PNAS
www.pnas.org

Supplementary Information for

Sustained ER stress promotes hyperglycemia by increasing glucagon action through the deubiquitinating enzyme USP14

Bin Liu, Zhijian Zhang, Yanyun Hu, Yan Lu, Duanzhuo Li, Jie Liu, Shengjie Liao, Min Hu, Yuxing Wang, Die Zhang, Yulu Chen, Qilan Qian, Xianfeng Lv, Duojiao Wu, Minjia Tan, Cheng Hu, Xuelian Xiong, Xiaoying Li

Xuelian Xiong
Email: xiong.xuelian@zs-hospital.sh.cn

Xiaoying Li
Email: li.xiaoying@zs-hospital.sh.cn

This PDF file includes:

Animal experiments

Eight weeks old male C57BL/6 and *ob/ob* mice were purchased from the Shanghai Laboratory Animal Company (SLAC) and Nanjing Biomedical Research Institute of Nanjing University respectively. Liver-specific ATF4 knockout mice were kindly provided by Dr. Feifan Guo (20) (Shanghai Institutes for Biological Sciences, Shanghai). All mice were housed at 21 ℃ ± 1 ℃ with 55% ± 10% humidity and a 12-hour light/dark cycle, and fed ad libitum with normal chow or high-fat diet (HFD; D12492; Research Diets) for 12 weeks. Sustained ER stress was induced with daily intraperitoneal injections of 0.2 mg/kg tunicamycin (654380, Sigma-Aldrich) for 14 days, and acute ER stress was induced with 1 mg/kg tunicamycin for 8 hr. The control animals were injected with equal volumes of saline.

Adenoviral infection

Recombinant adenoviruses for the overexpression of USP14 or knockdown of USP14 and FASN have been described previously (1). Briefly, Full-length USP14 cDNA was cloned into adenoviral plasmid (Invitrogen, Shanghai, China) to obtain USP14-overexpressing adenovirus, and 5×10^8 plaque-forming units were injected through the tail vein. Ad-shUSP14 particles were generated using pAD_BLOCK_IT_DEST vectors (Invitrogen, Grand Island, New York, USA). Adenoviruses expressing WT and dominant negative ATF4 were kindly provided by Dr. Feifan Guo (2) (Shanghai Institutes for Biological Sciences, Shanghai). The shRNA designed for the knockdown of IRE1α had the following target sequences: 5′-GCGAGAAGCAGCAGACTTTGT-3′. All viruses were purified by the cesium chloride method and dialyzed in PBS containing 10% glycerol prior to animal injection.

Pyruvate, glucose, insulin and glucagon tolerance tests

Pyruvate, glucose and glucagon tolerance tests were performed by intraperitoneally injecting the mice with 1.5 mg/kg pyruvate (Sigma, USA) or 2 mg/kg D-glucose (Sigma, USA) or 15μg/kg glucagon (Sigma) after a 16-hour fast. For the insulin tolerance test, the mice were injected with regular human insulin (Eli Lily, Indianapolis, Indiana, USA) at the dose of 0.75 U/kg body weight after a 6-hour fast. Blood glucose levels were then determined using a portable blood glucose meter (Lifescan, Johnson & Johnson, New

Brunswick, New Jersey, USA).

Isolation and culture of mouse primary hepatocytes (MPH)

MPHs were isolated from the liver of 10-weeks old C57BL/6 mice by collagenase perfusion as previously described, and purified by centrifugation. Fresh prepared MPHs were re-suspended in attachment media (Science Cell, USA), and seeded in 6-well plates at the final density of $5 * 10^5$ cells per well. To mimic the sustained and low-grade ER stress in mouse primary hepatocytes (MPHs), the dose for tunicamycin and thapsigargin is 0.25μg/ml and 50nM, respectively. The duration of both treatment is 48 hr.

In vitro glucose production assay

The MPHs were cultured till confluent, and the medium was replaced with DMEM supplemented with 2mM sodium pyruvate and 20mM sodium lactate. After 4h incubation, the medium was collected and glucose levels were measured using a colorimetric glucose assay kit (GAGO20; Sigma-Aldrich) according to the manufacturer's instructions. The readings were normalized to the total protein content of the whole-cell extracts.

RNA isolation and quantitative real-time PCR

Total RNAs from mouse liver or cultured mouse primary hepatocytes were extracted by using Trizol reagents (Invitrogen). The mRNAs were then reversed transcripted into cDNA using the Promega Reverse Transcription System (Madison, WI, USA). Oligo dT was used to prime cDNA synthesis. Quantitative real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Japan) on Light Cycler 480 (Roche, Switzerland) and data were analyzed by the comparative CT method. 36B4 mRNA levels were used as internal control. Primers used for qPCR analysis were available upon request. Transcriptomics analyses were performed using RNA-sequencing (RNA-seq) by Shanghai Cloud-seq Biotech Ltd. Co. The data have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession nos. GSE135372).

Immunoprecipitation (IP) and Western blotting

Hepatic tissues and cells were lysed in RIPA buffer containing 50mM Tris-HCl, 150mM NaCl, 5mM $MgCl_2$, 2mM EDTA, 1mM NaF, 1% NP40 and 0.1% sodium dodecyl sulfate (SDS). Lysates were cleared by centrifugation at 15,000 g for 20 minutes at 4 ℃ and then filtered through 0.45 μm spin filters (Millipore) to further remove cell debris. The resulting lysates were subjected to IP with antibodies as indicated. For western blotting, equivalent amounts of protein samples were denatured in the loading buffer, and resolved by 10-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in 5% non-fat milk for 1 h before incubation with primary antibody overnight at 4 ℃. Membranes were washed with phosphate-buffered saline with Tween-20 (PBST) five times and incubated with secondary antibody for 1 h. The signals of the proteins were then visualized by a electrochemiluminescence (ECL) system. The following primary antibodies were used: anti-ATF4 antibody at 1:2000 (#sc-390063, Santa Cruz Biotechnology, Santa Cruz, CA, USA),anti-CBP antibody at 1:2000 (#ab2832, Abcam, Cambridge, UK), anti-CREB antibody at 1:1000 (#9197, Cell Signaling Technology, Beverly, MA, USA), anti-CRTC2 antibody at 1:3000 (#ST1099, Millipore, Bedford, MA), anti-FASN antibody at 1:1000 (#3180, Cell Signaling Technology), anti-IRE1α antibody at 1:1000 (#3294, Cell Signaling Technology), anti-P300 antibody at 1:1000 (#sc-48343,Santa Cruz), anti-USP14 antibody at 1:2000 (#11931, Cell Signaling Technology), anti-β-actin antibody at 1:8000 (#4967, Cell Signaling Technology), anti-HA antibody at 1:2000 (#3724, Cell Signaling Technology).

Ubiquitination assay

The *in vivo* ubiquitylation assays were performed under denaturing conditions. Cells were grown in 10 cm plates to 80% confluence and then were transiently transfected with Flag-CBP and His-Ubiquitin, HA-USP14 WT/or HA-USP14 CI, using lipo2000 transfection reagent. The cells were treated with MG132 (20 μM) for 4 h. Cells were harvested, lysed in denaturing IP lysis buffer [1% SDS, 50 mM Tris, 10 mM DTT (pH 7.5)], and boiled at 95 ℃ for 5 min. The denatured proteins were diluted 10× in RIPA buffer containing 50mM Tris-HCl, 150mM NaCl, 5mM $MgCl_2$, 2mM EDTA, 1mM NaF, 1% NP40 and 0.1% sodium dodecyl sulfate (SDS) and then subjected to Flag IP. The input lysates and bound fractions were subjected to SDS-PAGE with antibodies

against Flag and His tags.

Cycloheximide (CHX) Chase assay

MPHs were seeded overnight in complete medium in 6-well plates to 60% confluence and then were transiently transfected Ad-GFP/Ad-WT USP14 or Con-shRNA/USP14-shRNA for 36 h, and followed by the addition of 25 μg/mL cycloheximide. Samples at the indicated times were harvested for immunoblot analysis.

Figures. S1 to S11

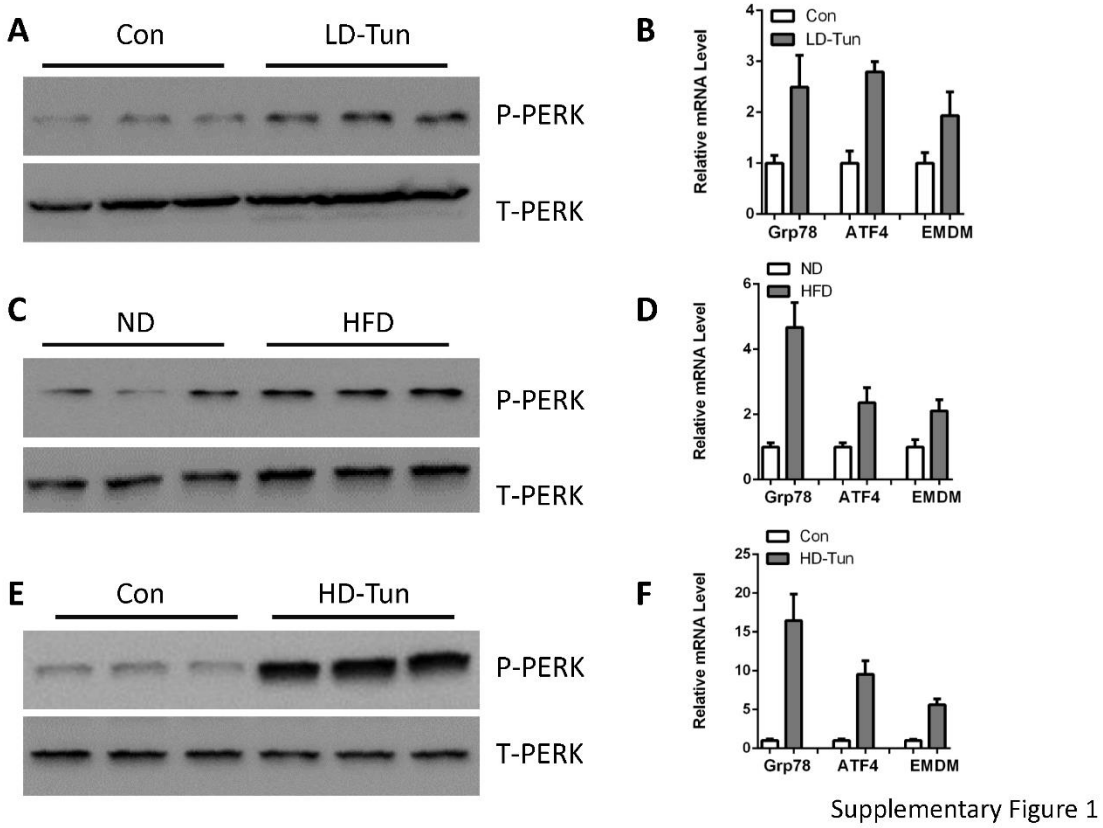


Fig. S1. (A) Protein levels of phosphorylated PERK in the liver of control and LD-Tun-treated

mice. Total PERK was set as a loading control. (B) Relative GRP78, ATF4 and EMDM mRNA levels in control and LD-Tun-treated mice. (C) Protein levels of phosphorylated PERK in the liver of mice fed a normal diet or high fat diet for 12 weeks. (D) Relative GRP78, ATF4 and EMDM mRNA levels in ND or HFD-fed mice. (E) Protein levels of phosphorylated PERK in the liver of control and HD-Tun-treated mice. (F) Relative GRP78, ATF4 and EMDM mRNA levels in control and HD-Tun-treated mice.

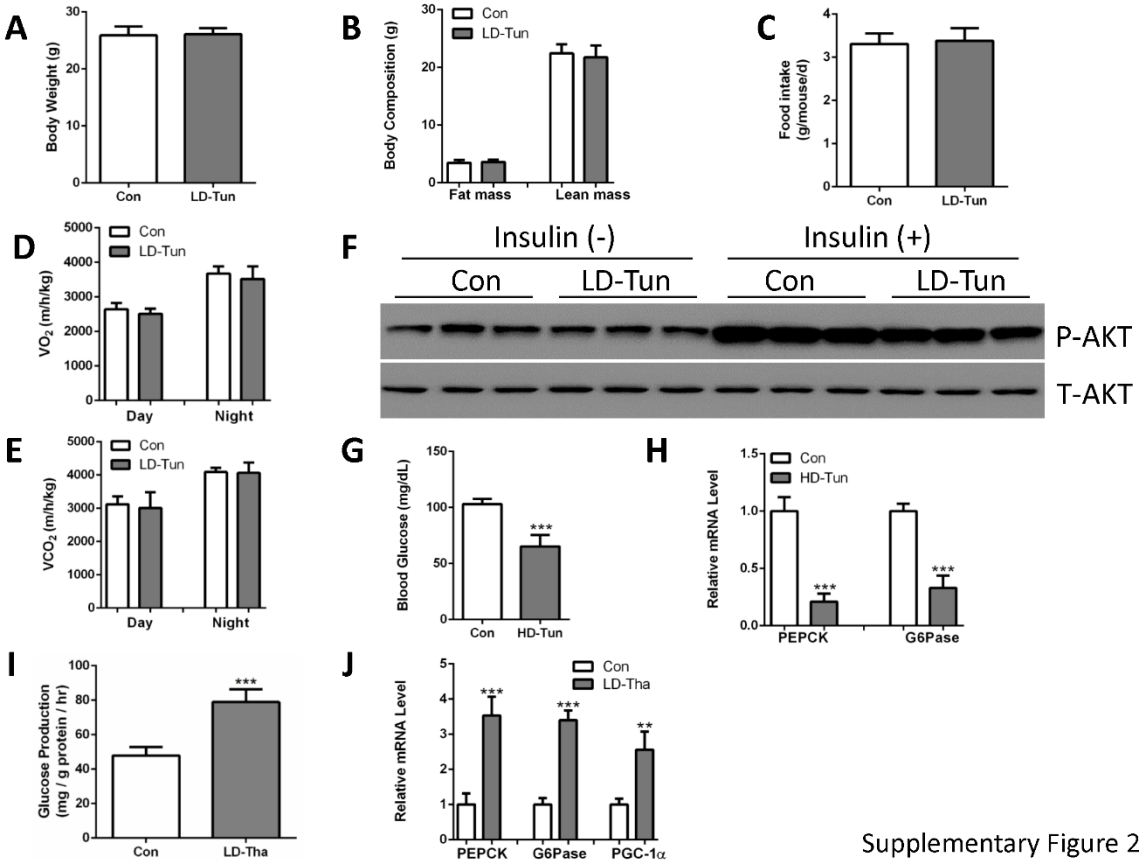


Fig. S2. (A-E) Body weight, body composition, food intake, O2 consumption and CO2 production in the above groups. (F) P-AKT (Ser473) levels in the liver of control and LD-Tun-treated mice before or after insulin stimulation (5 min, 0.75 U insulin/kg body weight). Total AKT was included as the loading control. (G-H) Blood glucose levels and mRNA expression of gluconeogenic enzymes in control and HD-Tun-treated mice. (I) Glucose levels in MPHs treated with LD-Tha or saline for 48 hr normalized to total protein levels. (J) Relative mRNA expression levels of gluconeogenic genes in the above groups.

A

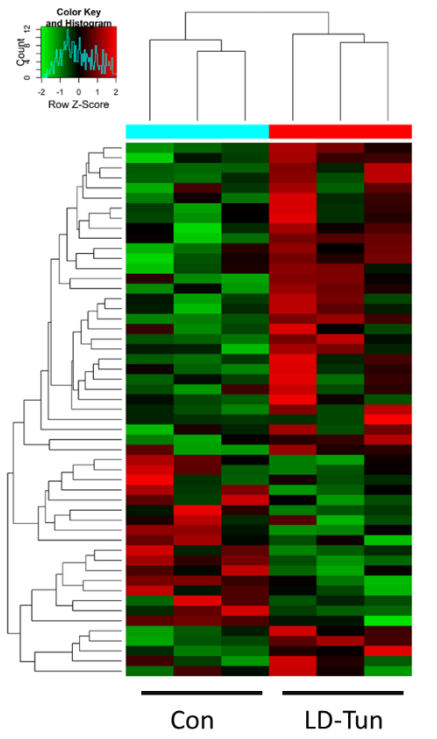


Fig. S3A. Heat map showing the fold change in mRNA levels in the LD-Tun-treated mouse liver relative to control.

B **List of mRNAs up-regulated in LD-Tun treated mice (*P* <0.05).**

Gene Symbol	*P* Value	x-Fold (LD-Tun/Con)
Clec10a	0.002	103.670864851
Rbm4	0.00005	81.866315978
Lpin2	0.00005	38.737274275
Luzp1	0.00005	25.112362279
USP14	**0.00105**	**23.181307881**
Tpra1	0.00005	14.236344752
Atxn2	0.0111	12.744893168
Gstt3	0.00005	12.272735467
Clpb	0.00005	10.797817814
Nacc1	0.00005	8.111678203
Unc119	0.04965	6.411364928
Celf2	0.00005	6.080586111
Uck1	0.00005	5.054062386
Cd36	0.00005	4.582556657
Bcam	0.00005	3.91672001
Cyp2c29	0.03805	3.82036132
As3mt	0.00005	3.401390458
Mdn1	0.00005	2.53525347
Dnase1	0.01245	2.516145838
Lphn1	0.00005	2.14977714

Supplementary Figure 3

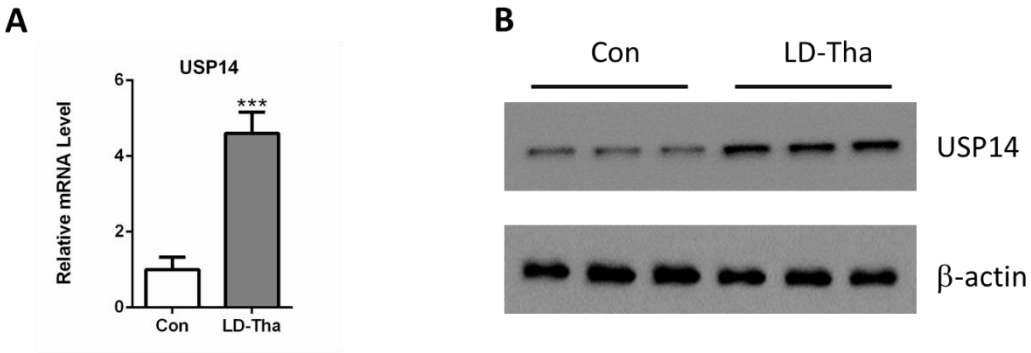
Fig. S3B. Up-regulated mRNAs in the liver of LD-Tun-treated mice (P < 0.05).

C List of mRNAs down-regulated in LD-Tun treated mice (p<0.05).

Gene Symbol	*P* Value	x-Fold (LD-Tun/Con)
Ces1b	0.02695	-2.166851072
Sptan1	0.00005	-2.226303066
Capn3	0.021	-4.115194015
Tmx2	0.00005	-4.743067265
Zfp598	0.00005	-5.348207666
Mup13	0.00005	-7.131309337
Eif4ebp3	0.0023	-7.145435983
Adh6-ps1	0.00005	-8.081971039
Gm6576	0.0006	-11.132801943
Rps13	0.00005	-13.24115722
Tstd1	0.00005	-15.151883607
Gcnt4	0.0112	-15.295963966
Gm10269	0.00005	-18.24377809
Cnot6	0.00005	-24.945615223
Gm21967	0.00005	-25.559833543
Cys1	0.00005	-25.91333176
Gm21887	0.00005	-32.872167725
Smim22	0.00005	-33.985470362
Mup15	0.00005	-45.016956543
Ing4	0.00435	-55.512855516

Supplementary Figure 3

Fig. S3C. Down-regulated mRNAs in the liver of LD-Tun-treated mice (P < 0.05).



Supplementary Figure 4

Fig. S4. (A-B) Relative expression of USP14 mRNA and protein in the liver of LD-Tha-treated and MPHs. MPHs were treated with thapsigargin (50nM) or vehicle control (Con) for 48 hr.

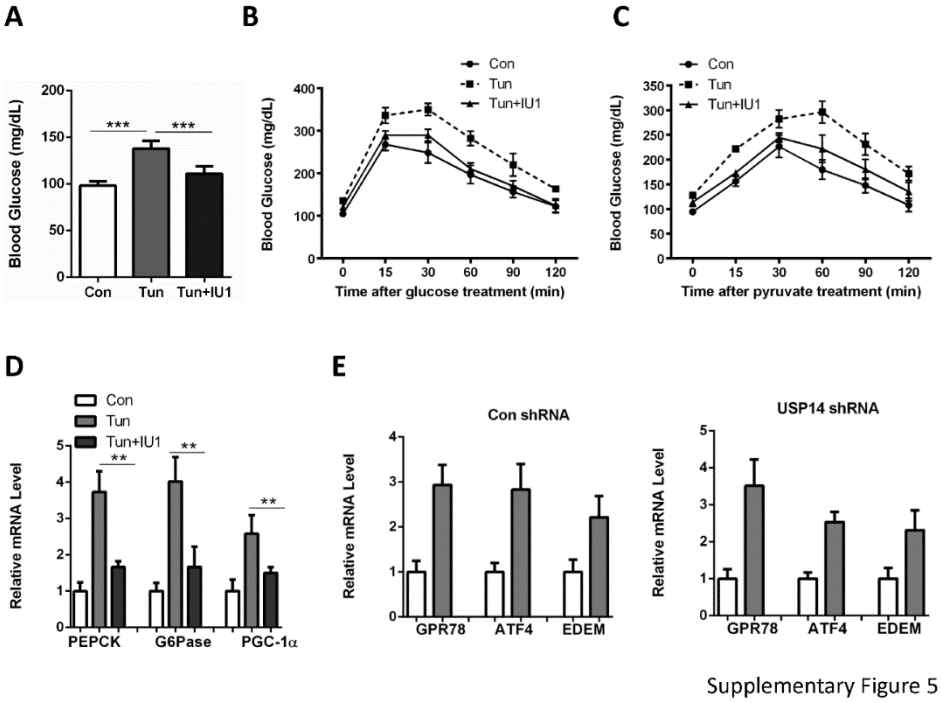


Fig. S5. (A) Blood glucose levels in mice treated with saline, LD-Tun or LD-Tun + IU1. (B-C) Glucose and pyruvate tolerance in the above groups. Relative mRNA levels of (D) gluconeogenic genes and (E) ER stress genes in the above groups.

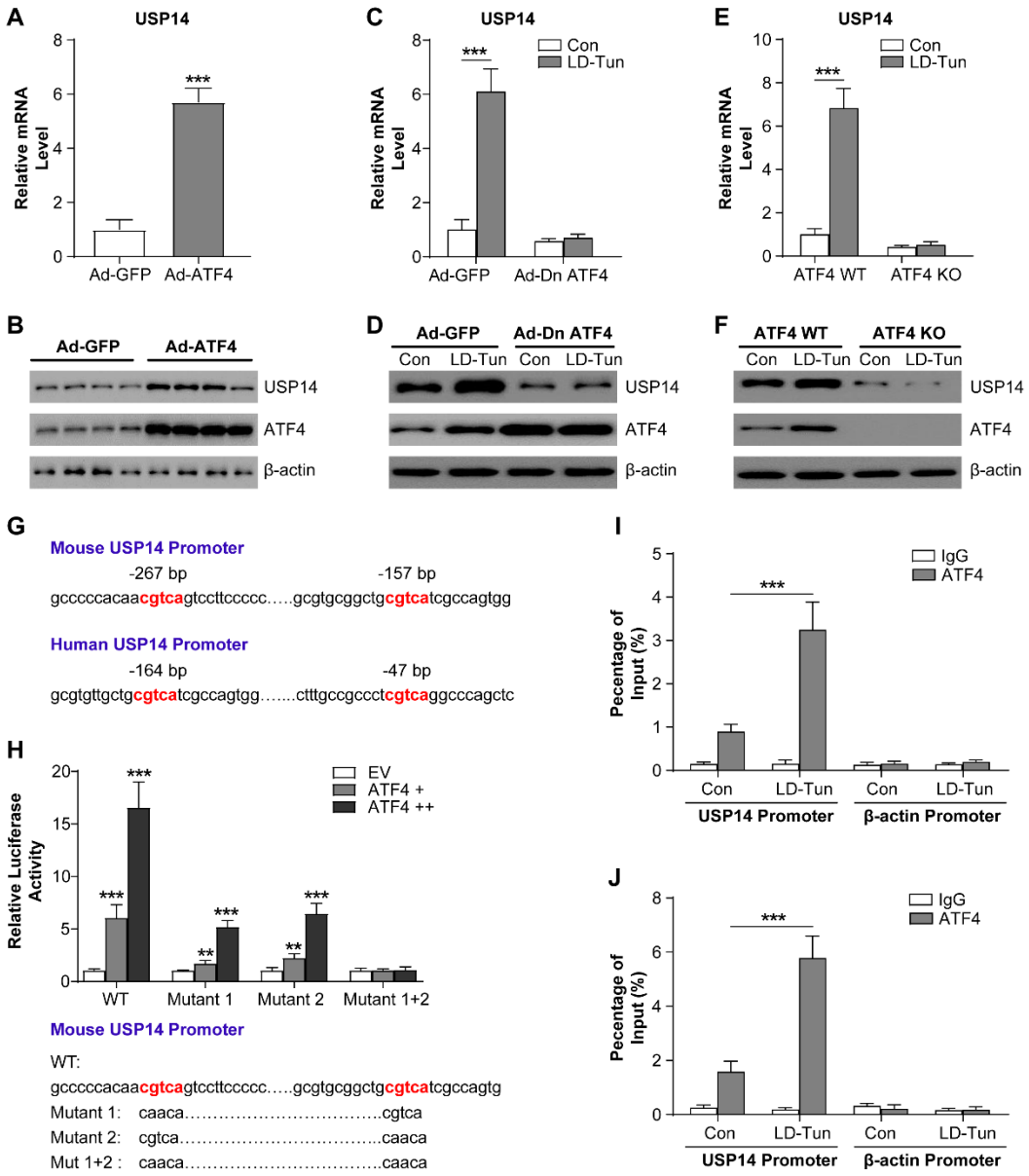
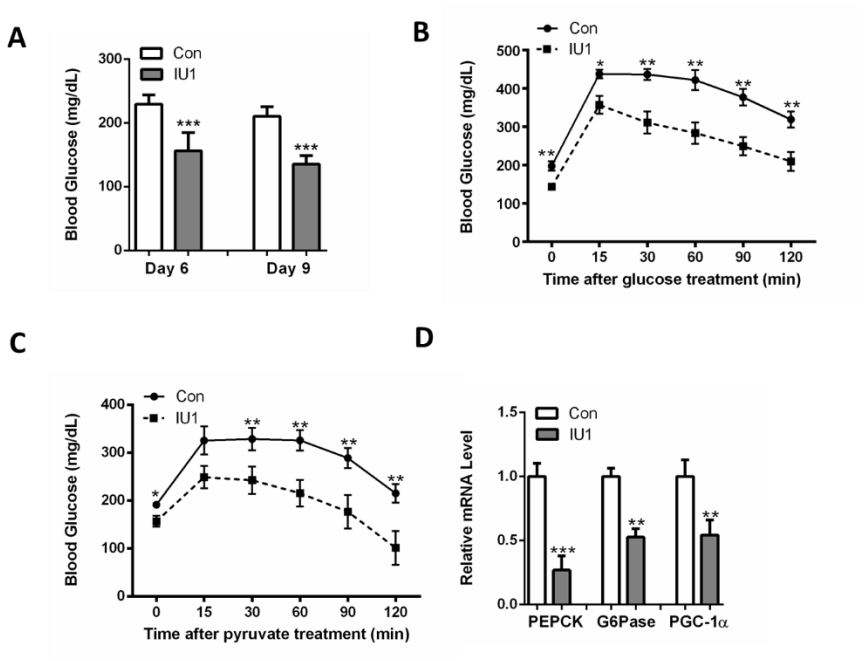


Fig. S6. (A-B) USP14 mRNA and protein levels in MPHs transfected with Ad-GFP or Ad-ATF4. n=4 per group. (C-D) USP14 mRNA and protein levels in MPHs transfected with Ad-GFP or Ad-Dn ATF4 and treated with (0.25μg/ml) or vehicle control (Con). n=4 per group. (E-F) USP14 mRNA and protein levels in MPHs isolated from hepatic ATF4 knockout mice or wild-type littermates. n=4 per group. (G) The proximal promoter region of human and mouse USP14 gene. The two potential ATF4 binding sites are highlighted in red. (H) Transcriptional activity of the wild-type and mutant mouse USP14 promoter in HEK293T cells transfected with luciferase reporter plasmid and increasing doses of the ATF4 expression plasmid (ATF4 +, ATF4 ++). (I-J) ChIP assay showing the binding affinity of ATF4 to USP14 promoter in the liver (I) and MPHs (J). n=6 per group, *** P<0.001.



Supplementary Figure 7

Fig. S7. (A) Blood glucose levels in HFD-fed obese mice at day 6 and day 9 after injection with IU1 or vehicle control. (B-C) Glucose and pyruvate tolerance in the above groups. (D) Relative mRNA levels of gluconeogenic genes in the above groups.

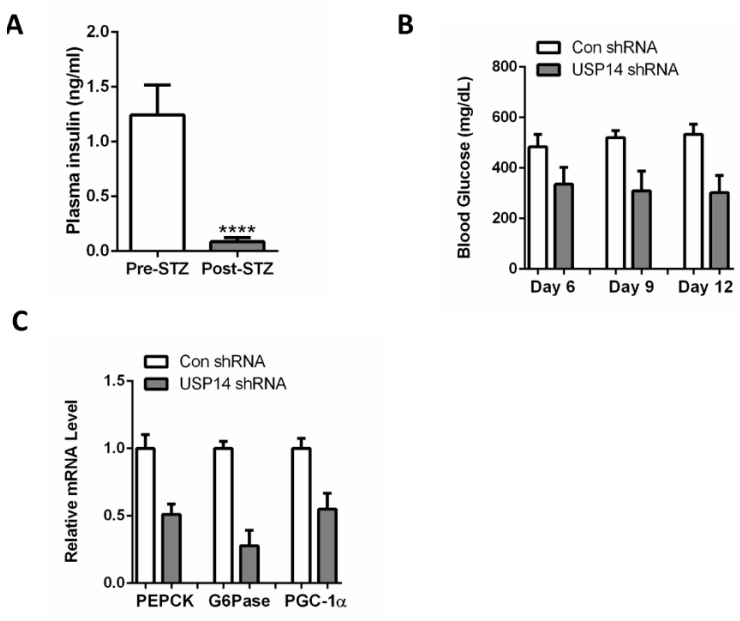


Fig. S8. (A) Plasma glucose levels in C57BL/6 mice before and after STZ treatment. (B) Blood glucose levels in STZ-treated mice treated with control or USP14 shRNA. (C) Relative mRNA levels of gluconeogenic genes in the above groups.

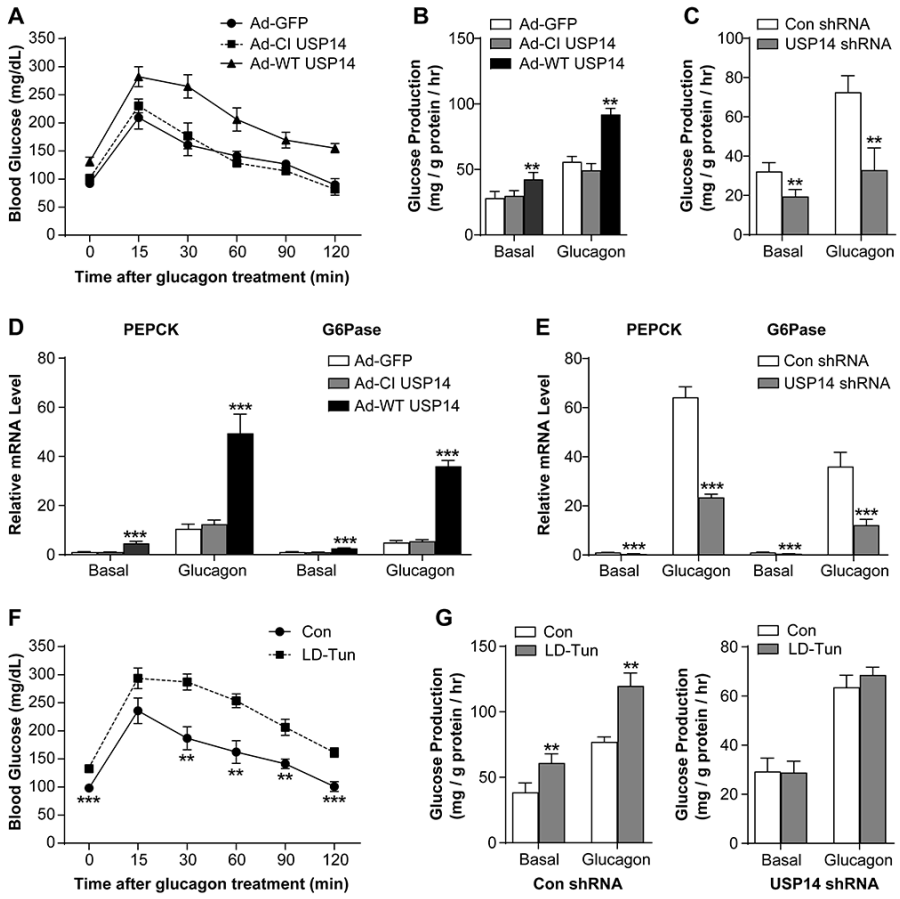


Fig. S9. (A) Glucagon tolerance in mice injected with Ad-GFP, Ad-CI USP14 or Ad-WT USP14. n=6 per group. (B) Glucose levels in MPHs transfected with Ad-GFP, Ad-CI USP14 or Ad-WT USP14, and treated with glucagon. (C) Glucose levels in MPHs transfected with control or USP14 shRNA and treated with glucagon. (D-E) Relative PEPCK mRNA levels in the above MPH groups. (F) Glucagon tolerance in mice treated with LD-Tun or saline. (G) Cellular glucose production in MPHs as indicated. ** P<0.01, *** P<0.001.

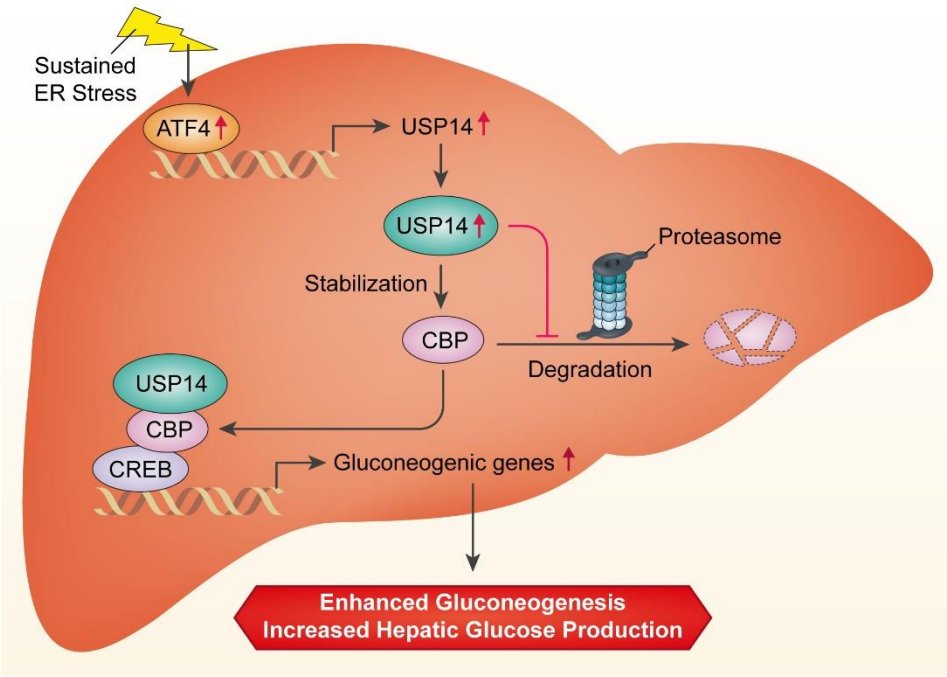


Fig. 10. Working model: Sustained ER stress induces the expression of USP14 through ATF4, which in turn promotes gluconeogenesis and glucose production through stabilization of CBP, a coactivator of CREB.

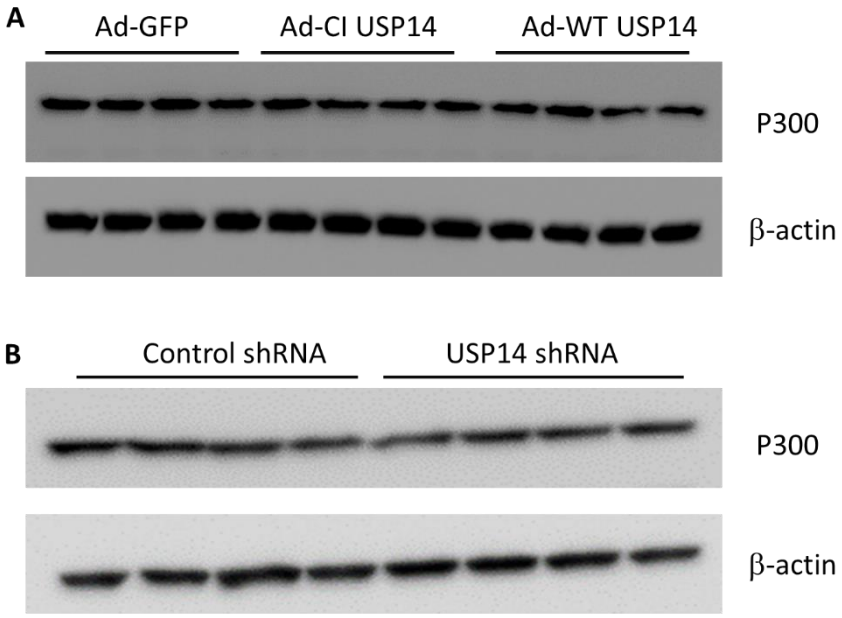


Fig. S11. (A) P300 protein levels in the liver of C57BL/6 mice infected with Ad-GFP, Ad-CI USP14 or Ad-WT USP14. (B) P300 protein levels in the liver of HFD-induced obese mice infected with control shRNA or USP14 shRNA.

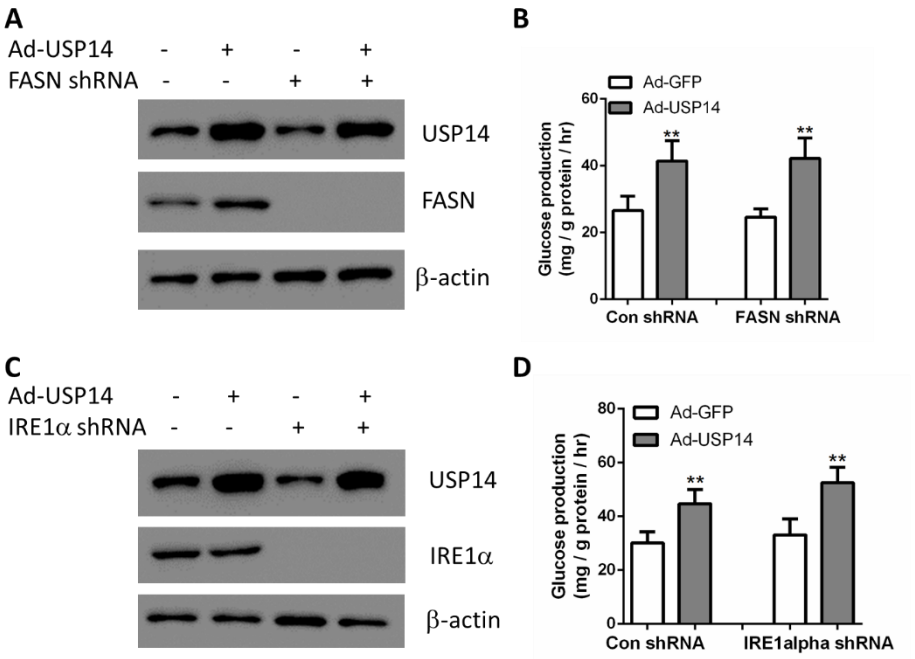


Fig. S12. (A) FASN and USP14 protein levels in MPHs transfected with Ad-USP14 or FASN shRNA. (B) Cellular glucose production in MPHs as indicated. (C) IRE1α and USP14 protein levels in MPHs transfected with Ad-USP14 or IRE1α shRNA. (D) Cellular glucose production in MPHs as indicated.

Supporting references

1. Liu B, *et al.* (2018) Proteome-wide analysis of USP14 substrates revealed its role in hepatosteatosis via stabilization of FASN. *Nature communications* 9(1):4770.
2. Zhang Q, *et al.* (2013) Central activating transcription factor 4 (ATF4) regulates hepatic insulin resistance in mice via S6K1 signaling and the vagus nerve. *Diabetes* 62(7):2230-2239.