***Science* Supporting Online Material**

Endoplasmic Reticulum Stress Links Obesity, Insulin Action, and Type 2 Diabetes

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Contents

Materials and Methods

Biochemical reagents. Anti-IRS-1, anti-phospho-IRS-1 (Ser^{307}) and anti-IRS-2 antibodies were from Upstate Biotechnology (Charlottesville, VA). Antibodies against phosphotyrosine, eIF2α, insulin receptor β subunit, and XBP-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-PERK, anti-Akt, and anti-phospho-Akt antibodies and c-Jun protein were from Cell Signaling Technology (Beverly, MA). Anti-phospho-eIF2α antibody was purchased from Stressgen (Victoria, British Columbia, Canada). Anti-insulin antibody and C-peptide RIA kit were purchased from Linco Research (St. Charles, MO). Anti-glucagon antibody was from Zymed (San Francisco, CA). PERK antiserum was kindly provided by Dr. David Ron (New York University School of Medicine). Texas red conjugated donkey anti-guinea pig IgG and fluorescein-conjugated (FITC-conjugated) goat anti-rabbit IgG were from Jackson Immuno Research Laboratories (West Grove, PA). Thapsigargin, tunicamycin, and JNK inhibitors were from Calbiochem (San Diego, CA). Insulin, glucose, and sulindac sulfide were from Sigma (St. Louis, MO). The Ultra Sensitive Rat Insulin ELISA kit was from Crystal Chem Inc. (Downers Grove, IL).

Cells. Rat Fao liver cells were cultured with RPMI 1640 (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS). At 70-80% confluency, cells were serum depleted for 12 hours prior to the experiments. Reagents including tunicamycin, thapsigargin, and JNK inhibitors were gently added to the culture dishes in the incubator to prevent any environmental stress due to vibration, temperature changes and light exposure. JNK inhibitors were added 1 hour before tunicamycin/thapsigargin treatment. The *XBP-1*$^{-/-}$ mouse embryonic fibroblasts (MEF) (*S1*), *IRE-1*$\alpha^{-/-}$ MEF cells (kindly provided by Dr. David Ron, New York University School of Medicine), and their WT controls were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) containing 10% FBS. A similar protocol was followed for experiments in MEF cells, except that the cells were serum depleted for only 6 hours

Overexpression of XBP-1s in MEFs. MEF-tet-off cells (BD Biosciences Clontech, Palo Alto, CA) were cultured in DMEM with 100 μg/ml G418 and 1 μg/ml doxycycline. The MEF-tet-off cells express exogenous tTA (tetracycline-controlled transactivator) protein, which binds to TRE (tetracycline response element) and activates transcription only in the absence of tetracycline or doxycycline. The cDNA of the spliced form of XBP-1 was ligated into pTRE2hyg2 plasmid (BD Biosciences Clontech, Palo Alto, CA). The MEF-

tet-off cells were transfected with the TRE2hyg2-XBP-1s plasmid, followed by selection in the presence of 400 μg/ml hygromycin B. Individual clones of stable transfectants were isolated and doxycycline-dependent XBP-1s expression was confirmed by immunoblotting in each experiment.

Northern blot analysis. Total RNA was isolated from mouse liver using Trizol reagent (Invitrogen, Carlsbad, CA), separated by 1% agarose gel, and then transferred onto BrightStar Plus nylon membrane (Ambion, Austin, TX). GRP78 cDNA probe was prepared from mouse liver total cDNAs by RT-PCR using the following primers: 5'-TGGAGTTCCCCAGATTGAAG-3' and 5'- CCTGACCCACCTTTTTCTCA-3'. The DNA probes were purified and labeled with ^{32}P-dCTP using random primed DNA labeling kit (Roche, Indianapolis, IN). Hybridization was performed according to the manufacturer's protocol (Ambion, Austin, TX) and visualized by Versa Doc Imaging System 3000 (BioRad, Hercules, CA).

Protein extracts from cells. At the end of each treatment, cells were immediately frozen in liquid nitrogen and kept at –80°C. Protein extracts were prepared with a lysis buffer containing 25 mM Tris-HCl (pH 7.4), 2 mM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM EGTA, 1 mM EDTA, 1% NP-40, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 10 nM okadaic acid, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Immunoprecipitations and immunoblotting experiments were performed with 750 μg and 75 μg total protein, respectively without any freeze-thaw cycles from individual aliquots.

Animal studies and obesity models. Adult (10-12 weeks of age) male *ob/ob* mice and their WT littermates were purchased from Jackson Labs. Mice used in the diet-induced obesity model were male C57BL/6. All mice were placed on high fat diet (HFD: 35.5% fat, 20% protein, 32.7% carbohydrates, Bio-Serve) immediately after weaning (at ~3 weeks of age). The XBP-1$^{+/-}$ and XBP-1$^{+/+}$ mice were on BALB/c genetic background. Insulin and glucose tolerance tests were performed as previously described (*S3*). Insulin and C-peptide ELISA were performed according to manufacturer's instructions using mouse standards (Crystal Chem Inc., Downers Grove, IL). Pancreas isolated from 16-week-old mice was fixed in Bouin's fluid and formalin, and paraffin sections were double-stained with guinea pig anti-insulin and rabbit anti-glucagon antibodies. Texas red dye conjugated donkey anti-guinea pig IgG and FITC conjugated Goat anti-rabbit IgG were used as secondary antibodies.

Insulin infusion and tissue protein extraction. Insulin was injected through the portal vein as previously described (*S2*, *S3*). Three minutes after insulin infusion, liver was removed and frozen in liquid nitrogen and kept at –80°C until processing. For protein extraction, liver tissue (~0.3g) was placed in 10 ml of lysis buffer containing 25 mM Tris-HCl (pH7.4), 10 mM Na_3VO_4, 100 mM NaF, 50 mM $Na_4P_2O_7$, 10 mM EGTA, 10 mM EDTA, 1% NP-40, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 10 nM okadaic acid, and 2 mM PMSF. After homogenization on ice, the tissue lysate was centrifuged at 4,000 rpm for 15 minutes at 4°C followed by 55,000 rpm for 1 hour at 4°C. One milligram of total tissue protein was used for immunoprecipitation and subsequent immunoblotting, whereas 100-150 μg total tissue protein was used for direct immunoblotting (*S3*).

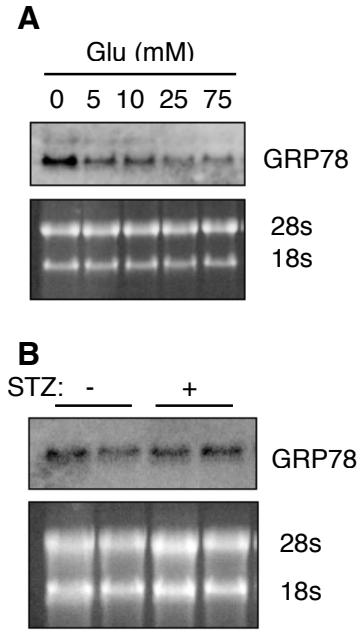

Fig. S1. Regulation of GRP78 expression by glucose *in vitro* and hyperglycemia *in vivo*. **(A)** Fao cells were treated with various doses of glucose (0, 5, 10, 25, and 75 mM) for 24 hours. The GRP78 mRNA level was examined by Northern blot using the total RNAs isolated from these cells. Ethidium bromide staining is shown as a control for loading and integrity of RNA. **(B)** Streptozotocin (STZ, 200 mg/kg) was injected intaperitoneally into 8-week-old male mice. Three days after injection, blood glucose levels were measured to confirm STZ-induced hyperglycemia (140.5±13.6 and 484.5±50.9 in controls and STZ injected diabetic animals, respectively). Livers were collected 10 days after injection and GRP78 expression was examined by Northern blot analysis using the liver total RNA.

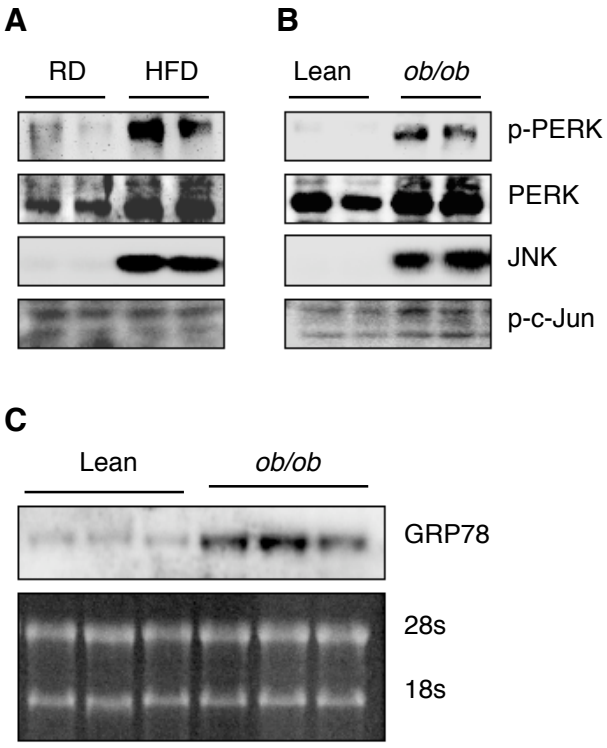

Fig. S2. ER stress indicators in adipose tissues of obese mice. Dietary (high fat diet-induced) and genetic (*ob/ob*) models of mouse obesity were used to examine markers of ER stress in adipose tissue compared with age and sex matched lean controls. **(A)** PERK phosphorylation (p-PERK) and JNK activity were examined in the adipose samples of the male mice (C57BL/6) that were kept either on regular chow diet (RD) or high fat diet (HFD) for 16 weeks. **(B)** PERK phosphorylation and JNK activity in the adipose tissues of male *ob/ob* and WT lean mice at the ages of 12-14 weeks. **(C)** The GRP78 mRNA level was examined by Northern blot analysis in the adipose tissues of WT lean and *ob/ob* animals. Ethidium bromide staining is shown as a control for loading and integrity of RNA.

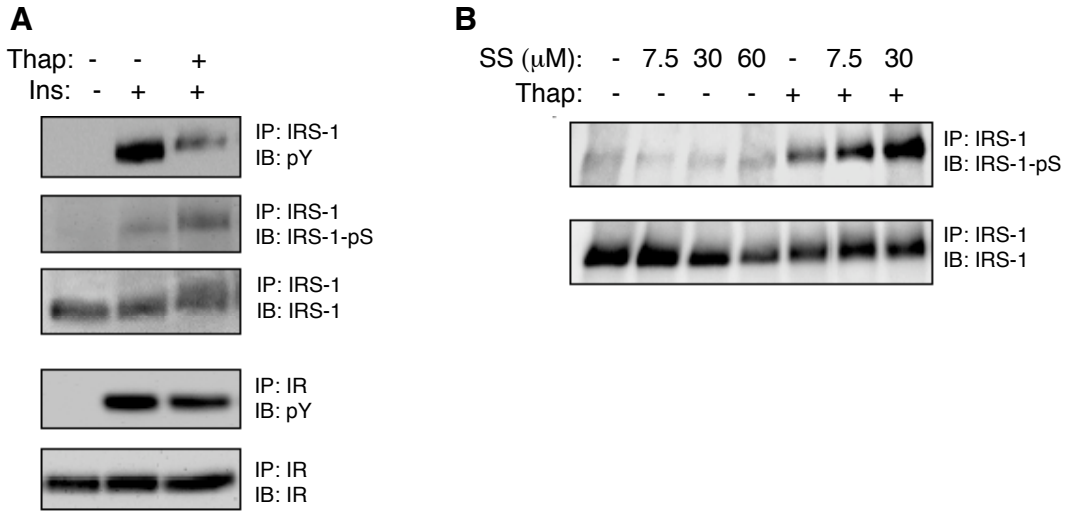

Fig. S3. Inhibition of insulin receptor signaling by thapsigargin-induced ER stress and the role of calcium levels in IRS-1 serine phosphorylation. **(A)** ER stress was induced in Fao cells by 1-hour treatment with 300 nM thapsigargin (Thap), and cells were subsequently stimulated with insulin (Ins). IRS-1 tyrosine phosphorylation (pY) and serine phosphorylation (pSer307), insulin receptor (IR) tyrosine phosphorylation, and total protein levels were examined using either immunoprecipitation (IP) followed by immunoblotting (IB) or direct immunobloting. **(B)** Fao cells were treated with sulindac sulfide (SS: 0, 7.5, 30, and 60 μM) for 45 minutes with or without an additional hour of exposure to 300 nM thapsigargin (Thap). IRS-1 serine phosphorylation and total IRS-1 protein levels were examined as described above.

These results demonstrate that treatment of cells with thapsigargin, an agent that induces ER stress by inhibiting calcium ATPase, also increased IRS-1 serine phosphorylation and suppressed insulin receptor signaling. The use of sulindac sulfide to block calcium influx to the cytosol from the extracellular compartment addresses whether thapsigargin-induced inhibition of insulin action was simply due to alterations in cellular calcium levels. Treatment with sulindac sulfide alone had no effect on Ser307 phosphorylation of IRS-1. Furthermore, in the presence of thapsigargin, sulindac sulfide did not interfere with IRS-1 serine phosphorylation indicating that the effect of thapsigargin on Ser307 phosphorylation of IRS-1 and inhibition of insulin receptor signaling is mediated through ER stress.

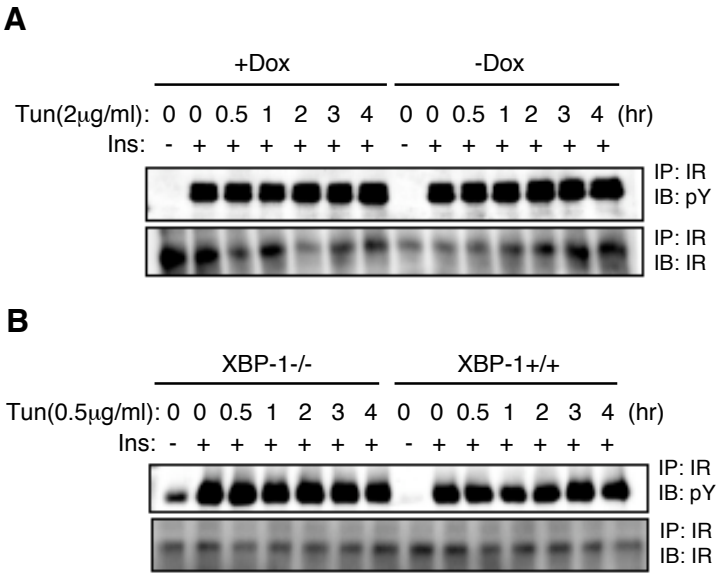

Fig. S4. Insulin-induced insulin receptor autophosphorylation in XBP-1 overexpressing and XBP-1-deficient cells. **(A)** XBP-1 overexpressing fibroblasts (-Dox) and their controls (+Dox) were treated with 2 μg/ml tunicamycin (Tun) for indicated periods (0, 0.5, 1, 2, 3, and 4 hours). Insulin-induced insulin receptor (IR) tyrosine phosphorylation (pY) and total IR levels were examined in those cells using immunoprecipitation (IP) with IR antibody followed by immnunoblotting (IB) with antibodies against IR or phospho tyrosine (pY). **(B)** XBP-$1^{-/-}$ MEF cells and their WT controls were treated with 0.5 μg/ml tunicamycin for indicated periods (0, 0.5, 1, 2, 3, and 4 hours). Insulin-induced insulin receptor (IR) tyrosine phosphorylation (pY) and total IR levels were examined as in panel A.

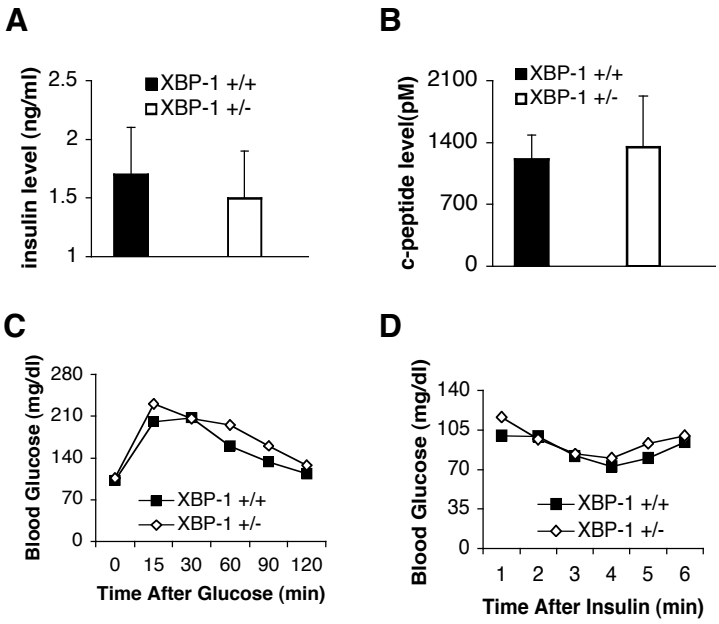

Fig. S5. Insulin sensitivity in lean XBP-1$^{+/-}$ and XBP-1$^{+/+}$ mice. In XBP-1$^{+/-}$ and XBP-1$^{+/+}$ mice placed on regular chow diet, blood insulin **(A)** and c-peptide **(B)** levels were measured, and glucose **(C)** and insulin tolerance tests **(D)** were performed at 16 weeks of age. On chow diet, there was no difference in blood glucose levels, C-peptide levels, and insulin sensitivity measured by glucose and insulin tolerance tests between genotypes followed up to 18 weeks of age.

At the onset of the HFD experiment, there was also no difference in glucose metabolism between XBP-1$^{+/-}$ and XBP-1$^{+/+}$ mice as determined by fasting and fed blood glucose, insulin and C-peptide levels, and by intraperitoneal glucose and insulin tolerance tests. Serum levels of leptin (26.2±2.5 and 25.2±1.8 ng/ml in XBP-1$^{+/+}$ and XBP-1$^{+/-}$, respectively), adiponectin (15.5±1.8 and 16.6±1.6 ng/dl in XBP-1$^{+/+}$ and XBP-1$^{+/-}$, respectively) and triglycerides (67.7±5.5 and 62.8±3.4 mg/dl in XBP-1$^{+/+}$ and XBP-1$^{+/-}$, respectively) did not exhibit any statistically significant differences between the genotypes measured after 16 weeks of HFD. Blood insulin levels in XBP-1$^{+/+}$ mice were significantly lower than those in XBP-1$^{+/-}$ littermates (0.89±0.25 and 2.27±0.32 ng/ml in XBP-1$^{+/+}$ and XBP-1$^{+/-}$ after 20 weeks on HFD, respectively, $p<0.05$). C-peptide levels were also significantly higher in XBP-1$^{+/-}$ animals than in WT controls (772.91±132.24 and 1374.11±241.8 ng/ml in XBP-1$^{+/+}$ and XBP-1$^{+/-}$ ($p<0.05$).

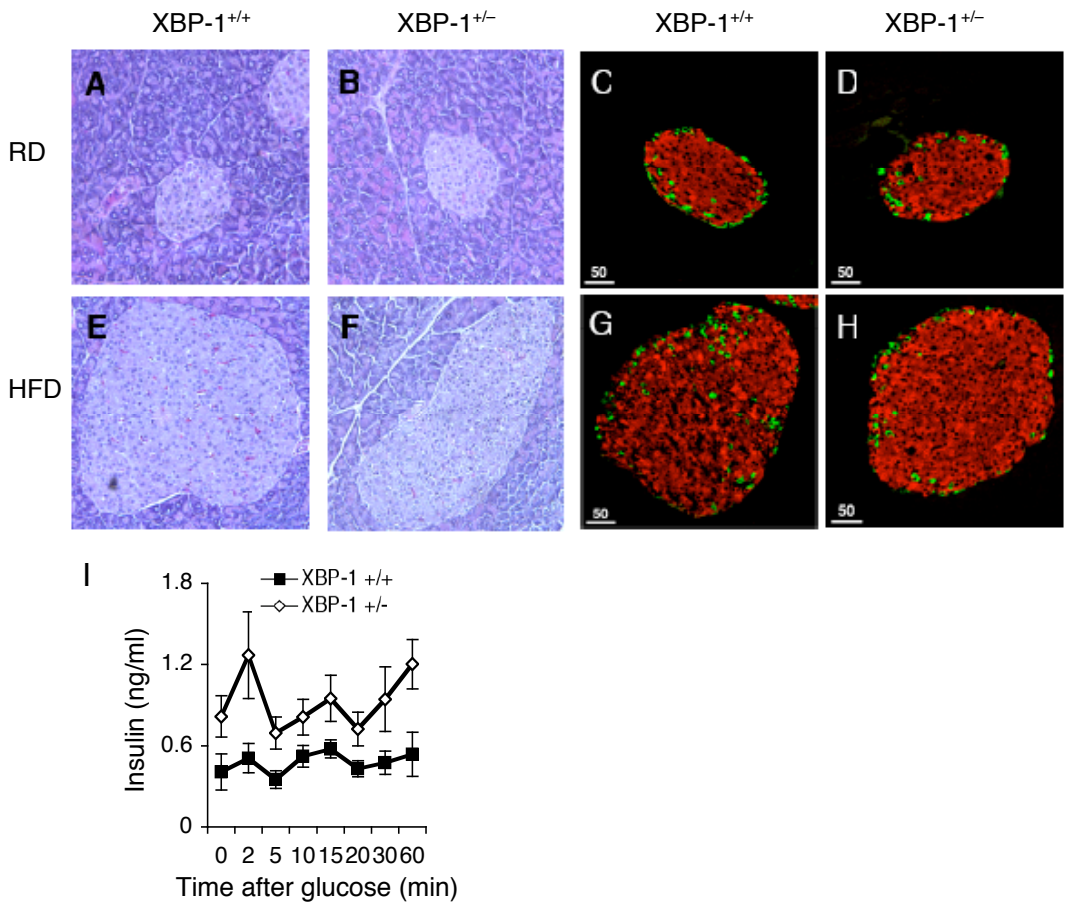

Fig. S6. Characterization of pancreatic islets in XBP-1$^{+/-}$ and XBP-1$^{+/+}$ mice. **(A-H)** Islet morphology, size and immuno-histochemical staining for insulin (red) and glucagon (green) in pancreatic sections obtained from XBP-1$^{+/-}$ and XBP$^{+/+}$ mice on either regular chow **(A-D)** or high fat **(E-H)** diet. **(I).** Glucose-stimulated insulin secretion in XBP-1$^{+/-}$ and WT mice. Glucose was administered intraperitoneally to mice in each genotype following 16 weeks of high fat diet and blood samples are collected at the indicated times for insulin measurements.

In these experiments, there was no detectable abnormality in the XBP$^{+/-}$ islets and no difference was evident between genotypes under standard conditions. On HFD, both the XBP-1$^{+/-}$ and XBP-1$^{+/+}$ mice exhibited islet hyperplasia. This anticipated response to HFD was similar between genotypes and the hyperplastic component (islet size >150 μM) comprised 40% of all islets in XBP-1$^{+/-}$ and 43% of all islets in WT mice on HFD. In experiments examining glucose-stimulated insulin secretion in XBP-1$^{+/-}$ and WT mice on HFD, the XBP-1$^{+/-}$ mice responded to glucose with even a stronger insulin secretory response, which effectively eliminates the possibility of an isolated islet defect underlying their phenotype. Hence, these data indicate that the phenotype of the XBP-1$^{+/-}$ mice cannot be attributed to defective islets and even after 16 weeks on HFD, the islets appear indistinguishable between genotypes. However, our data does not rule out involvement of XBP-1 in islet function during obesity and diabetes.

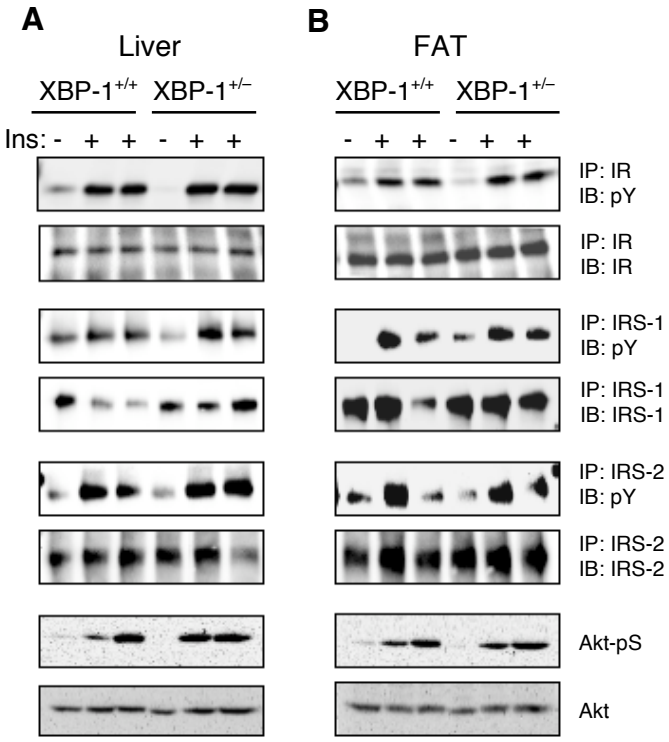

Fig. S7. Intact insulin receptor signaling in liver and adipose tissues of XBP-1$^{+/-}$ and XBP-1$^{+/+}$ mice on regular chow diet. After infusion of insulin (1U/kg) through portal vein, insulin receptor (IR) tyrosine phosphorylation (pY), IRS-1 tyrosine phosphorylation**,** IRS-2 tyrosine phosphorylation**,** Akt Ser473 phosphorylation**,** and their total protein levels were examined in livers **(A)** and adipose tissues **(B)** of XBP-1$^{+/-}$ and XBP$^{+/+}$ mice on regular chow diet.

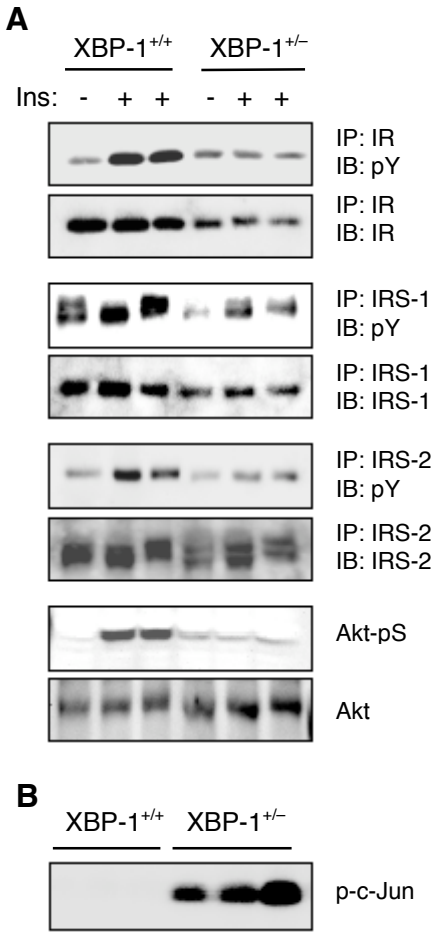

Fig. S8. Reduced insulin receptor signaling in adipose tissues of XBP-1$^{+/-}$ and XBP-1$^{+/+}$ mice on high fat diet. **(A)** After infusion of insulin (1U/kg) through portal vein, insulin receptor (IR) tyrosine phosphorylation (pY), IRS-1 tyrosine phosphorylation**,** IRS-2 tyrosine phosphorylation**,** Akt Ser473 phosphorylation**,** and their total protein levels were examined in adipose tissues of XBP-1$^{+/-}$ and XBP$^{+/+}$ mice on high fat diet for 16 weeks. **(B)** JNK kinase assay was performed in adipose tissues of XBP-1$^{+/-}$ and XBP$^{+/+}$ mice on high fat diet for 16 weeks.

References and Notes

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S2. J. Hirosumi *et al.*, *Nature* **420**, 333 (2002).

S3. K. T. Uysal, S. M. Wiesbrock, M. W. Marino, G. S. Hotamisligil, *Nature* **389**, 610 (1997).