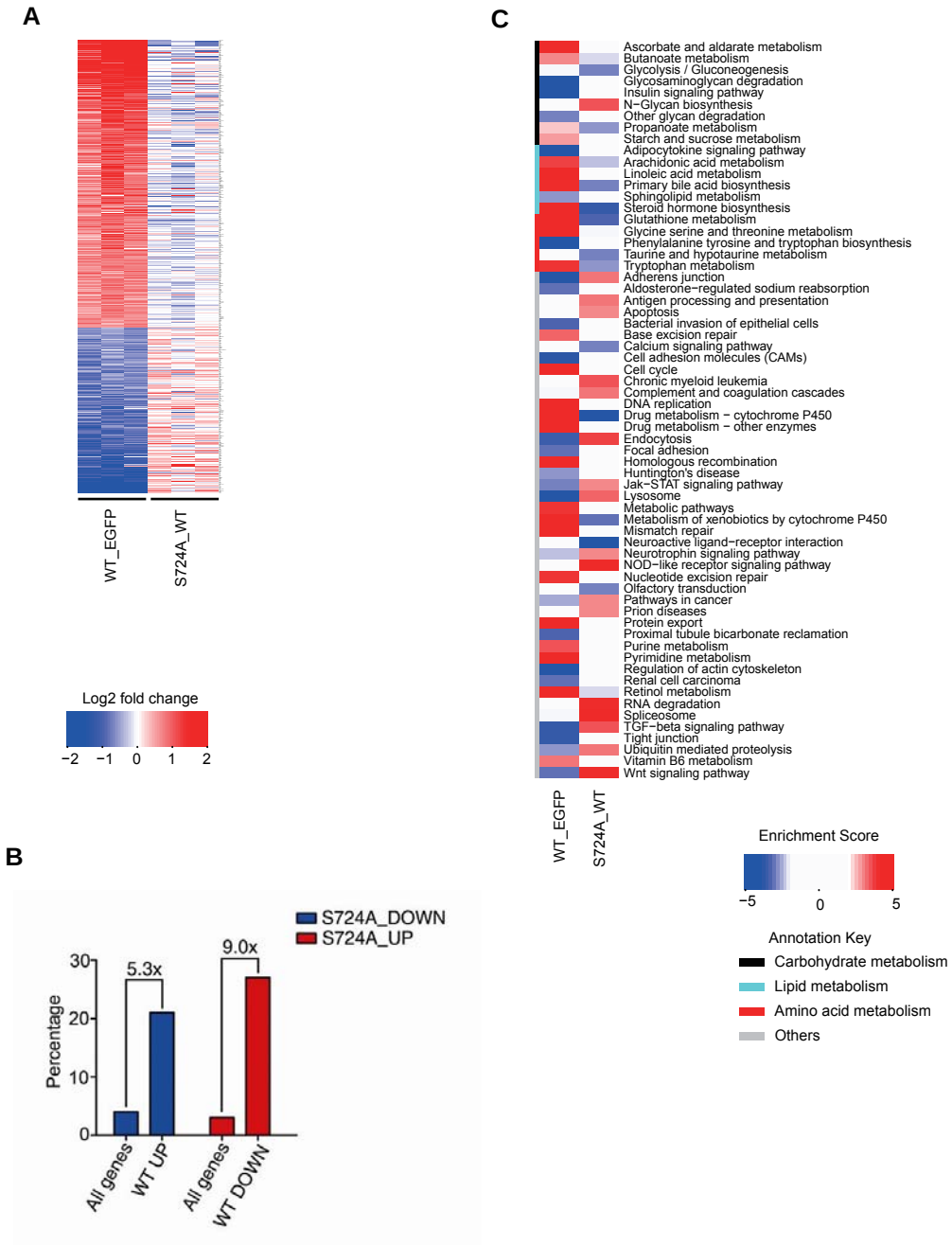
A
WT_EGFP
S724A_WT
Log2 fold change
−2 −1 0 1 2
B
S724A_DOWN
S724A_UP
5.3x
9.0x
Percentage
30
20
10
0
All genes
WT UP
All genes
WT DOWN
C
Ascorbate and aldarate metabolism
Butanoate metabolism
Glycolysis / Gluconeogenesis
Glycosaminoglycan degradation
Insulin signaling pathway
N−Glycan biosynthesis
Other glycan degradation
Propanoate metabolism
Starch and sucrose metabolism
Adipocytokine signaling pathway
Arachidonic acid metabolism
Linoleic acid metabolism
Primary bile acid biosynthesis
Sphingolipid metabolism
Steroid hormone biosynthesis
Glutathione metabolism
Glycine serine and threonine metabolism
Phenylalanine tyrosine and tryptophan biosynthesis
Taurine and hypotaurine metabolism
Tryptophan metabolism
Adherens junction
Aldosterone−regulated sodium reabsorption
Antigen processing and presentation
Apoptosis
Bacterial invasion of epithelial cells
Base excision repair
Calcium signaling pathway
Cell adhesion molecules (CAMs)
Cell cycle
Chronic myeloid leukemia
Complement and coagulation cascades
DNA replication
Drug metabolism − cytochrome P450
Drug metabolism − other enzymes
Endocytosis
Focal adhesion
Homologous recombination
Huntington's disease
Jak−STAT signaling pathway
Lysosome
Metabolic pathways
Metabolism of xenobiotics by cytochrome P450
Mismatch repair
Neuroactive ligand−receptor interaction
Neurotrophin signaling pathway
NOD−like receptor signaling pathway
Nucleotide excision repair
Olfactory transduction
Pathways in cancer
Prion diseases
Protein export
Proximal tubule bicarbonate reclamation
Purine metabolism
Pyrimidine metabolism
Regulation of actin cytoskeleton
Renal cell carcinoma
Retinol metabolism
RNA degradation
Spliceosome
TGF−beta signaling pathway
Tight junction
Ubiquitin mediated proteolysis
Vitamin B6 metabolism
Wnt signaling pathway
WT_EGFP
S724A_WT
Enrichment Score
−5 0 5
Annotation Key
Carbohydrate metabolism
Lipid metabolism
Amino acid metabolism
Others

Fig. S7. Transcriptomic analysis of the effect of blocking Ser^{724} phosphorylation on IRE1α-evoked gene expression profiles in liver cells. Primary hepatocytes were infected for 48 hours with adenoviruses expressing EGFP, IRE1α or IRE1α-S724A. Total cellular RNA was subjected to whole-genome microarray analysis using Affymetrix Mouse Genome 430 2.0 Arrays. (A) Individual heatmaps from three independent experiments showing differentially expressed genes elicited by overexpressed IRE1α-WT, which were aligned with changes of these genes caused by S724A mutation. Included are genes exhibiting >1.25-fold alterations or trends of significant changes as determined by RankProd with proportion of false positive (pfp) < 0.1. Gene labels are also indicated. (B) Percentage of all arrayed-probed genes (All genes) that were down- or up-regulated by S724A mutant as compared with percentage of IRE1α (WT)-upregulated (WT UP) or -downregulated (WT DOWN) genes which were down- or up-regulated by S724A. (C) Heatmaps showing IRE1α-evoked changes of cellular pathways or biological processes that were influenced by S724A mutation. Parametric Analysis of Gene Set Enrichment (PAGE) was performed using the KEGG database (http://www.genome.jp/kegg/), with false discovery rate (FDR) < 0.01. Pathways related to metabolism of carbohydrates, lipids and amino acids are indicated.

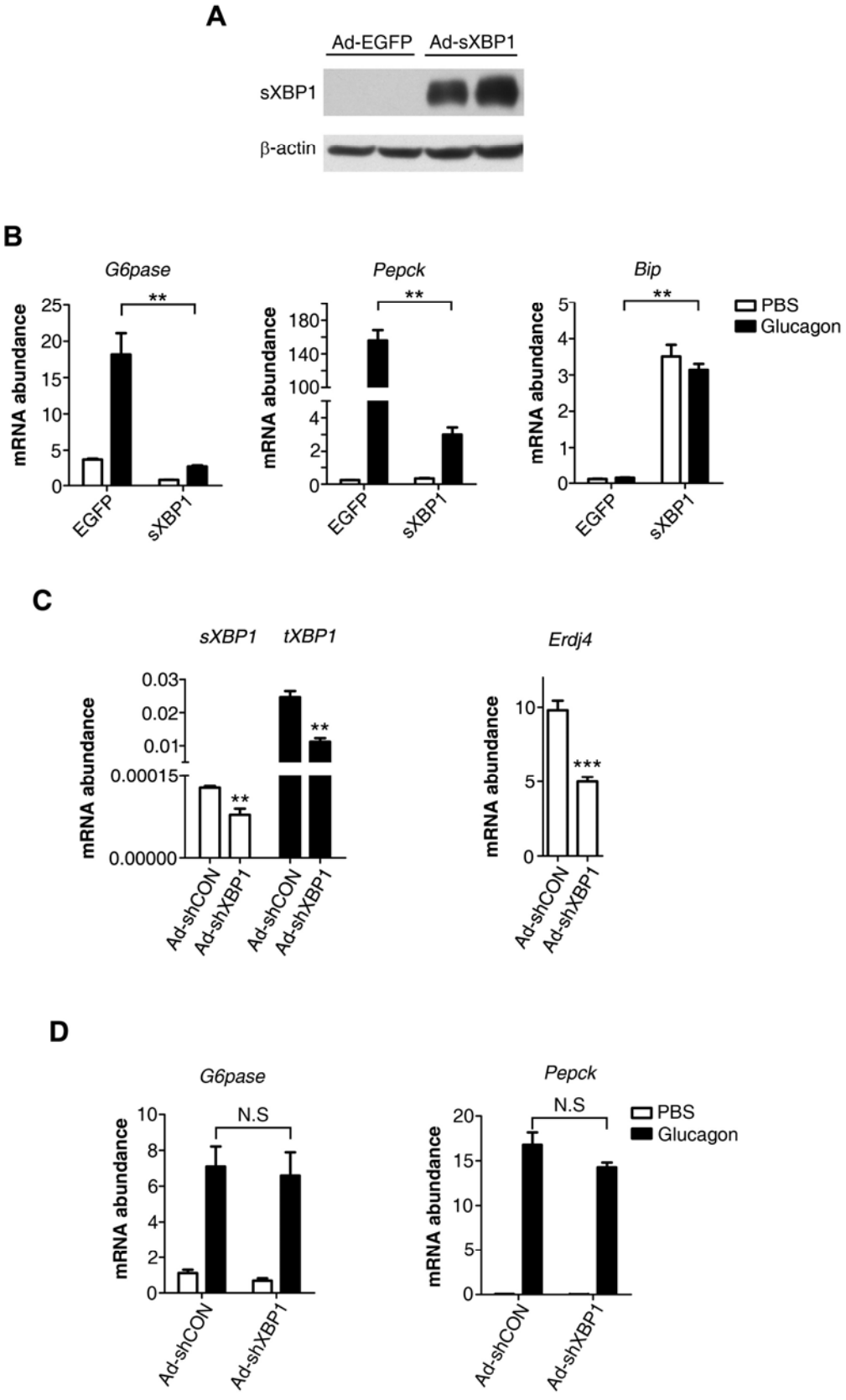
A
Ad-EGFP
Ad-sXBP1
sXBP1
β-actin
B
G6pase
Pepck
Bip
mRNA abundance
**
PBS
Glucagon
EGFP
sXBP1
C
sXBP1
tXBP1
Erdj4
mRNA abundance
**
***
Ad-shCON
Ad-shXBP1
D
G6pase
Pepck
N.S
mRNA abundance
PBS
Glucagon
Ad-shCON
Ad-shXBP1

Fig. S8. Effects of sXBP1 overexpression or XBP1 knockdown on the expression of gluconeogenic genes. (A and B) Adenoviral overexpression of the spliced form of XBP1 suppressed the expression of *G6pase* and *Pepck* while inducing the expression of *Bip*. Primary hepatocytes were infected for 48 hours with recombinant adenoviruses expressing EGFP or Flag-tagged spliced (s) form of XBP1. Infected cells were then treated with PBS or 100 nM glucagons for 1 hour. (A) sXBP1 protein was analyzed by immunoblotting using the Flag antibody. Actin was shown as the loading control. (B) The mRNA abundance of *G6pase*, *Pepck* and *Bip* was determined by quantitative real-time RT-PCR using *actin* as an internal control. $^{**}P < 0.01$ by two-way ANOVA. (C and D) Adenoviral knockdown of the expression of XBP1 had no effect on glucagon-stimulated expression of *G6pase* and *Pepck*. Primary hepatocytes were infected with the control (CON) or XBP1 shRNA adenoviruses for 72 hours. (C) The mRNA abundance of *sXBP1* and *tXBP1* was analyzed for infected cells without treatment, and the expression of *Erdj4*, a XBP1 target gene, was assessed for cells upon treatment with 1 μM thapsigargin for 1 hour by quantitative real-time RT-PCR. (D) The mRNA abundance of *G6pase* and *Pepck* was determined for cells treated with PBS or 100 nM glucagon for 4 hours by quantitative real-time RT-PCR. The mRNA expression of *actin* was used as an internal control. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus shCON by *t*-test. All data are shown as the mean ± SEM (n=3 independent experiments).

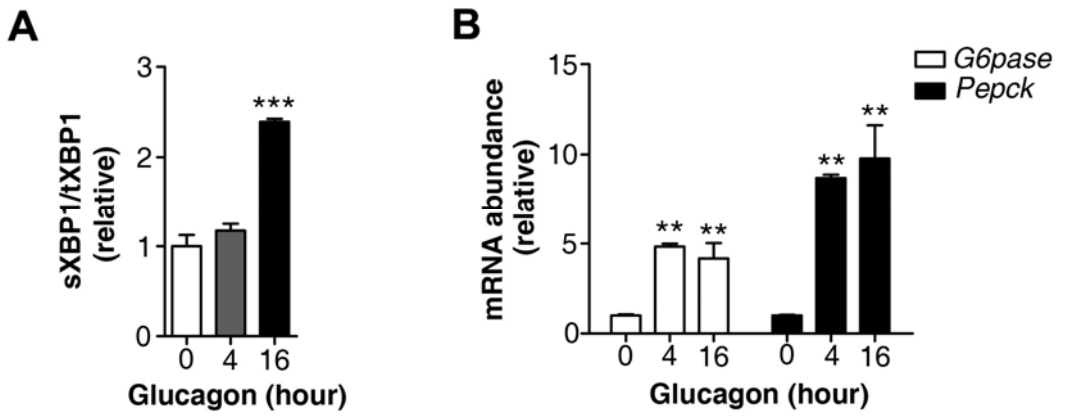

Fig. S9. Effect of acute versus chronic exposure to glucagon on XBP1 mRNA splicing and the expression of gluconeogenic genes. Primary hepatocytes were treated with 100 nM glucagon for 4 or 16 hours. The medium was changed every 4 hours to keep the glucagon activity during the 16-hour treatment. (A) The ratios of spliced (s) to total (t) XBP1 mRNA and (B) the mRNA abundance of *G6pase* and *Pepck* were determined by real-time RT-PCR analysis, using *actin* as an internal control. Results were normalized to the value at 0 min. Data are shown as the mean ± SEM (n=3 independent experiments). **P < 0.01 and ***P < 0.001 versus 0 time point by one-way ANOVA.

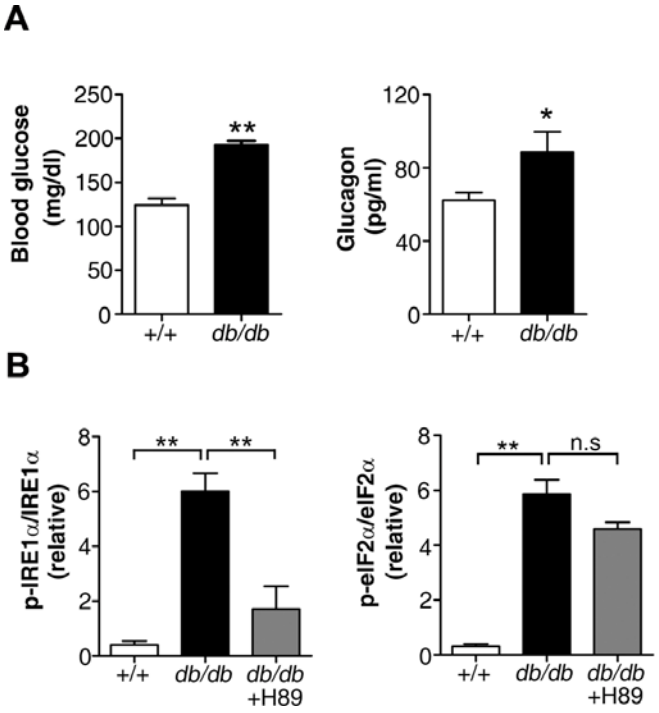

Fig. S10. (A) Elevated blood glucose and glucagon levels in *db/db* mice. After a 6-hour fast, blood samples were collected from male C57BL/6J *db/db* mice or their wild-type (+/+) littermates and supplemented with glucagon inhibitor aprotonin. Glucose concentrations were measured by a glucometer and glucagon levels determined by RIA. Data are presented as the mean ± SEM (n=5/genotype). *P < 0.05, **P < 0.01 versus wild-type littermates by *t*-test. **(B) Increased phosphorylation of hepatic IRE1α is PKA-dependent in *db/db* mice.** Male *db/db* mice were treated for 2 hours with PBS or H89 (5 mg/kg body weight) through i.p. injection. Phosphorylation of liver IRE1α and eIF2α was analyzed by immunoblotting as shown in Fig. 4A. Relative p-IRE1α/IRE1α and p-eIF2α/eIF2α ratios were determined from densitometric quantifications of the immunoblots and are shown as the mean ± SEM (n=3/group). **P < 0.01 by one-way ANOVA.

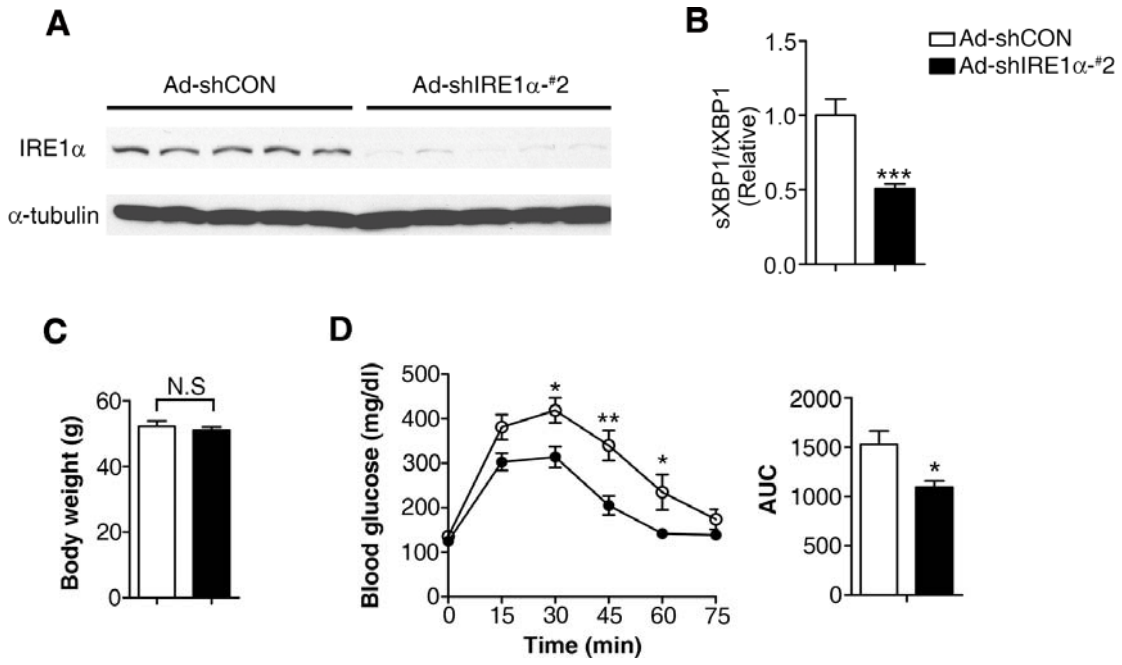

Fig. S11. Effects of hepatic IRE1α suppression on XBP1 mRNA splicing, body weight and the hyperglycemic response to glucagon in *db/db* mice. Male *db/db* mice were infected with adenoviruses Ad-shCON or Ad-shIRE1α-$^{\#}$2 through tail vein injection (n=5/group). (A) Immunoblotting analysis of liver IRE1α from individual mice at 21 days post infection. (B) The spliced (s) and total (t) XBP1 mRNA in the liver was determined by real time RT-PCR after a 6-hour fast. (C) Knockdown of hepatic IRE1α expression did not affect body weight. Body weight was determined for *db/db* mice infected for 21 days. (D) Hepatic IRE1α knockdown reduced glucagon-induced elevations of blood glucose. Glucagon challenge test was performed in mice infected for 11 days through administration i.p. of 150 μg/kg glucagon after a 15-hour fast. Blood glucose was measured at the indicated time points. *P < 0.05 and **P < 0.01 by two-way ANOVA. The bar graph indicates the areas under the curve (AUC) for the glucose levels during the test, shown as the mean ± SEM (n = 5/group). *P < 0.05 by t-test.

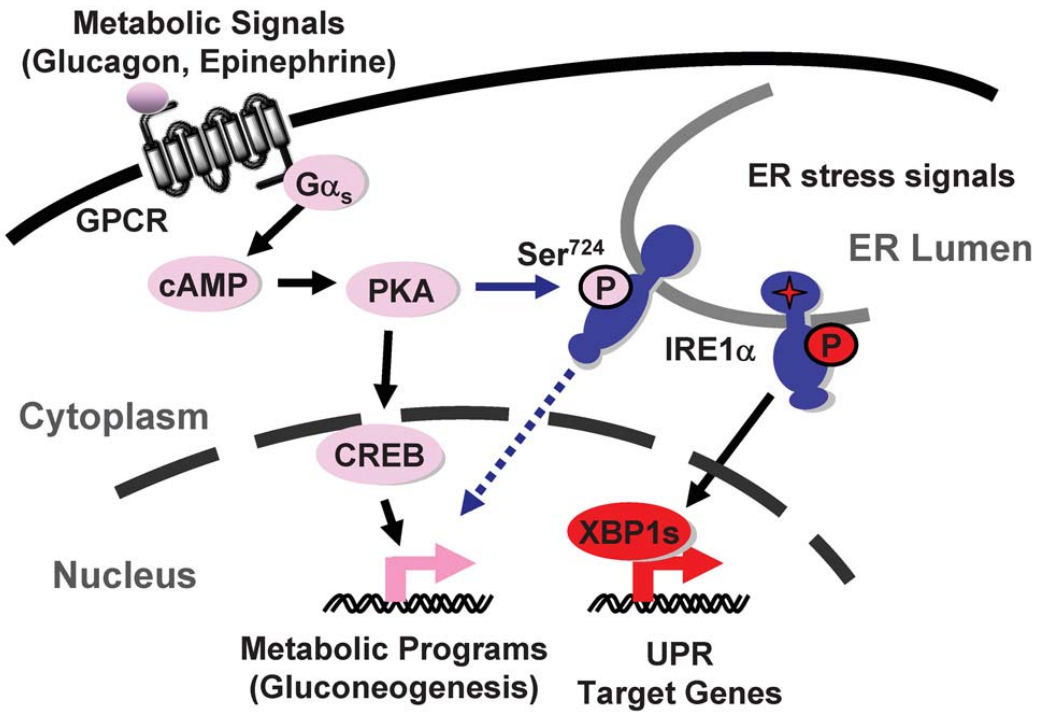

Fig. S12. Schematic model for the PKA-dependent mechanism that links the IRE1α branch of the UPR with the GPCR signaling pathway. Metabolic signals such as GPCR agonists (glucagon or epinephrine) stimulates PKA, which in turn directly phosphorylates IRE1α at Ser^{724}, a critical regulatory site within the activation segment of IRE1α. Phosphorylation activation of IRE1α as such plays an important role in glucagon-regulated metabolic programs in the liver, e.g. promoting the expression of gluconeogenic genes in an XBP1-independent fashion. Under obesity-associated metabolic stress, dysregulation of PKA activity, in addition to aberrant ER lipid and calcium metabolism, contributes to increased phosphorylation of hepatic IREα, which constitutes a critical component in perturbation of glucose homeostasis. Thus, IREα also integrates metabolic signals through phosphorylation by PKA of the GPCR pathway in liver cells and is implicated in the control of glucose metabolism.

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