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Supplemental Information

An Hsp20-FBXO4 Axis Regulates Adipocyte Function through Modulating PPARγ Ubiquitination

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice

Procedures involving mice were approved by the IACUC of the University of Cincinnati and were conducted according to National Institutes of Health guidelines. Hsp20 knockout mice and adipose tissue-specific overexpression mice were generated by the Division of Developmental Biology at Cincinnati Children's Hospital Medical Center, male C57BL/6J mice and FVB/NJ mice were purchased from the Jackson Laboratory and allowed to acclimate in the animal facility for 2 weeks before being enrolled in studies. Unless specified, male mice were used for experiments. Mice were housed under a 12-hour light-dark cycle at constant temperature (23℃) and given regular chow diet (7022, Envigo). For cold challenge experiments, 8-12 weeks old male mice were housed at 4℃ environment for various time periods in groups of two mice per cage. Axillary temperature were monitored at indicated times using a TH-5 thermometer (Physitemp). For DIO studies, 6-week-old male mice were placed on a HFD (D12492, Research diets) for a total period of 16 weeks.

Indirect calorimetry

Mice were housed in chambers with integrated control of ambient temperature and simultaneous measurement of food intake, locomotor activity and EE by indirect calorimetry (TSE Systems, Chesterfield, MO, USA). Mice were monitored at 23°C or 29°C to compare energy balance at standard room temperature or at thermoneutrality, respectively.

Noradrenaline (norepinephrine) stimulation of oxygen consumption

Animals were adapted to 29°C overnight (14–16 hr) prior to experiments. Energy expenditure, oxygen consumption and carbon dioxide were analyzed in response to s.c. (1 mg kg−1) injection of noradrenaline (norepinephrine; Sigma-Aldrich, St Louis, MO, USA) as previously described (Heppner et al. 2015).

Glucose tolerance and insulin tolerance tests

For IPGTT, mice were fasted overnight and injected intraperitoneally with 20% dextrose at a dose of 1-1.5 g per kg body weight. Glucose levels in tail blood were measured at indicated intervals using glucometer. Serum insulin was measured during the GTT at the 0, 30 min time points by using mouse insulin ELISA kit (Mercodia). For IPITT, mice were fasted for 6 hours before experiments, Insulin (1U/kg for chow diet and 1.25U/kg for HFD, Sigma-Aldrich) was administered intraperitoneally, and glucose levels in tail blood were subsequently monitored at 15, 30, 60, 90 and 120 min post-injection.

Measurement of metabolic factors

Serum adiponectin levels were tested by a mouse adiponectin ELISA kit (EMD Millipore) while leptin level was estimated by a mouse leptin ELISA kit (Crystal Chem Inc.). Serum total cholesterol, triglyceride levels and Nonesterified fatty acid (NEFA) were tested with a Wako LabAssay NEFA kit (Wako).

Histology

Tissues were extracted, fixed in 10% neutral-buffered formalin (Sigma-Aldrich) for 48 hr at 4℃, embedded in paraffin and sectioned at 5 μm. Then, tissue sections were stained with H&E using standard techniques. Immunohistochemistry was done using UCP1 antibody (1:500, catalog ab10983, Abcam). Immunofluorescent double staining was done using insulin (1:100, catalog ab7842, Abcam) and glucagon antibody (1:100,

catalog ab10988, Abcam), following the manufacturer's instructions. Sections were visualized and photographed using a Nikon Eclipse microscope.

Flow cytometry analysis

Isolation of SVCs have been described previously(Cho, Morris, and Lumeng 2014). Briefly, inguinal white adipose tissue (iWAT) were minced and subjected to Type II collagenase (Sigma-Aldrich) for 45 min at 37℃ with constant shaking. After digestion, the cell slurry was filtered through a 100-μm cell strainer (BD Biosciences) and centrifuged at 500 g for 10 min at 4℃. The SVC pellets were collected and resuspended in RBC lysis buffer (ebioscience), 5 min later, centrifuged at 500 g for 10 min at 4℃. Cell pellets were resuspended in FACS buffer and incubated on ice before staining with antibody. For flow cytometry analysis of macrophages, $1x10^6$ SVCs were stained with DAPI (Sigma-Aldrich, 0.2 mg/ml), F4/80 PE (0.2 μg/10^6 cells, catalog 12-4801-82, eBioscience), CD11b APCeFluor780 (0.16 μg/10^6 cells, catalog 47-0112-80, eBioscience), CD11c PE-Cy7 (0.2ug/10^6 cells, catalog 25-0114-82, eBioscience), CD45.2 PerCP-Cy5.5 (0.2 μg/10^6 cells, catalog 45-0454-82, eBioscience), CD301 Alexa Fluor647 (0.2 μg/10^6 cells, catalog MCA2392A647T, AbD Serotec), or their isotype controls (eBioscience) on ice for 30 min in dark. After washing, cells were fixed in 0.1% PFA (Sigma-Aldrich) and analyzed with Flow cytometer (FACSCanto II; BD Biosciences).

Cell culture

For primary adipocytes, SVCs from iWAT of 5- to 6-week-old male mice were prepared and differentiated as previously described(Kajimura et al. 2009). iWAT was minced and digested in digestion medium [1.5U/ml Collagenase D (Roche) and 2.4U/ml Dispase II (Roche) in HBSS] for 45 min at 37℃ with constant shaking. Then the cell suspension was passed through a 100-μm cell strainer (BD Biosciences) and

centrifuged at 700 g for 10 min at room temperature. The medium was aspirated and SVCs were resuspended in DMEM/F12 (Thermofisher) supplemented with 10% FBS, 100 IU /ml penicillin and 100 µg/ml streptomycin, and then cells were plated in 100mm culture dishes. For differentiation assays, primary adipocytes and 3T3-L1 cells were cultured to 98% confluence and the growth medium was changed to induction medium (day 0) containing insulin (0.5 mg/ml; Sigma-Aldrich), dexamethasone (2 µg/ml; Sigma-Aldrich), isobutylmethyl-xanthine (0.5mM; Sigma-Aldrich), and 10% FBS. After 48 hr (day 2), medium was changed to maintenance medium containing insulin (0.5 mg/ml; Sigma-Aldrich) and 10% FBS until cells were ready for harvest (generally day 7-8).

Generation of Hsp20-overexpression and FBXO4-knockdown cell lines

Fbxo4 AAV shRNA pooled virus was purchased from Applied Biological Materials Inc. The sequences are as follows: 417-CCCAATTCTCTGGAGATACTTTCTGTTTC; 768-ACAGATTGACGGTATTGGATCTGGAGTCA; 971-AGAAAGTGTGTGAGGTTGTAGATGGGTTT; 1252-ATTGAGTGGATTCTTGA AGAAGTAGAATC. The adenovirus vector carrying Hsp20 cDNA (Ad.Hsp20) was generated by using the AdEasy system. Adenovirus and AAV Infection were performed according to the manufacturer's instructions.

Quantitative real-time PCR and RNA-Seq

Quantitative Real-time PCR (qPCR) was performed as described previously(Ma et al. 2015). Total RNA was extracted from cultured cells or tissues using the RNeasy kit (Qiagen) in accordance with the manufacturer's instructions. cDNA was synthesized from 1.0 ug RNA using Superscript II Reverse Transcriptase (Invitrogen). qPCR was performed in triplicate with the ABI PRISM 7900HT sequence detection system (ABI) using SYBR green (Applied Biosystems). Relative mRNA levels were calculated using

the delta delta CT method and normalized to GAPDH as internal control. Directional polyA RNA-seq was performed by the Genomics, Epigenomics and Sequencing Core (GESC) at the University of Cincinnati. The GEO accession number for RNA-Seq data reported in this paper is GSE94436.

Western-blot analysis

For insulin stimulation, 10U insulin (Sigma-Aldrich) was injected intraperitoneally as described previously(McClung et al. 2004). 8 minutes later, samples of liver, skeletal muscle and iWAT were dissected and immediately frozen in liquid nitrogen. Tissues or cells used for western blot analysis were homogenized in RIPA buffer (Thermofisher) supplemented with phenylmethylsulfonyl fluoride (Sigma-Aldrich), phosphatase inhibitor (Cell signaling) and complete protease inhibitor cocktail (Roche). Tissue or cell lysates were then centrifuged at 14,000 g for 15 min at 4℃ and protein concentration was determined using BioRad Protein Assay Reagent (Bio-Rad). Proteins were separated by SDS-PAGE, transferred to a (NC) membrane (GE Healthcare Life Sciences). Immunoblotting was performed using the following primary antibodies: β-actin (1:1000, catalog 4970s, Cell Signaling Technology); GAPDH (1:1000, catalog 5174s, Cell Signaling Technology); Ucp1 (1:10000, catalog ab10983, Abcam); Hsp20 (1:2000, catalog ab13491, Abcam; 1:2000, catalog 10R-H111a, Fitzgerald); AKT (1:2000, catalog 2920s, Cell Signaling Technology); p-AKT (1:2000, catalog 4060s, Cell Signaling Technology); PPARγ (1:1000, catalog 2443s, Cell Signaling Technology); PhosphoSer112- PPARγ (1:2000, catalog MAB3632, Millipore); PhosphoSer273-PPARγ (1:2000, catalog orb158188, Biorbyt); CDK5 (1:1000, catalog 12134s, Cell Signaling Technology); CDK9 (1:1000, catalog 2316s, Cell Signaling Technology); FBXO4(1:1000, catalog ab153803, Abcam. 1:1000, catalog 100401963, Rockland); SIAH2 (1:1000, catalog NB110-88113SS, Novusbio); TRIM23 (1:1000, catalog NB100-78620, Novusbio); MKRN1 (1:1000, catalog A300-

990A-T, Bethyl); Erk1/2 (1:1000, catalog 4695s, Cell Signaling Technology); and Phospho-Erk1/2 (1:1000, catalog 9101s, Cell Signaling Technology);. For secondary antibody incubation, anti-rabbit (catalog AF1800, R&D) or anti-mouse HRP (catalog MAB1799, R&D) was diluted 1:5,000. Results were visualized with enhanced chemiluminescence (ECL) western blotting substrates (Pierce) and quantified using the NIH ImageJ software.

Lipidomics assay

Lipidomics assay was performed by the Lipidomics Research Program at the University of Cincinnati. Briefly, samples of inguinal white adipose tissue (20-25 mg), liver (25-30 mg), and plasma (100 ul) were snap-frozen in liquid nitrogen. Samples were placed in a 20 ml glass vial into which 4 ml of 0.5N methanolic sodium hydroxide was added, and the sample heated at 80°C for 5 min. Following a 10 min cooling period, 3 ml of boron trifluoride in methanol was added to methylate the sample. After an additional 5 minutes of heating in a heat block (80°C), the sample vial was allowed to cool, and 2 ml of a saturated solution (6.2 M) of sodium chloride and 5 ml of hexane was added. The samples were then mixed by vortex for 1 minute. The hexane fraction was transferred into a 20 ml vial containing 10 mg of sodium sulfate to dry the sample. The hexane solution was removed for gas chromatography analysis with a Shimadzu GC-2014 (Shimadzu Scientific Instruments Inc., Columbia MD). The column was a DB-23 (123-2332): 30m (length), I.D. (mm) 0.32 wide bore, film thickness of 0.25 µM (J&W Scientific, Folsom CA). The carrier gas was helium with a column flow rate of 2.5 ml/min. Fatty acid identification was determined using retention times of authenticated fatty acid methyl ester standards (Matreya LLC Inc., Pleasant Gap PA).

Immunoprecipitation and ubiquitination assay

Cells were harvested and lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, leupeptin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 1mM Na3VO4, 1mM Na2EDTA, 1mM EDTA, 10 mM NaF, 2.5 mM sodium pyrophosphate, and complete protease inhibitor cocktail (Roche). The cell lysates were centrifuged at 14,000 g for 15 min at 4°C, and the resulting supernatant was incubated with antibodies overnight at 4°C, followed by incubation with Protein G Agarose Beads (Cell Signaling Technology) for 3 h. The beads were then separated by centrifugation, washed 5 times with ice-cold lysis buffer, and then boiled in SDS sample buffer for 5 min. In the ubiquitination assay, cells were incubated with MG132 at a final concentration of 5-10 μM for 2-4 hr, washed two times with PBS, collected into a cell pellet at 14,000 g and then resuspended in lysis buffer (no Triton X-100) plus with 2mM NEM (Sigma-Aldrich). The extract was denatured by adding 1% SDS to the lysate and boiled for 10 min, followed by dilution to 0.1% SDS by adding lysis buffer, protease inhibitors, and NEM. Lysed samples were immunoprecipitated with PPARγ antibodies, followed by Western-blotting analysis.

Differential gene expression and functional enrichment analysis

Differential gene expression analysis of RNA-seq data was performed by the Laboratory for Statistical Genomics and Systems Biology at the University of Cincinnati using the standard pipeline. Specifically, sequence alignment was done by Tophat(Trapnell, Pachter, and Salzberg 2009) and differential expression analysis was done by edgeR(Robinson, McCarthy, and Smyth 2009). Functional enrichment (p value 0.05 FDR) analysis of significantly differentially expressed genes in cold treatment or Hsp20 KO mice was done using the ToppFun application of ToppGene Suite(Chen et al. 2009). Network representation of select significantly enriched GO biological processes was done using Cytoscape(Shannon et al. 2003).

Statistics

Student's t test was used for single comparisons. Two-way ANOVA (repeated measurement) was used for IPGTT and IPITT. The correlation between energy expenditure, oxygen consumption and carbon dioxide production and lean mass for WT and KO mice was analyzed by linear regression to assess intercept using GraphPad Prism 7 for Mac (GraphPad Software, La Jolla California USA). Analysis of fatty acid methyl esters was based on areas calculated with Shimadzu Class VP 4.3 software, and data are expressed as weight percent of total fatty acids (mg fatty acid/100 mg fatty acids). Unless specified, Data are represented as mean ± SEM. *P< 0.05, and not significant (n.s.) P> 0.05.

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Figure S1, related to Figures 1 and 2

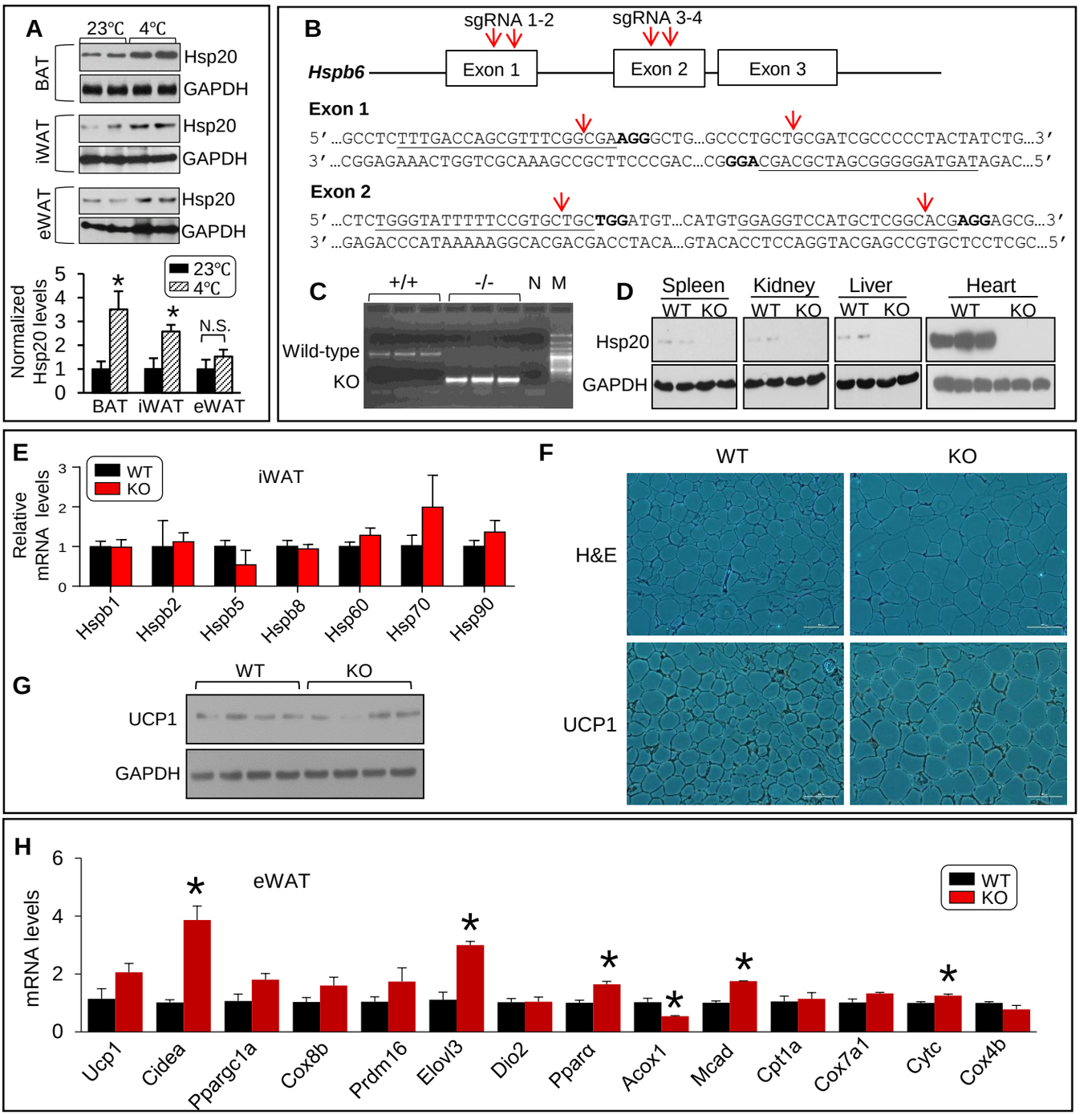

Figure S1. Generation and Characterization of Hsp20-knockout Mice. **Related to Figure 1 and Figure 2.** (A) Immunoblot analysis with quantification of Hsp20 expression in the BAT, iWAT, and eWAT of mice housed at 23℃ (as Ctl) or 4℃ for 48 hr (n=5 per group). (B) To generate knockout mice of Hsp20, a total of 4 gRNAs were selected to inject with Cas9 mRNA into one-cell embryos, with two gRNAs targeting to exon 1 and the other two to exon 2. Because of the Cas9 activity, sequence between the gRNA targeting sites were deleted. (The PAM of each gRNA targeting site is highlighted in bold. The spacer sequence is underlined. The cutting site of Cas9 is indicated by arrow). (C) Genotyping results of Hsp20 KO mice (+/+, WT; -/- , KO; N, negative Ctl; M, DNA molecular marker). (D) Western blot analysis of Hsp20 in various tissues of WT and KO mice (n = 4 per genotype). (E) Hsp20 ablation did not alter mRNA expression levels of other HSPs in iWAT. (F) Representative H&E and UCP1 staining of eWAT sections of WT and KO mice housed at RT (scale bar, 100 um; n=6 per genotype). (G) Immunoblot analysis of UCP1 in eWAT of WT and KO mice housed at RT (n = 8 per genotype). (H) Quantitative RT-PCR analysis of thermogenic, mitochondrial and fatty acid oxidation genes in eWAT of WT and KO mice. Data are represented as the mean ± SEM.

Figure S2, related to Figure 3

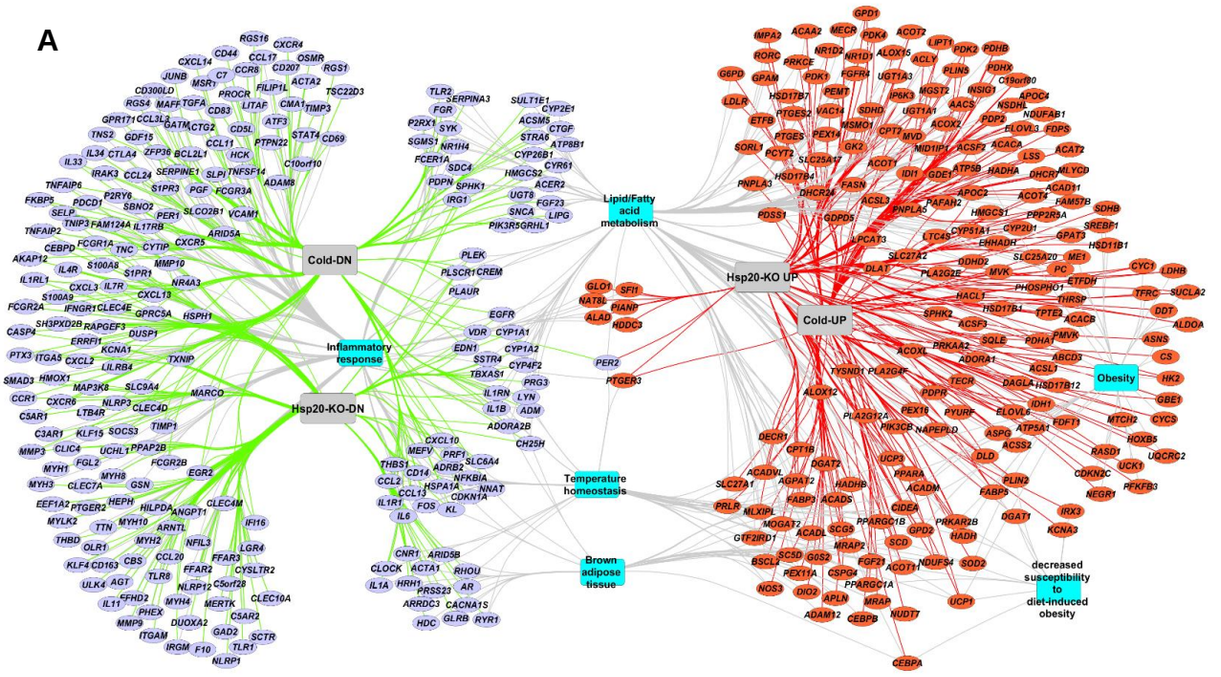

Figure S2. Gene Enrichment Analyses from iWAT Reflect a Comparable Role of Hsp20 Deletion on Lipid Metabolism and Inflammation with Cold Treatment. Related to Figure 3. (A) Gene enrichment analyses from the iWATs of KO and WT mice with or without cold exposure reflect a network representation of select enriched (*p* value<0.05 FDR) biological processes from ToppFun using the Cytoscape software.

Figure S3, related to Figure 3

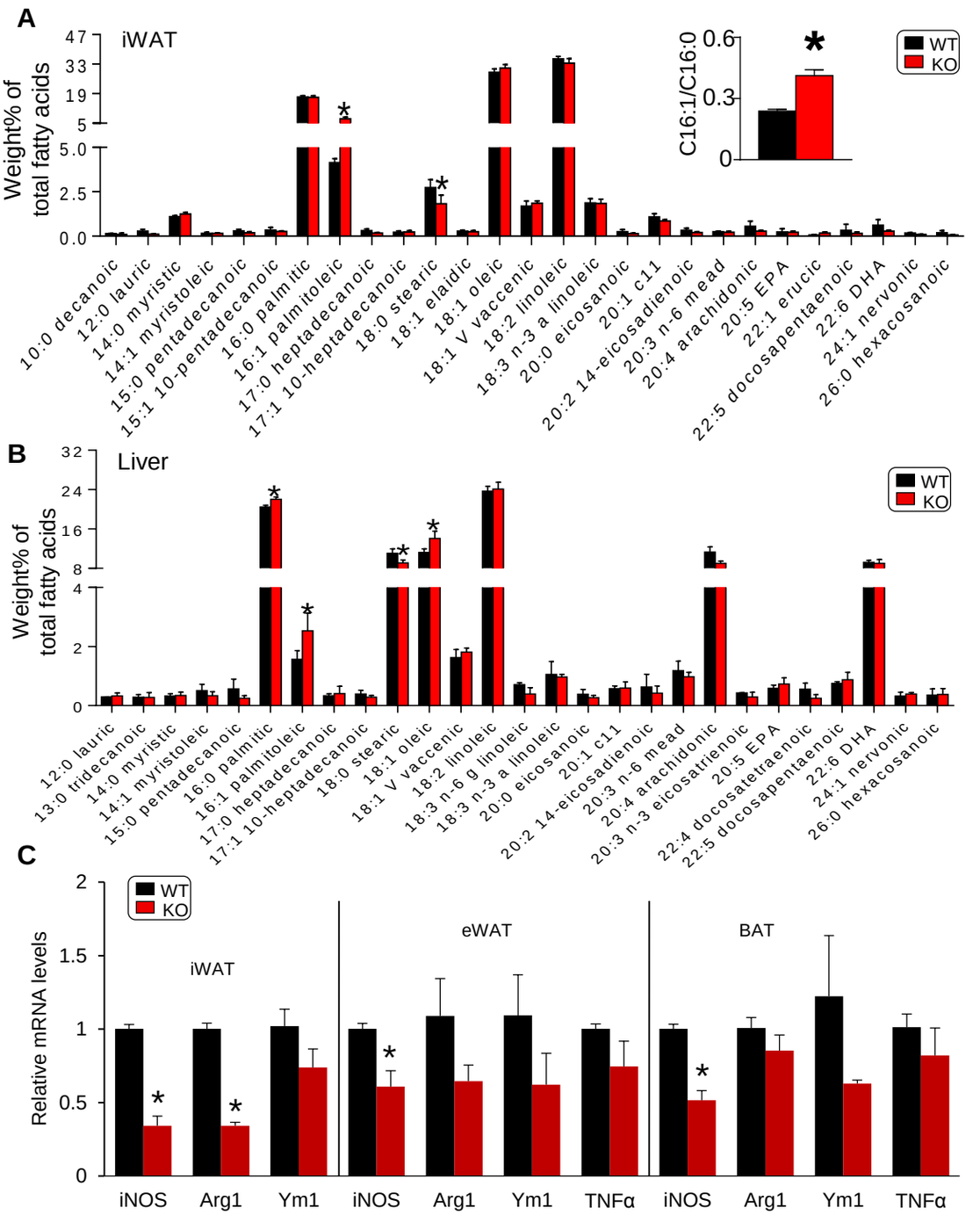

Figure S3. iWAT and liver lipidomic analysis and macrophage polarization characterization in different adipose depots. Related to Figure 3. (A and B) Total fatty acid composition in the iWAT (B) and the liver (C) collected from 16-week-old WT and KO mice fed with CD (n=4 per genotype). (C) mRNA expression of macrophage polarization marker genes in different adipose tissues. Data are represented as the mean ± SEM.

Figure S4, related to Figure 4

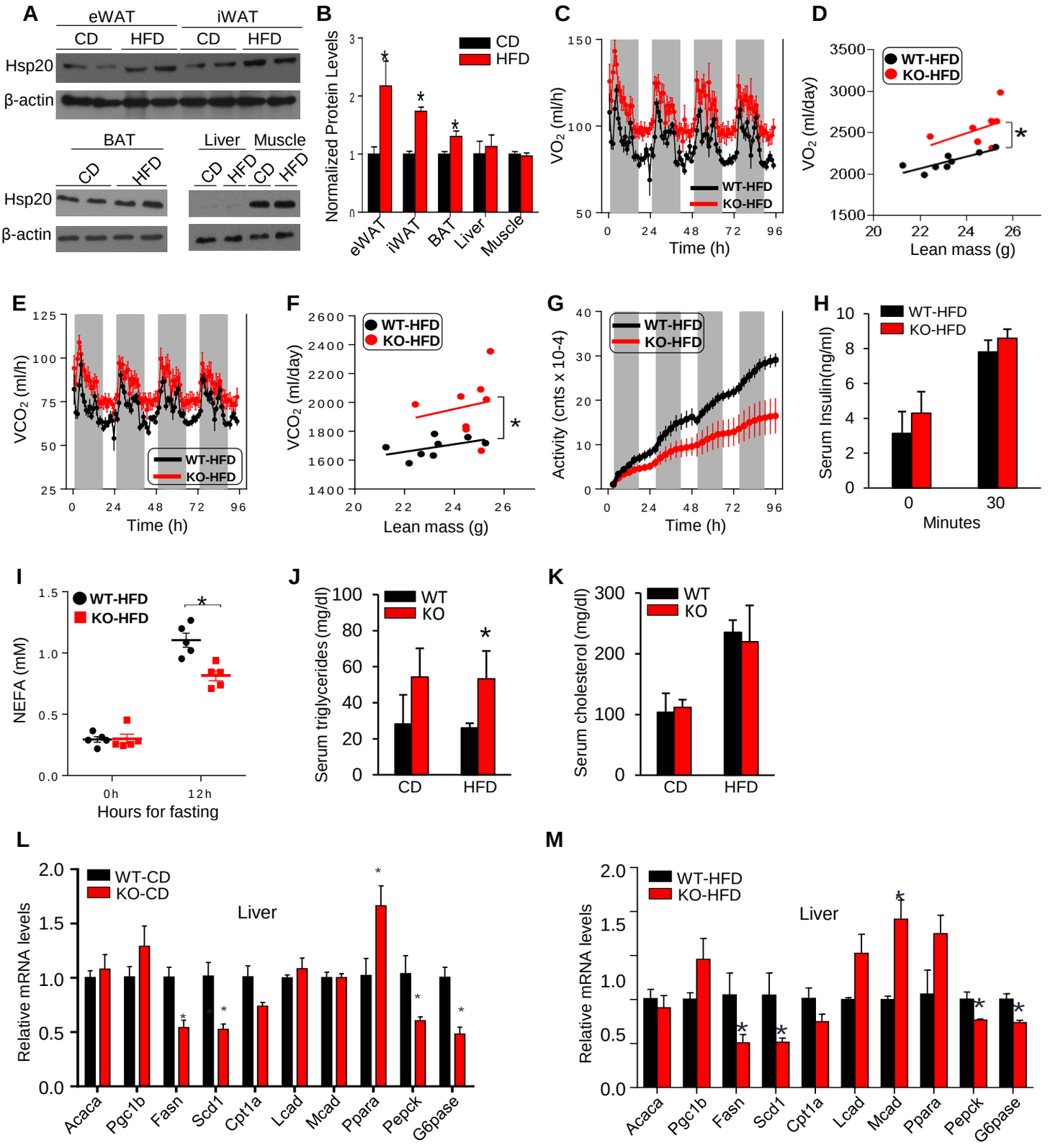

Figure S4. Metabolically Characterizations of KO Animals Upon HFD. Related to Figure 4. (A and B) Immunoblot of Hsp20 in various tissue of WT mice fed with CD and HFD for 16 weeks (n=5 per group). (C) VO_2 and (D) the linear regression analysis of the VO_2 against lean body mass in mice after 12 weeks of HFD. (E) VCO_2 and (F) the linear regression analysis of the VCO_2 against lean body mass in mice after 12 weeks of HFD. (G) Quantification of total activity in WT and KO mice fed with HFD for 12 weeks. n=8 mice per genotype for C-G. (H) Insulin levels in the blood of mice fed with HFD (n = 4). (I) Serum NEFA levels at baseline, or after 12-hr fasting (n=5). (J) Serum triglycerides and (K) cholesterol of WT and KO mice fed with CD or HFD (16-week) after 6-hr fasting (n=3). (L and M) mRNA expression of genes involved lipid oxidation and lipogenesis in the liver from WT and KO mice fed with CD (L) and HFD (M) for 16 weeks (N=4). Data are represented as the mean ± SEM.

Figure S5, related to Figure 5

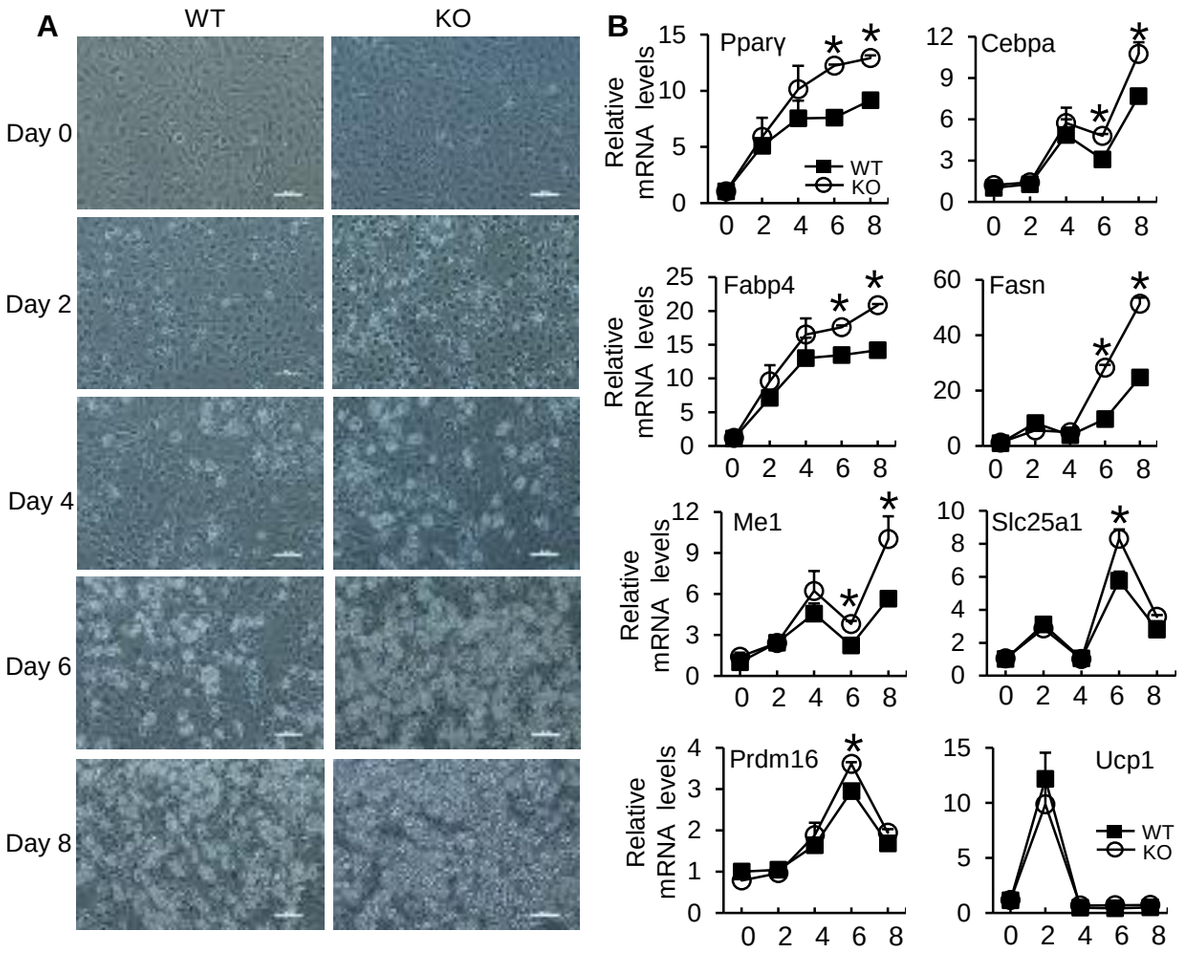

Figure S5. Ex vivo characterization of adipocyte differentiation. Related to Figure 5. (A) Representative images of SVC differentiation at indicated time points of differentiation. (B) mRNA expression of marker genes at indicated time points of differentiation. Data are represented as the mean ± SEM.

Figure S6, related to Figure 5

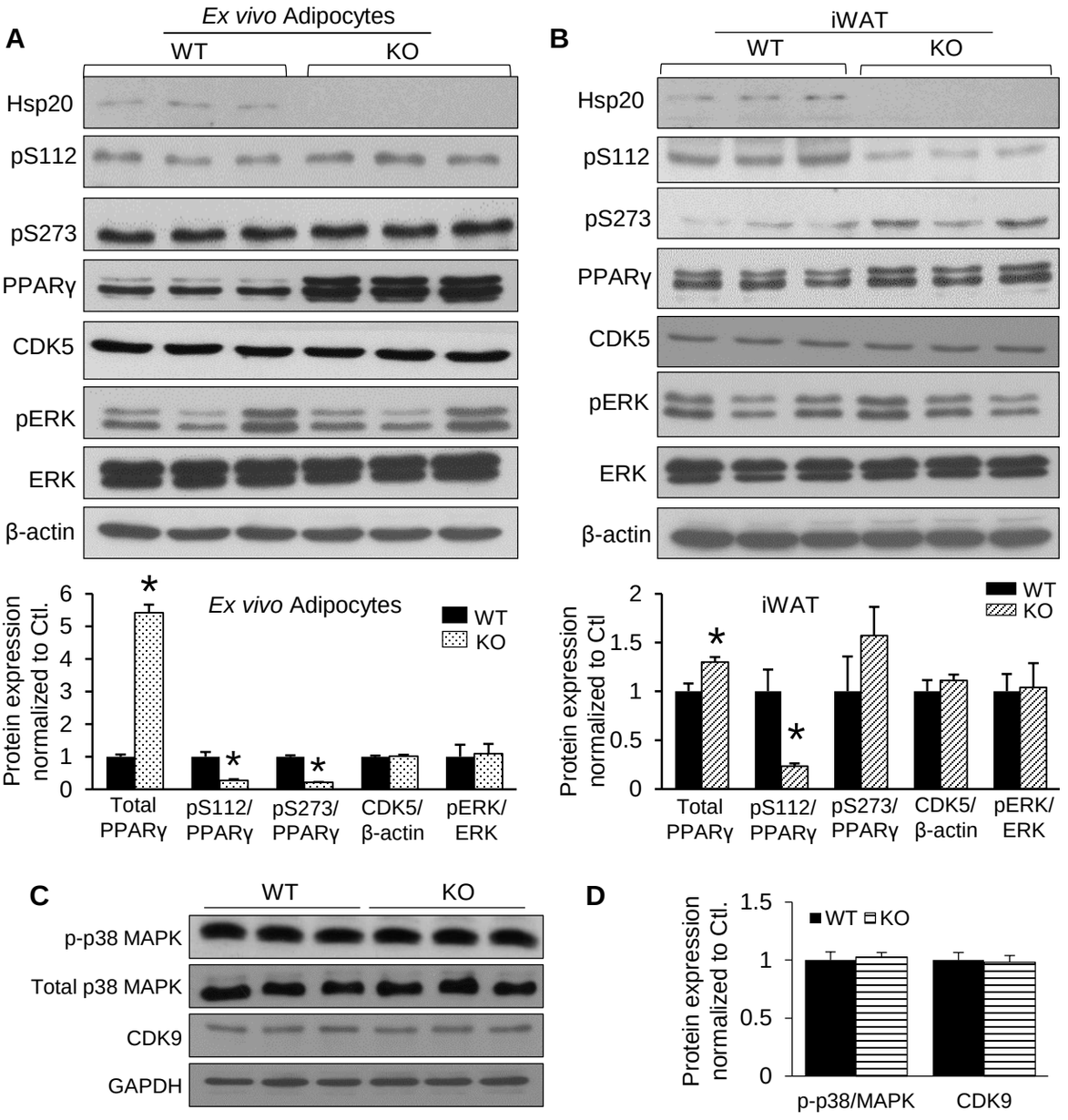

Figure S6. Hsp20 Regulates PPARγ Protein Ubiquitination and Stability in Isolated Adipocytes. Related to Figure 5. (A) Immunoblot analysis and quantification of total PPARγ, phospho-PPARγ at Ser-112 and Ser-273, and its upstream kinases including ERK and CDK5 at day 8 of SVCs differentiation. (B) Western blot analysis and quantification for total PPARγ, phospho-PPARγ at Ser-112 and Ser-273, pERK and CDK5 in the iWAT of WT and KO mice (n=3 per genotype). (C and D) Western blot analysis (C) and quantification (D) of phosphorylated MAPK and CDK9 in iWAT of WT and KO mice. Data are represented as the mean ± SEM.

Figure S7, related to Figure 6

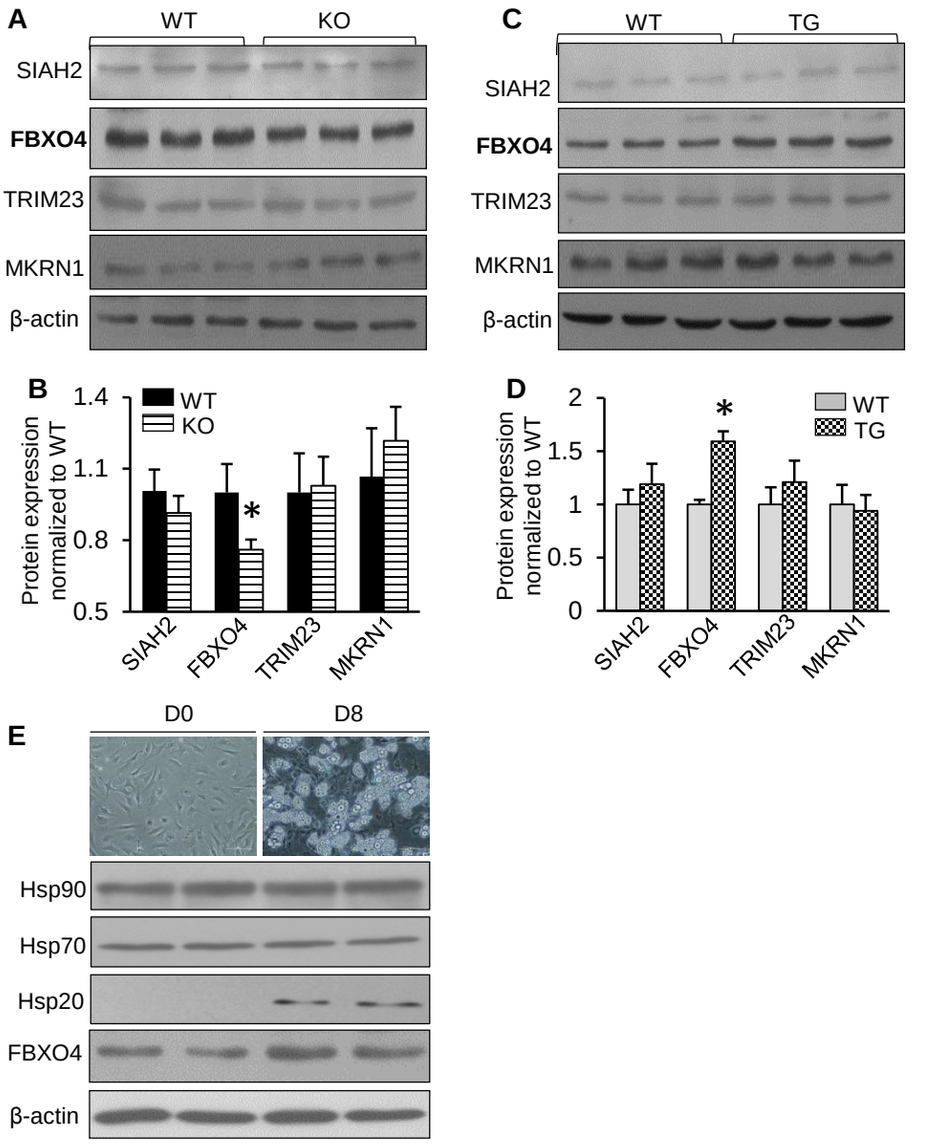

Figure S7. FBXO4 is Required for Hsp20-Mediated Control of PPARγ Stability. Related to Figure 6. (A-D) Representative immuno-blots and the quantitation analysis of E3 ligases in the iWAT of WT, KO or TG mice. (E) Representative immuno-blots of Hsps and FBXO4 in SVC-differentiated adipocytes. Data are represented as the mean ± SEM.