P38 MAPK REGULATED XBP1s NUCLEAR TRANSLOCATION AND mRNA STABILITY ARE CRUCIAL FOR MAINTENANCE OF GLUCOSE HOMEOSTASIS IN OBESITY

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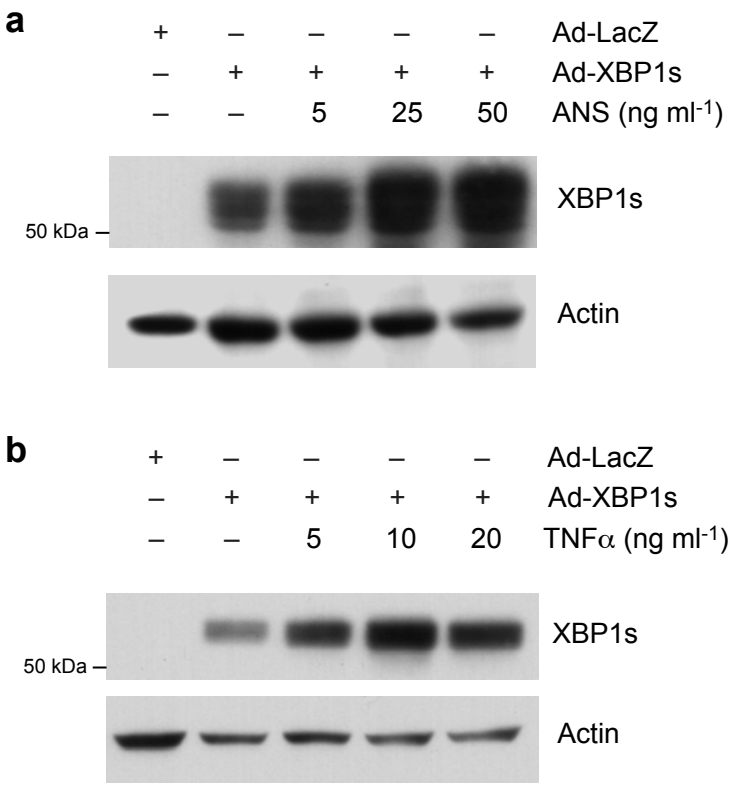
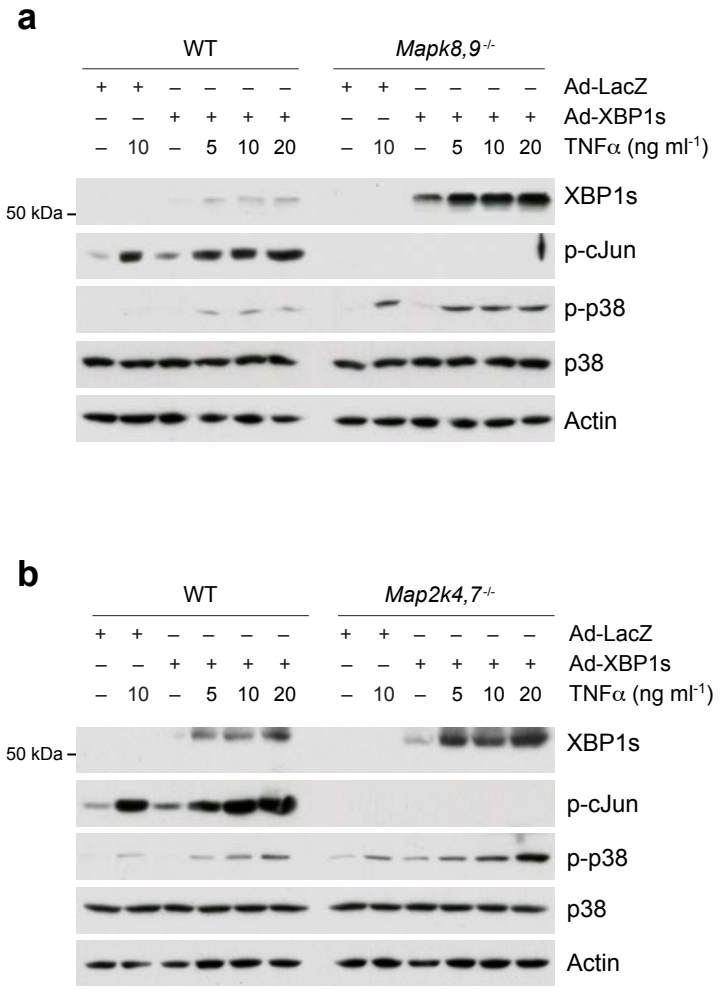
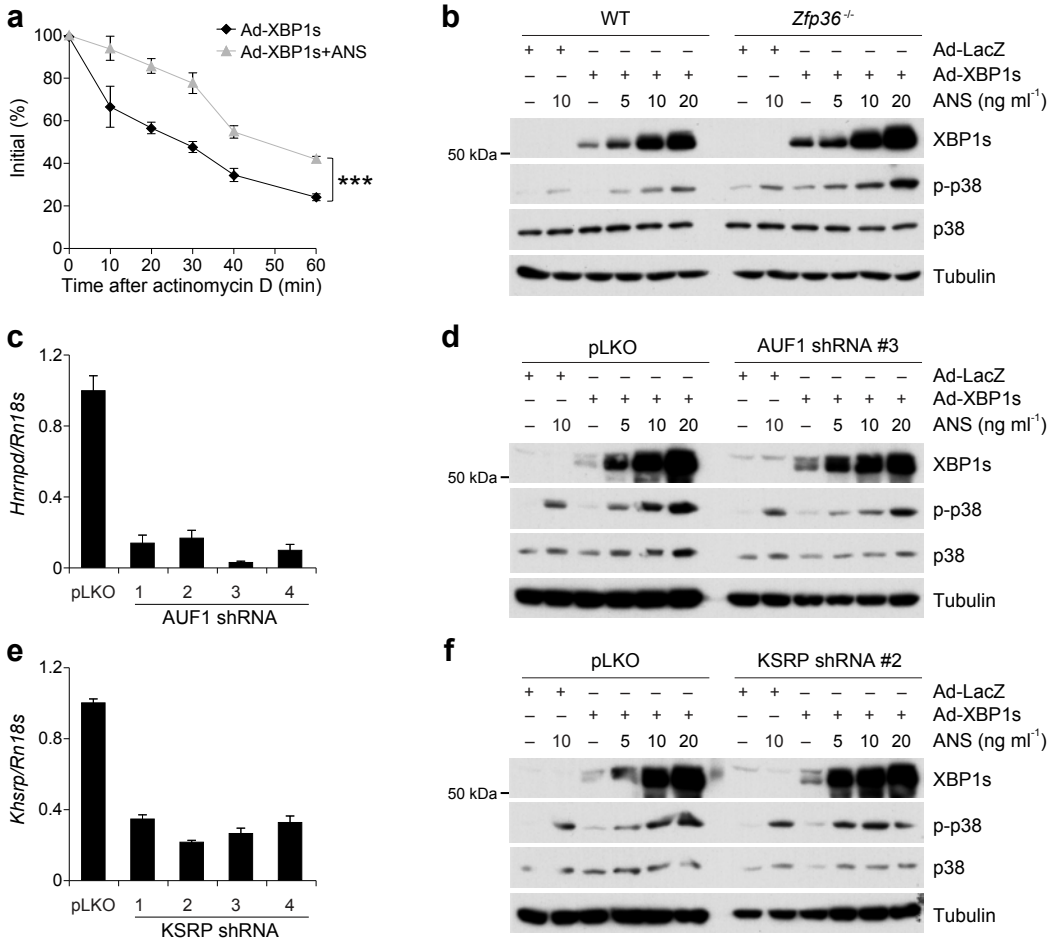
Supplementary Figures



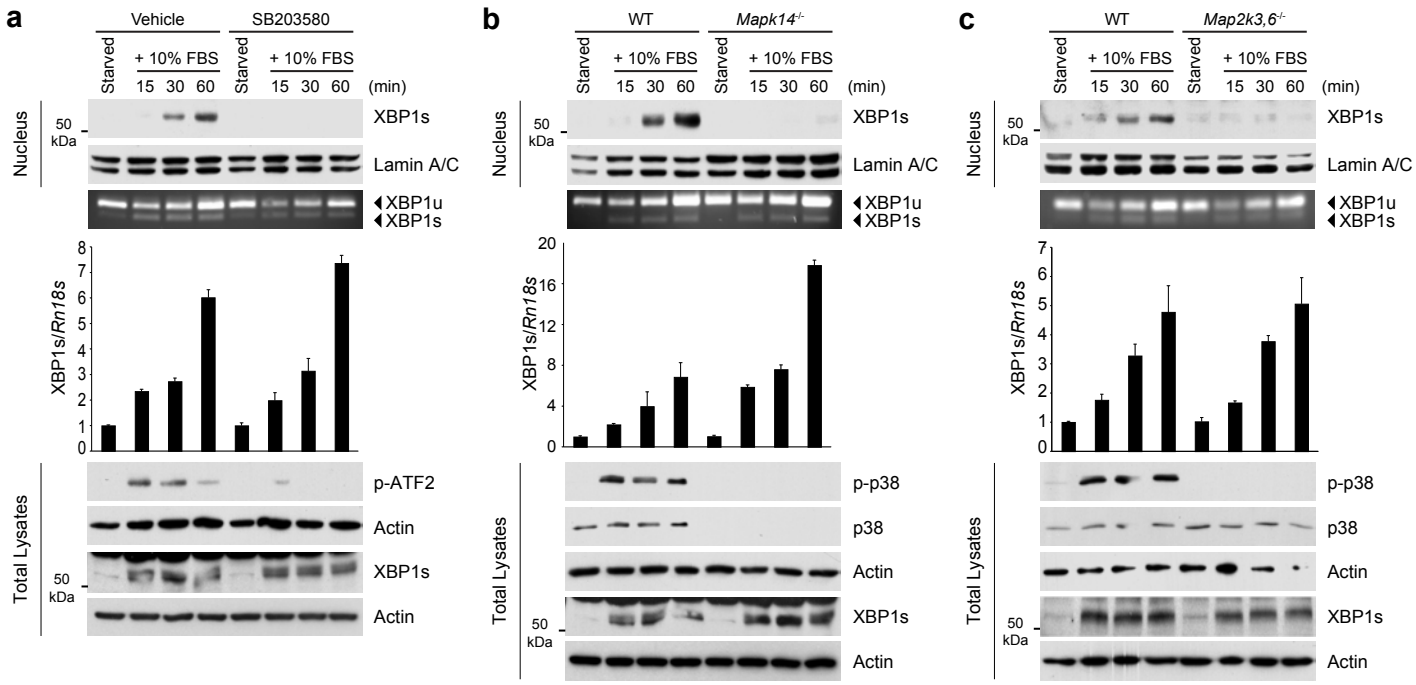
Supplementary Figure 1. Anisomycin or TNFα increases XBP1s protein levels. Fao cells were infected with Ad-LacZ or Ad-XBP1s. Subsequently, cells were stimulated with (**a**) ANS or (**b**) TNFα for 2 h at the indicated concentrations. XBP1s protein levels were determined via immunoblotting. Each experiment was independently reproduced three times.



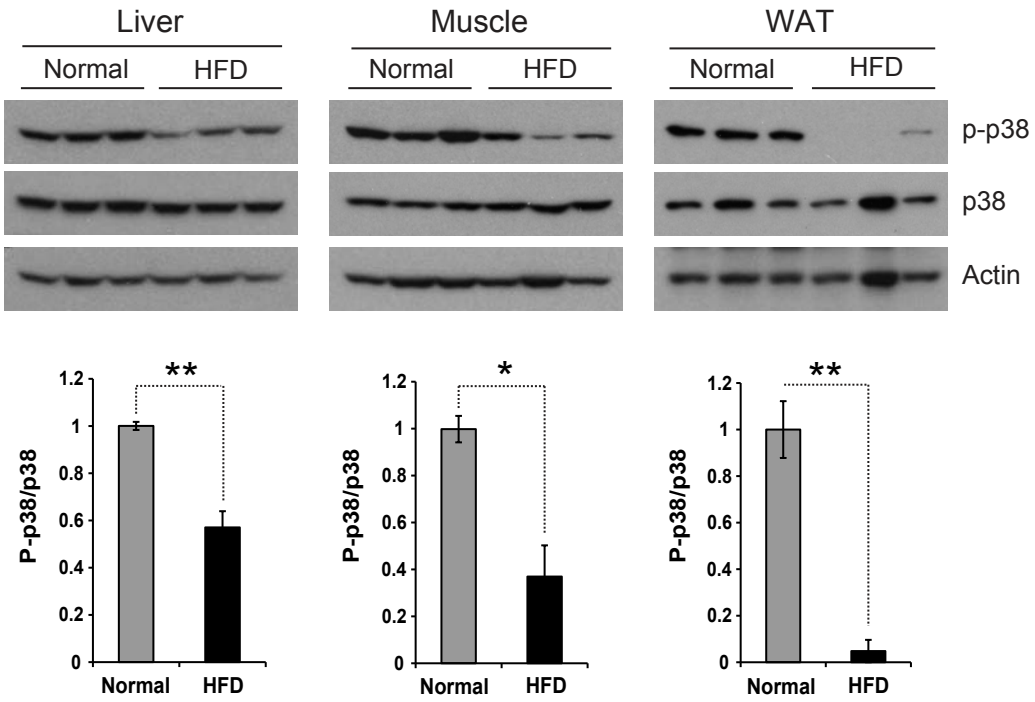
Supplementary Figure 2. Effect of TNFα on XBP1s protein levels in *Mapk8,9*$^{-/-}$ or *Map2k4,7*$^{-/-}$ cells. (**a**) *Mapk8,9*$^{-/-}$ and wild-type (WT) cells or (**b**) *Map2k4,7*$^{-/-}$ and WT cells were infected with Ad-LacZ or Ad-XBP1s and subsequently stimulated with TNFα for 2 h at the indicated concentrations. XBP1s, p-cJun, p-p38, p38 and actin levels were detected via immunoblotting. Each experiment was independently reproduced three times.



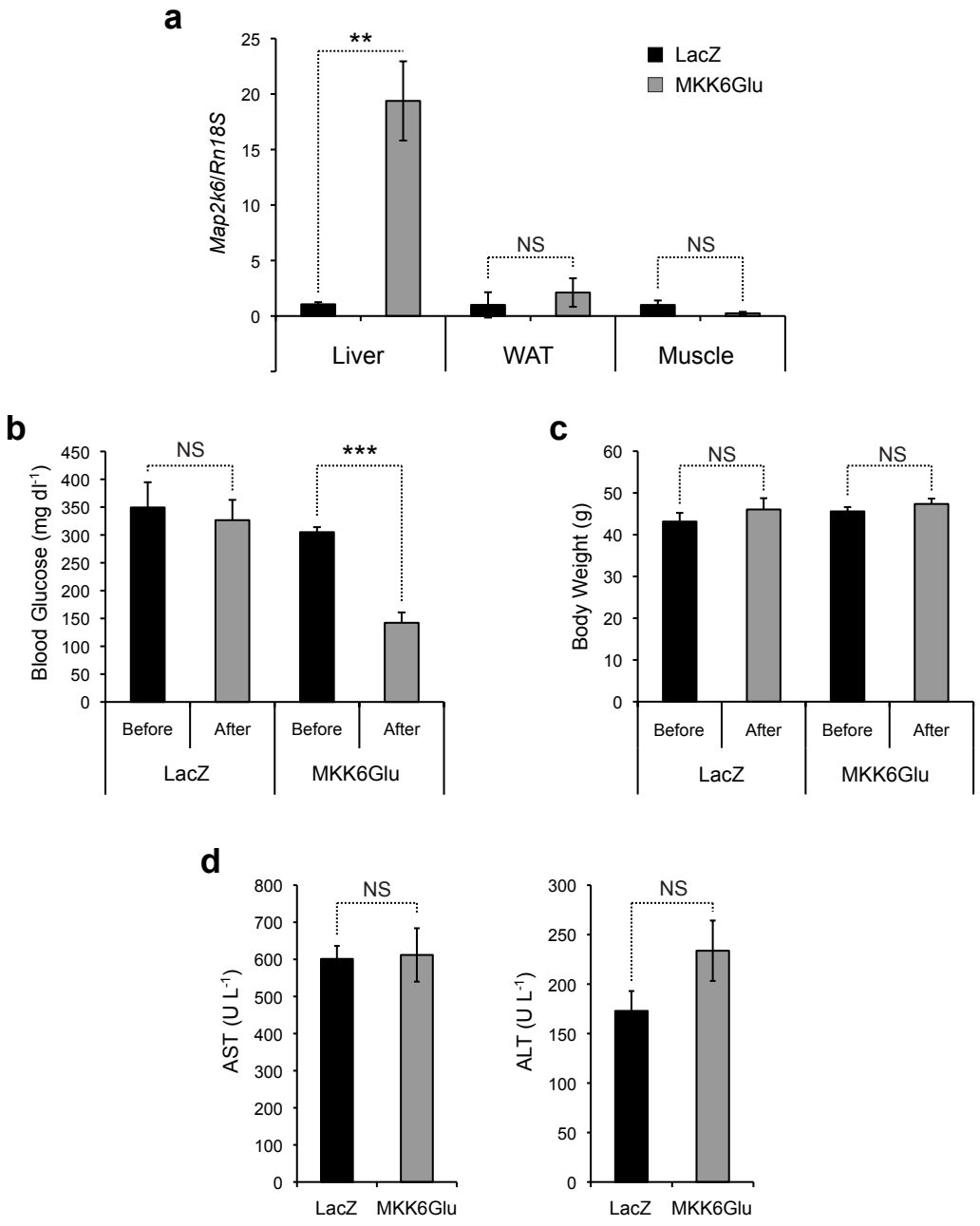
Supplementary Figure 3. TTP, AUF1 and KSRP do not play a role in p38 MAPK-mediated up-regulation of XBP1s mRNA stability. **(a)** TTP knockout (*Zfp36*$^{-/-}$) cells were infected with Ad-XBP1s and subsequently treated with ANS (25 ng ml^{-1}) for 1 h followed by incubation with actinomycin D (10 μg ml^{-1}). mRNA levels of XBP1s were determined at the indicated time points. **(b)** WT and *Zfp36*$^{-/-}$ cells were infected with Ad-LacZ or Ad-XBP1s, and then treated with ANS for 2 h at the indicated concentrations. XBP1s protein levels were determined via western blotting. **(c)** Four different lentiviral shRNAs were tested for silencing AUF1 (*Hnrnpd*) expression. #3 AUF1 lentiviral-shRNA was chosen. Procedures regarding lentivirus preparation, transduction and screening are described in supplementary methods. **(d)** Control (pLKO) and AUF1 knockdown cells were infected with Ad-LacZ or Ad-XBP1s, and subsequently treated with ANS at the indicated concentrations for 2 h. XBP1s, p-p38, p38 and tubulin levels were analyzed with western blotting. **(e)** Four different lentiviral shRNAs were tested for silencing KSRP (*Khsrp*) expression. #2 KSRP lentiviral-shRNA was chosen. **(f)** pLKO and KSRP knockdown cells were infected with Ad-LacZ or Ad-XBP1s and subsequently treated with ANS at indicated concentrations for 2 h. XBP1s, p-p38, p38 and tubulin levels were analyzed with western blotting. Each experiment was independently performed three times. Error bars are ±S.E.M. Significance was determined by two-way ANOVA with Bonferroni multiple-comparison analysis (***p<0.001).



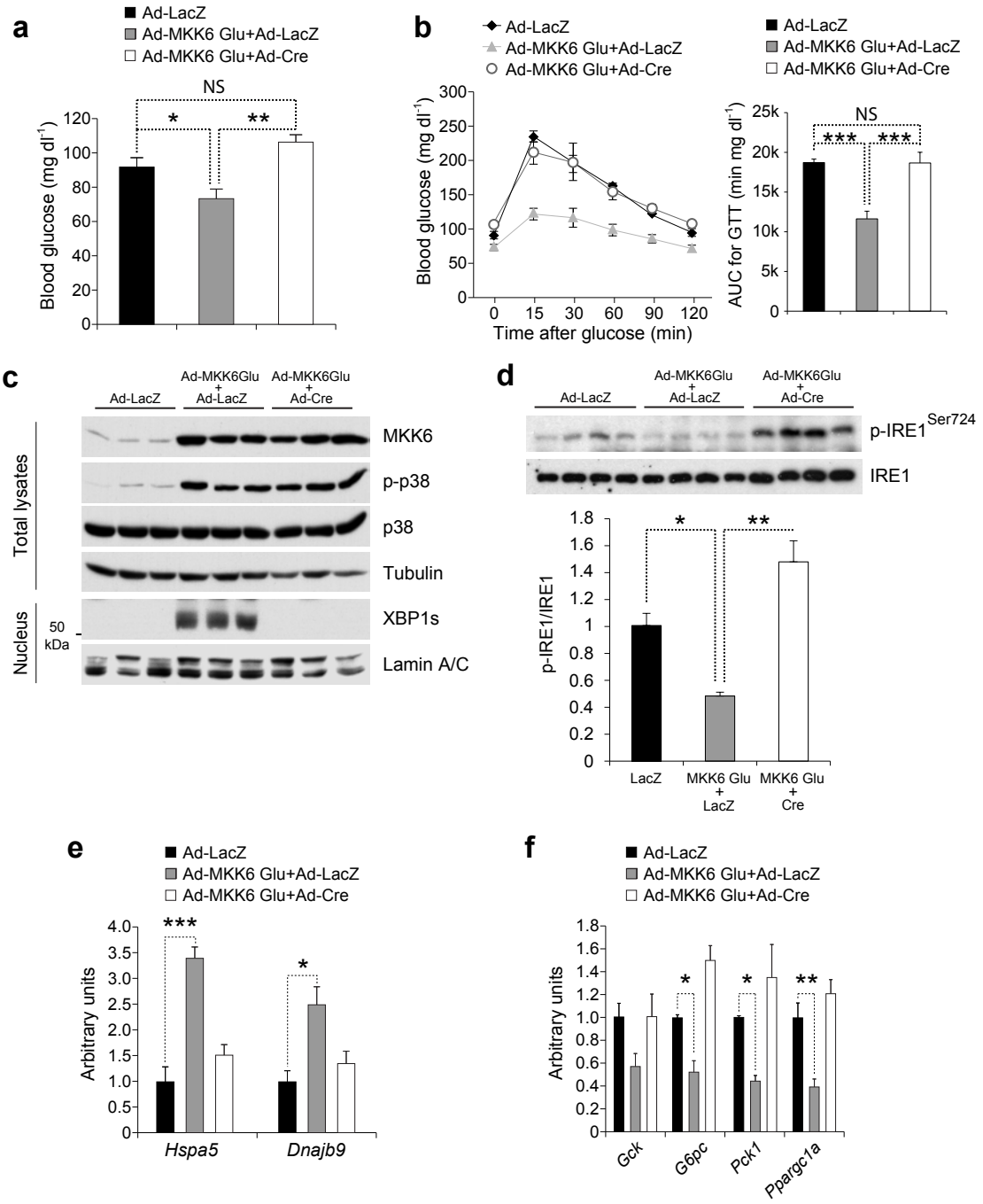
Supplementary Figure 4. Inhibition of p38 MAPK blocks XBP1s nuclear translocation. (**a**) MEFs were starved in serum-free medium in the absence or presence of p38 MAPK inhibitor SB203580 (10 μM). Following 16 h of starvation, cells were reincubated in medium containing 10% FBS for 15, 30 and 60 min. Nuclear levels of XBP1s and lamin A/C and total levels of p-ATF2, actin, XBP1s were determined via immunoblotting. XBP1 splicing and XBP1s mRNA levels were also analyzed. (**b-c**) (**b**) *Mapk14*$^{-/-}$ and WT cells (**c**) *Map2k3,6*$^{-/-}$ and WT cells were subjected to the same starvation, serum-stimulation procedure described in **a**, and indicated parameters were analyzed.



Supplementary Figure 5. Reduced p38 MAPK activity in diet-induced obese mice. Phospho-p38 MAPK, total p38 MAPK, and actin levels in the liver, muscle, and adipose (WAT) tissues from age-matched wild-type male mice under normal diet or high fat diet (HFD) for 8 wk. Error bars are ±S.E.M. Statistical significance was determined by Student's *t*-test (*p<0.05, **p<0.01). Reduced p38 MAPK activity was observed independently from different groups of high fat diet-fed mice compared with age-matched mice under normal diet.



Supplementary Figure 6. Adenoviral expression of MKK6Glu in the liver of obese and diabetic mice. (**a-d**) 8-week-old male *ob/ob* mice were injected with Ad-LacZ (LacZ) or Ad-MKK6Glu (MKK6Glu) (8×10^6 pfu g^{-1} for each virus) through the tail vein. (**a**) mRNA level of MKK6 (*Map2k6*) was measured from the liver, muscle, and adipose (WAT) tissues. (**b**) Fed blood glucose was measured from mice before and 4 d after adenovirus injection. (**c**) Body weight of mice before and 4 d after virus injection. (**d**) Aspartate transaminase (AST) and alanine transaminase (ALT) levels in the blood. Error bars are ±S.E.M. and statistical significance was determined by Student's *t*-test (**p<0.01, ***p<0.001). NS: Non-significant.



Supplementary Figure 7. XBP1 plays a major role in p38 MAPK-mediated enhancement of glucose homeostasis. (**a-f**) *Xbp1*$^{flox/flox}$ mice that were kept on HFD-feeding for 16 wk were injected with Ad-LacZ (1.58 x 10^8 PFU g^{-1}) (n=5) or with Ad-MKK6Glu (0.08 x 10^8 PFU g^{-1}) plus Ad-LacZ (1.5 x 10^8 PFU g^{-1}) (n=5) and the third group with Ad-MKK6Glu (0.08 x 10^8 PFU g^{-1}) and Ad-Cre (1.5 x 10^8 PFU g^{-1}) (n =5) through the tail vein. (**a**) Six-hour fasting blood glucose levels on post-injection day 13. (**b**) GTT performed 15 d after the adenovirus injections. (**c-f**) On post-injection day 17 mice were sacrificed after 6-h fasting and liver tissues were extracted. (**c**) MKK6, p-p38, p38 and tubulin levels in whole liver extracts and XBP1s and lamin A/C in the nuclear extracts. (**d**) p-IRE1^{Ser724} and total IRE1 levels. (**e-f**) mRNA levels of (**e**) *Hspa5* and *Dnajb9*, (**f**) *Gck*, *G6pc*, *Pck1* and *Ppargc1a* in livers. Three independent groups of mice (n=15 total in each group) were used in the experiments. Error bars are ±S.E.M. Significance was determined by one-way ANOVA with Bonferroni multiple-comparison analysis (*p<0.05, **p<0.01, ***p<0.001).

Supplementary Methods

Biochemical Reagents

Anti-XBP1, anti-phosphotyrosine (PY99), anti-β-actin, anti-insulin receptor (IR), and HRP-conjugated goat anti-mouse or goat anti-rabbit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p38, anti-phospho-p38, anti-phospho-c-Jun, anti-phospho-Akt at Thr308 or Ser473, anti-Akt, anti-phospho-ATF2, anti-MAPKAPK2 (anti-MK2), anti-β-Gal, anti-lamin A/C and anti-α-tubulin were purchased from Cell Signaling Technology (Beverley, MA). Rabbit polyclonal anti-phospho-XBP1s Thr48 and Ser61 were raised against CRAAGSEASGT(p)PQARKRQR and CKRQRLTHLS(p)PEEKALRRKLK respectively, by Covance (Denver, PA). Anti-phospho-IRE1 and anti-IRE1 were from Novus Biologicals (Littleton, CO). Anti-IRS1 was obtained from Millipore (Billerica MA). Anti-Flag antibody, TNFα and polybrene were from Sigma Aldrich (St. Louis, MO). JNK inhibitor VIII, SB203580, cycloheximide, actinomycin D, anisomycin and puromycin were from Calbiochem (La Jolla, CA). Fetal bovine serum, Dulbecco's Modified Eagle Medium, Opti-MEM, streptomycin, penicillin, Lipofectamine 2000, LR Clonase II Enzyme Mix and Trizol were from Invitrogen (Carlsbad, CA). Restriction endonucleases and T4 DNA ligase were from New England Biolabs (Ipswich, MA). Nuclear extraction kit for tissues was from Thermo Scientific (Rockford, IL). Nuclear extraction kit for cells was from Active Motif (Carlsbad, CA). cDNA Synthesis Kit, SYBR Green Supermix and Detergent-compatible protein assay kit were from BIO-RAD (Hercules, CA). BM Chemiluminescence Blotting Substrate was from Roche (Indianapolis, IN).

Cell Culture

JNK1,2 knockout cells (*Mapk8,9*$^{-/-}$), MKK3,6 knockout cells (*Map2k3,6*$^{-/-}$), MKK4,7 knockout cells (*Map2k4,7*$^{-/-}$) and p38α knockout cells (*Mapk14*$^{-/-}$), as well as their wild type control cells were kindly provided by Dr. Roger J. Davis (University of Massachusetts Medical School). MK2 knockout (*Mapkapk2*$^{-/-}$) cells and their control cell lines were kindly provided from Dr. Matthias Gaestel (Hannover Medical School, Germany). TTP knockout (*Zfp36*$^{-/-}$) and its control cell lines were generous gifts from Dr. Perry J. Blackshear (National Institute of Environment Health Sciences). 293A and 293T cells were from Invitrogen. HEK293 and MEF cells were from American Type Tissue Collection (ATCC). These cells were grown in Dulbecco's Modified Eagel Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 U ml^{-1} penicillin and 1μg ml^{-1} streptomycin at 37°C and 5% CO_2. Fao cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 10 U ml^{-1} penicillin and 1μg ml^{-1} streptomycin.

Plasmids

MKK6Glu and MKK7-JNK1-expressing plasmids were obtained from Addgene (Cambridge, MA). For producing adenovirus expressing MKK6Glu, MKK6Glu in pcDNA3.1 was subcloned into pENTR3C (Invitrogen) at the sites of Not I and Kpn I. Primer sequences are: 5'-TTAAGGGTACCGGCGCCATGTCTCAGTCGAAAGGCAA-3' (forward); 5'-TTAAGGCGGCCGCTTATCATTAGTCTCCAAGAATCAG-3' (reverse). The resulting MKK6Glu in pENTR3C was further subcloned into pAD

(Invitrogen) by using LR clonase (Invitrogen) for generating adenovirus. Plasmids for amino acid substitution mutants for XBP1s (T48A, S61A and T48A/S61A) were generated by PCR-based mutagenesis using pcDNA3.1-XBP1s or pENTR-XBP1s as a template with a kit from Stratagene (La Jolla, CA). Primer sequences for T48A are: 5'-GGGTCGGAGGCGAGCGGGGCACCGCAGGCTCGCAAGCGG- 3'(forward); 5'-CCGCTTGCGAGCCTGCGGTGCCCCGCTCGCCTCCGACCC-3' (reverse). Primer sequences for S61A are: 5'-CAGCGGCTCACGCACCTGGCCCCGGAGGAGAAAG CGC-3' (forward); 5'-GCGCTTTCTCCTCCGGGGCCAGGTGCGTGAGCCGCTG-3' (reverse). Mutations at specific positions were confirmed by sequencing. The resulting mutated XBP1s in pcDNA3.1 (Invitrogen) were used for transient transfection assays and mutated XBP1s in pENTR3C were further subcloned into pAD for producing adenovirus.

Gene silencing experiments with lentiviral shRNAs

shRNAs in pLKO vector targeting AUF1 and KSRP were from Sigma-Aldrich. The target sequences for AUF1 are: TGAATGGAAGTATGACGTT (shRNA #1); AGTGGTTATGGGAAAGTAT (shRNA #2); GAGAGTGTAGATAAGGTCA (shRNA #3); CAATGTTGGTCTTAGTAAA (shRNA #4).

The target sequences for KSRP are: CTGAGAAGATTGCTCACAT (shRNA #1); TTGGGAAGAGTATTACAAA (shRNA #2); AGCAGATTGACCATGCAAA (shRNA #3); GCATCCAGTTCAAGCAAGAT (shRNA #4). For generating lentiviral particles, pLP1, pLP2, VSVG and either pLKO empty vector or pLKO-containing AUF1 or KSRP shRNA insert were transfected into 293T packaging cells by using Lipofectamine (Invitrogen). The medium was changed 24 h post-transfection and the viral supernatants

were collected three days post-transfection, aliquoted and stored at -80°C. For enhancing infection efficiency of lentivirus, MEF cells were pretreated with polybrene (8 μg ml^{-1}, Sigma-Aldrich) overnight, and then cells were infected with lentivirus and medium was changed 24 h after the infections. After 48 h post-infection, puromycin (2 μg ml^{-1}, Sigma-Aldrich) was added for selecting stably-infected cells and only puromycin-resistant cells were used in experiments. Cells infected with the lentivirus containing empty vector pLKO used as controls.

Total Protein Extraction from Cells

Cells were lysed in lysis buffer (25 mM Tris-HCl, pH 7.4; 10 mM NaF; 10 mM $Na_4P_2O_7$; 2 mM Na_3VO_4; 1 mM EGTA; 1 mM EDTA; 1% NP-40; 10 μg ml^{-1} Leupeptin; 10 μg ml^{-1} Aprotinin; 1 mM PMSF and 20 nM Okadaic acid). After 20 min-rotation at 4°C, cell lysates were centrifuged at 13,200 rpm for 20 min at 4°C. Supernatants were collected and protein concentration was quantified by using Protein Assay Kit (Bio-Rad). The concentrations of protein were normalized with lysis buffer to have equivalent amounts of protein and volume. Protein was denatured by boiling at 100°C for 5 min in Laemmli buffer. The lysates were cooled to room temperature before loading for western blot analysis.

Total Protein Extraction from Tissue

Tissues were homogenized with a bench-top homogenizer (Polytron, PT2100) in ice-cold tissue lysis buffer (25 mM Tris-HCl, pH 7.4; 100 mM NaF; 50 mM $Na_4P_2O_7$; 10 mM Na_3VO_4; 10 mM EGTA; 10 mM EDTA; 1% NP-40; 10 μg ml^{-1} Leupeptin; 10 μg ml^{-1}

Aprotinin; 2 mM PMSF and 20 nM Okadaic acid). After homogenization, lysates were rotated for 1 h at 4°C and then subjected to centrifugation at 13,200 rpm for 20 min at 4°C. The lipid layer was removed and the supernatant was transferred into Eppendorf tubes for centrifugation. This process was repeated for 2 times to get rid of lipid completely. Protein concentration was quantified by using Protein Assay Kit (Bio-Rad). Equivalent protein concentration in each sample was prepared and boiled at 100°C for 5 min in Laemmli buffer. The lysates were cooled to room temperature before loading for western blot analysis.

Cytoplasmic and Nuclear Protein Extraction

Cytoplasmic and nuclear protein fractions were extracted from cells by using nuclear protein extraction kit from Active Motif (Carlsbad, CA). Cells were maintained in 10 cm tissue culture dishes for nuclear/cytoplasmic extraction. After removal of the media, cells were washed with ice-cold phosphate buffered saline (PBS) containing phosphatase inhibitors. Subsequently, 3 ml of ice-cold PBS with phosphatase inhibitors was added and then cells were scrapped out of the dish. Collected cells were separated from PBS by centrifugation for 5 min at 500 rpm and resuspended with 500 µl of 1X hypotonic buffer. After 15-min incubation in hypotonic buffer on ice, 25 µl of supplied detergent was added and cells were vortexed 10 sec. Cells were centrifuged for 30 sec at 14,000 x g and supernatant (cytoplasmic fraction) was saved for further analysis. Remaining pellet, which contains nuclei, were resuspended with 50 µl of provided complete lysis buffer with 1 mM dithiothreitol (DTT), vortexed 10 sec, and incubated for 30 min on ice. After

30-sec vortexing and 10-min centrifugation at 14,000 x g, the supernatant was collected and analyzed as nuclear fraction.

For liver tissue, a kit from Thermo Scientific (Rockford, IL) was used according to the manufacturer's instruction. Liver tissues were cut into small pieces, washed with PBS, and separated from PBS by centrifugation at 500 x g for 5 min. Collected tissues were resuspended by company-supplied CER I buffer and homogenized with a Dounce homogenizer. Homogenized tissues were vortexed and incubated on ice for 10 min. Following CER II buffer addition, tissues were vortexed for 5 sec, incubated for 1 min on ice, vortexed again, and then centrifuged for 5 min at maximum speed in a microcentrifuge. The supernatant (cytoplasmic fraction) was saved for later analysis. The pellets were resuspended with supplied NER buffer and undergone a series of multiple vortexing (15 sec) and incubation on ice (10 min) for a total of 40 min. After 10-min centrifugation, the supernatant (nuclear fraction) was collected and analyzed with immunoblotting.

Western Blot Analysis

Samples from cell or tissue lysates were resolved by SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membrane. After 1 h blocking at room temperature using 10% blocking reagent (Roche), membrane was incubated overnight with primary antibody in Tris-buffered saline solution/Tween (TBST) containing 10% blocking reagent at 4°C. After the incubation, membrane was washed three times in TBST and incubated with secondary antibody for 1 h at room temperature. After three-time washing

in TBST, membrane was developed using a chemiluminescence assay system (Roche) and exposed to Kodak films. Relative protein levels were quantified by Image J program. For stripping, membrane was vigorously shaken in stripping buffer (62.5 mM Tris-HCl, pH 6.7; 2% SDS; 100 mM 2-mecaptomethanol) at 50°C for 20 min. After stripping, membrane was washed three times in TBST.

Adenovirus Production and Infection

Adenovirus expressing XBP1s T48A, XBP1s S61A, XBP1s T48A/S61A and MKK6Glu were produced with ViraPower Adenoviral Expression System (Invitrogen) according to manufacturer's instruction. Briefly, pAD-XBP1s T48A, pAD-XBP1s S61A, pAd-XBP1s T48A/S61A and pAd-MKK6Glu were linearized by restriction endonuclease digestion with PacI and transfected to 293A cells by Lipofectamine. The media was changed with fresh media every other day until the cytopathic effect was observed. When cytopathic effect reached to 80%, cells were collected by centrifugation. The pellet was resuspended in PBS and subjected to freezing and thawing cycles at -80°C and 37°C for 4 times. The supernatant containing the virus was prepared by centrifugation at 4,000 rpm for 20 min at room temperature. Ad-XBP1s were generated as described previously[1]. For infection, cells were incubated with adenovirus in reduced volume of medium containing 1% FBS and antibiotics. Cells were gently rocked every 15 min for 1 h to increase efficiency of infection, and then fresh medium were added and cells were incubated for additional 15 h or 23 h.

Real-Time Quantitative PCR

Total RNA was extracted from cells or animal tissues using Trizol reagent (Invitrogen) and transcribed into cDNA using cDNA synthesis kit (Bio-Rad). The gene expression analysis was performed with iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) with SYBR Green Supermix (Bio-Rad). The mRNA level was normalized to 18S as a house keeping gene. The primer sequences used were:

Rn18S forward: 5'-AGT CCC TGC CCT TTG TAC ACA-3';

Rn18S reverse: 5'-CGT TCC GAG GGC CTC ACT-3';

Dnajb9 forward: 5'-CCC CAG TGT CAA ACT GTA CCA G-3';

Dnajb9 reverse: 5'-AGC GTT TCC AAT TTT CCA TAA ATT-3';

Hspa5 forward: 5'-TCA TCG GAC GCA CTT GGA A-3';

Hspa5 reverse: 5'-CAA CCA CCT TGA ATG GCA AGA-3';

G6pc forward: 5'-CCG GTG TTT GAA CGT CAT CT-3';

G6pc reverse: 5'-CAA TGC CTG ACA AGA CTC CA-3';

Pck1 forward: 5'-ATC ATC TTT GGT GGC CGT AG-3';

Pck1 reverse: 5'-ATC TTG CCC TTG TGT TCT GC-3';

Ppargc1a forward: 5'-TGA TGT GAA TGA CTT GGA TAC AGA CA-3';

Ppargc1a reverse: 5'-CAA TGC CTG ACA AGA CTC CA-3';

Gck forward: 5'-GAA AAG ATC ATT GGC GGA AA-3';

Gck reverse: 5'-CCC AGA GTG CTC AGG ATG TTA AG-3';

XBP1s forward: 5'-GGTCTGCTGAGTCCGCAGCAGG-3';

XBP1s reverse: 5'-AGGCTTGGTGTATACATGG-3'.

XBP1 Splicing Assay

XBP1 splicing assay was performed by PCR with cDNA as template. The PCR conditions were as follows: 94°C for 3 min; 29 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec; and 72°C for 3 min. The primer sequences are:

Forward: 5'-ACACGCTTGGGAATGGACAC-3';

Reverse: 5'-CCATGGGAAGATGTTCTGGG-3'.

Protein Degradation and mRNA Stability Analysis

MEF cells were infected with Ad-XBP1s. After 24 h post-infection, cells were treated with anisomycin at 25 ng ml^{-1} or vehicle (DMSO) for 2 h. Translation initiation inhibitor cycloheximide (10 μg ml^{-1}, Sigma) was added to the medium. Cells were flash frozen in liquid nitrogen at various time points. Protein levels were determined via immunoblotting. For mRNA stability determination, MEF cells were infected with Ad-XBP1s. Sixteen hour after the infection, cells were treated with anisomycin (25 ng ml^{-1}) or vehicle (DMSO) for 1 h. Actinomycin D (10 μg ml^{-1}, Sigma) was added to the medium. Cells were flash frozen in liquid nitrogen at various time points. mRNA levels were determined via Q-PCR.

Mice

Leptin deficient (*ob/ob*) and wild type mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *Xbp1*$^{flox/flox}$ mice were kindly provided by Dr. Laurie H. Glimcher (Harvard School of Public Health). Mice were fed with normal chow or high fat diet (45% calorie from fat) with a free access to water. All animal procedures used in this

study were approved by the Animal Care and Use Committee at Children's Hospital Boston.

Blood Glucose and Plasma Insulin Measurements

Mice were fasted for 6 h, after which their blood was analyzed for glucose measurement with a glucose meter (Bayer, Mishawaka, IN). For insulin analysis, blood was collected from tail vein and plasma was separated by centrifugation at 2,000 x g, 4 °C for 20 min. Plasma insulin were measured with an Ultra Sensitive Mouse Insulin ELISA kit from Crystal Chem (Downers Grove, IL).

Blood Alanine Transaminase (ALT) and Aspartate Transaminase (AST) Measurements

The blood from mice before and after adenoviral injection was collected and blood ALT and AST levels were measured with ALT Color Endpoint Assay and AST Color Endpoint Assay kits from Bioo Scientific (Austin, TX) as instructed by provided manuals.

1. Park, S.W.*, et al.* The regulatory subunits of PI3K, p85alpha and p85beta, interact with XBP-1 and increase its nuclear translocation. *Nat Med* **16**, 429-437 (2010).