Supplemental Materials

(1) Supplemental Materials and Methods

Plasmid construction

Human PAQR3, PPARα and various truncations were cloned into the CS2MT (6xMyc) and p3Xflag-CMV-10 vectors, respectively. For the plasmids used in the GST-Pulldown assay, the cDNA regions encoding NH2-terminal 1-71 amino acids domain of human PAQR3 and 101-173 amino acids domain of human PPARα were cloned into the pGEX-4T-1 and pET-28a(+) vectors, respectively. All the constructs were confirmed by DNA sequencing. For the plasmids used in lentiviral shRNA-mediated knockdown, the target sequences for the shRNAs were as follows: shGFP: TTCTCCGAACGTGTCACGT; shPAQR3: GGACAACCCGTACATCACC; shHUWE1-1: ACACTTTCACAGATACTAT; and shHUWE1-2: GTCTGTGTTGTGGAGGTTT.

Cell lines and treatment

HEK293T, HepG2 and Hepa1-6 cells were purchased from the American Type Culture Collection (ATCC) and maintained in DMEM containing 10% FBS and 1% penicillin–streptomycin. PAQR3 and HUWE1 were knocked down in HEK293T and/or HepG2 cells using a lentiviral-based shRNA strategy. To examine the degradation of PPARα via proteasome-dependent or proteasome-independent protein degradation, HEK293T stable cells expressing control or PAQR3-specific shRNA were transfected with PPARα expression plasmids. At 24 h after the transfection, cells were incubated with 20 μM CQ (chloroquine diphosphate salt, Sigma-Aldrich), a lysosomal inhibitor, for 4 h or 10 μM MG132 (Millipore Corp, Billerica, MA, USA), a proteasome inhibitor, for 6 h. Polyetherimide (PEI) Transfection Reagent (Sigma-Aldrich) and lipo2000 (Invitrogen, Frederick, MD, USA) were used in transient transfection for HEK293T cells and HepG2/Hepa1-6 cells, respectively.

Construction of lentivirus

Lentivirus-based gene silencing by shRNA was proceeded as previously reported (1). Briefly, the shRNA targeting human PAQR3 was inserted into the pBS-SKII-hU6 vector downstream of the hU6 promoter. The shRNA expression cassette was then subcloned into the FG12 vector and confirmed by DNA sequencing. The shRNAs targeting human HUWE1 were purchased from Shanghai Genechem Co., LTD (Shanghai, China). Lentiviral stocks were prepared by Polyetherimide (PEI) co-transfection of the lentiviral expression and the packaging plasmids psPAX2 and pMD2.G into HEK293T cells. Supernatants were

collected at 48 h and 72 h after transfection, and then the virus were filtered through a 0.45 μm filter (Millipore Corp, Billerica, MA, USA). Recombinant lentiviruses were used to infect HEK293T and HepG2 cells (4 μg/ml polybrene). Selection of stable clones was carried out using puromycin (Sigma-Aldrich).

Adenovirus construction and injection

Adenoviruses of either scrambled sequence or shRNA sequence specific targeted for mouse PAQR3 (5'-GCTTTCCTGTTCTACATTTCC-3') were generated using the BLOCK-iT Adenoviral RNAi Expression System (Invitrogen, Frederick, MD, USA) according to the manufacturer's instructions. Adenoviruses were delivered into mice by tail vein injection ($5x10^8$ p.f.u. viruses per mouse). One week post-injection, the mice were subjected to different experiment procedures.

Immunoblot analysis and antibodies

Cells were lysed by RIPA buffer and centrifuged at 13,200 rpm for 15 min at 4°C. The supernatant was harvested and protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Identical amounts of protein were subjected to SDS–PAGE electrophoresis and transferred to pre-activated polyvinylidene fluoride membranes (Millipore Corp, Billerica, MA, USA). Antibodies used in immunoblotting were as follows: anti-Myc (sc-40, 1:1000), anti-HA (sc-805, 1:1000), anti-GFP (sc-8334, 1:1000), and anti-ubiquitin (sc-8017, 1:1000) from Santa Cruz Biotechnology; anti-Flag (F3165, 1:5000) and anti-tubulin (T6199, 1:5000) from Sigma-Aldrich; anti-PPARα (ab8934, 1:500) from Abcam; anti-HUWE1 (5695, 1:1000) from Cell Signaling Technology; anti-PAQR3 (PA5-24654, 1:500) from Thermo Scientific Pierce.

Histological analysis of tissues

Livers embedded in optimum cutting temperature compound (Tissue-Tek; Laborimpex, Torrance, CA, USA) were used for Oil Red O staining for the assessment of hepatic steatosis as previously reported (2).

RNA isolation and relative quantitative RT-PCR

Total RNA was extracted from cultured cells or mouse tissues with Trizol reagent (Life Technologies, Carlsbad, CA, USA). 1.5 μg RNA were reverse transcribed with a high capacity cDNA reverse transcription kit (Tiangen, Shanghai, China) and subjected to RT-PCR by using SYBR green mixture (TOYOBO, Osaka, Japan) with ABI 7900 RT-PCR Systems according to the manufacturer's protocol. Relative mRNA levels were quantified using the

comparative ΔCT method, normalized to actin with the sequences of primers described in Table S1.

Measurement of blood parameters

Total lipids were extracted from 50 mg of liver as previously described (3). Blood samples were collected by heart puncture from isoflurane-anesthetized mice. Serum was obtained after centrifugation (30 min, 3,000 rpm, 4°C) and serum indices were measured respectively. Triglycerides (TG), non-esterified fatty acids (NEFAs) and β-hydroxybutyrate were measured with a TG kit (SHENSUO UNF, Shanghai, China), FFAs kit (Wako Chemicals, Osaka, Japan), FGF21 kit (Li Ka Shing Faculty of Medicine, The University of Hong Kong) and β-hydroxybutyrate colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA), respectively, according to the manufacturer's instructions. The concentrations of liver TG and NEFAs were normalized to protein concentrations.

Luciferase reporter assay

Luciferase activity was measured with a luciferase assay system (Promega, Madison, WI, USA). HepG2 cells stably expressing PAQR3 or PAQR3-shRNA were transiently transfected with 0.1 μg PPRE-luciferase reporter, 0.075 μg β-galactosidase and 0.3 μg PPARα in 24-well plates. At 24 h after the transfection, cells were incubated with or without PPARα agonists as indicated for 24 h and then the luciferase and β-galactosidase activities were measured based on manufacturer's instruction. The expression plasmid for 3xPPRE reporter was kindly provided by Dr. Feifan Guo (Institute for Nutritional Sciences, SIBS, Chinese Academy of Sciences).

Fatty acid β-oxidation assay

As for the activities of fatty acid β-oxidation from liver homogenates, 100 mg liver was homogenized using a Potter-Elvejhem homogenizer in 1 ml of ice-cold homogenization buffer (220 mM mannitol, 70 mM sucrose, 2 mM HEPES, and 0.1 mM EDTA, pH 7.4). 200 μl homogenate were added to 800 μl of reaction medium (50 mM sucrose, 150 mM Tris-HCl, 20 mM KH_2PO_4, 10 mM $MgCl_2$-$6H_2O$, 2 mM EDTA, 1 mM L-carnitine, 1 mM Palmitate complexed to fatty acid-free albumin at a molar ratio of 5:1, 0.3 μCi of [9,10-^{3}H(N)]-palmitate, pH 7.4). After incubation for 30 min at 37°C, the reactions were terminated by addition of 0.5 ml of 3 M perchloric acid. After incubation for 1 h at room temperature, the precipitated protein was removed by centrifugation at 13,200 rpm for 15 min at 4°C, and 100 μl supernatant was mixed with 0.8 ml of scintillation liquid for radioactivity measurement using the liquid scintillation counter (PerkinElmer).

To determine β-oxidation activities in hepatocytes, primary hepatocytes were preincubated with 125 μM palmitic acid/BSA for overnight in the presence or absence of 30 μM WY14643 (Tocris Bioscience, MN, USA) or 50 μM fenofibrate (MCE, Monmouth Junction, NJ, USA) in low glucose medium, followed by incubation with 125 μM palmitic acid/BSA and 1 mM carnitine in glucose free DMEM medium for an aditional 4 h. Cells were then incubated for 1 h at 37°C in 0.25 ml of β-oxidation medium (119 mM NaCl, 5mM KCl, 2.6 mM KH_2PO_4, 2.6 mM $MgSO_4$, 2 mM $CaCl_2$, 24.6 mM $NaHCO_3$, 10 mM HEPES, 8 mM palmitate bound to fatty acid-free albumin at a molar ratio of 5:1 and 0.1 μCi [9,10-^{3}H(N)]-palmitate, pH 7.4), and 240 μl supernatant was subsequently mixed with 96 μl of 1.3 M perchloric acid. Precipitated protein was removed by centrifugation at 13,200 rpm for 15 min at 4°C, and 300 μl supernatant was mixed with 0.8 ml of scintillation liquid for radioactivity measurement.

Cycloheximide (CHX) pulse-chase assay

Cells were seeded in 12-well plates for culturing overnight. HEK293T stably transfected cells expressing control shRNA or PAQR3-specific shRNA were transiently transfected with the indicated PPARα expression plasmids. Twenty-four hours after the transfection, the cells were incubated with 100 μg/ml cyclohexamide (CHX) dissolved in DMSO for the indicated time points. The total protein lysate were harvested and subjected to immunoblotting. For the HepG2 cells, endogenous PPARα was detected by treating with 100 μg/ml of cycloheximide (CHX) for different length of time and then harvested for immunoblotting with the antibodies as indicated.

GST pulldown assay

Briefly, the bacterial expression plasmids coding NH2-terminal 1-71 amino acids of PAQR3 and 101-173 amino acids domain of PPARα were transformed into BL21 codon plus (Stratagene). To induce the protein expression, the *E. coli* was induced overnight with 0.5 mM IPTG when the optical density reached 0.6-0.8 at 600 nm (OD_{600}) at 18°C. Cells were resuspended in PBS containing 0.5% TritonX-100, 2 mM EDTA, 5 mM β-mercaptoethanol, 1 mM PMSF, and protease inhibitor cocktail followed by ultrasonication. Glutathione–Sepharose 4B beads (GE Healthcare) and Ni-NTA (QIAGENE) beads were added into the extracts and the protein-loaded beads were eluted by 20 mM reduced glutataione and 250 mM imidazole respectively after incubation at 4°C for 2 h. Then, the bacterial purified proteins were mixed equally with 50 μl Glutathione–Sepharose 4B beads followed by incubation at 4°C for an additional 2 h. Finally, the centrifugal beads were eluted by 20 mM reduced glutataione, and the elution was subjected to SDS–PAGE and stained using

Commassie Blue Staining Solution.

BODIPY staining

Lipid droplets were stained by incubating cells with BODIPY (Invitrogen, Frederick, MD, USA) for 30 min, fixed and processed for immunofluorescence as described previously (4).

Cellular oxygen consumption

Cellular oxygen consumption was measured using the Seahorse XF24 analyzer (Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's protocol.

References

1. Qin XF, An DS, Chen IS, Baltimore D. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc Natl Acad Sci U S A 2003;100:183-188.

2. Wang L, Wang X, Li Z, Xia T, Zhu L, Liu B, Zhang Y, et al. PAQR3 has modulatory roles in obesity, energy metabolism, and leptin signaling. Endocrinology 2013;154:4525-4535.

3. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497-509.

4. Xu D, Wang Z, Zhang Y, Jiang W, Pan Y, Song BL, Chen Y. PAQR3 modulates cholesterol homeostasis by anchoring Scap/SREBP complex to the Golgi apparatus. Nat Commun 2015;6:8100.

(2) Supplemental Figures

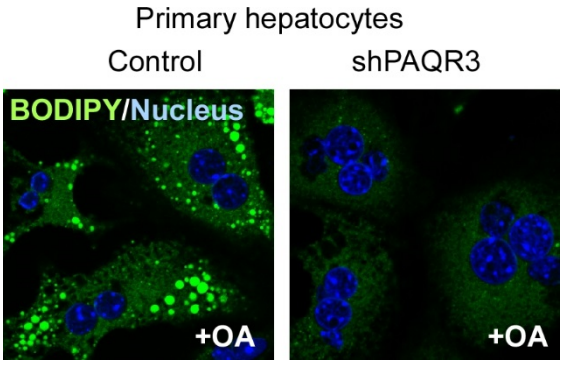

Figure S1 *Paqr3* deficiency decreases lipid accumulation in primary hepatocytes

Representative BODIPY staining of primary hepatocytes isolated from wild-type mice infected with adenoviruses containing control shRNA or PAQR3-specific shRNA and then incubated in the presence of oleic acid (OA, 200 μM) for 18 h. Co-localization of BODIPY (green) with nuclei (blue) in hepatocytes. Nuclei are highlighted with Hoechst 33342 (Molecular Probes).

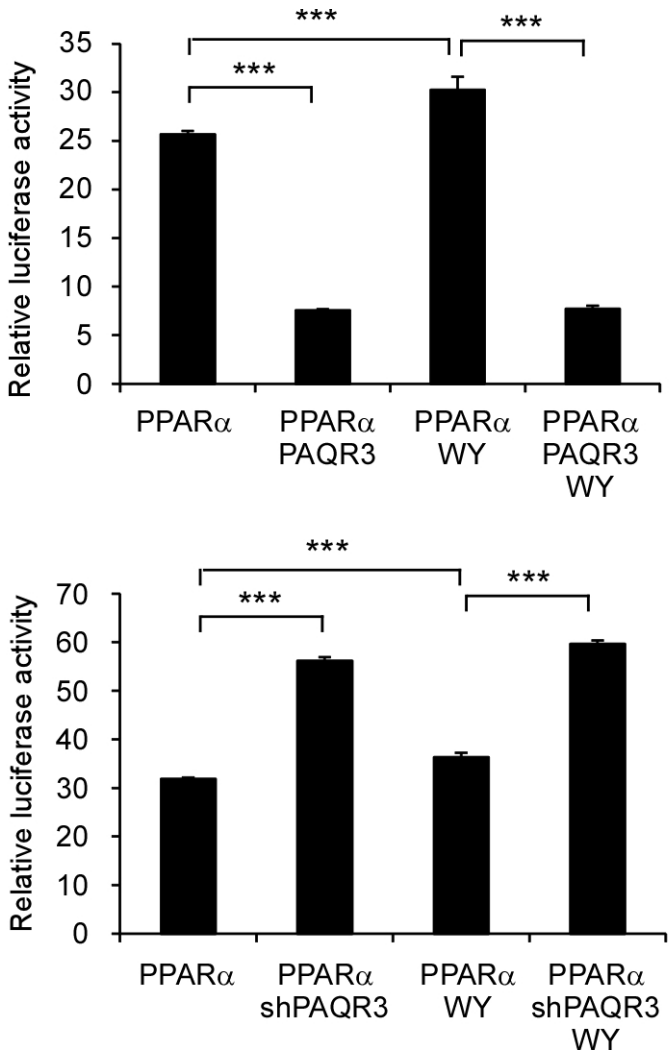

Figure S2 PAQR3 regulates ligand-dependent PPARα transactivation in HEK293T cells

HEK293T cells that stably expressed ectopic PAQR3 (upper panel) or PAQR3-shRNA (lower panel) as well as their individual controls by lentivirus were transiently transfected with PPAR-responsive luciferase reporter together with PPARα expression plasmid, followed by treatment with WY14643 (WY) for 24 h as indicated. Luciferase activity was measured with these cells. The data are presented as mean ± SD, *** for $p < 0.001$.

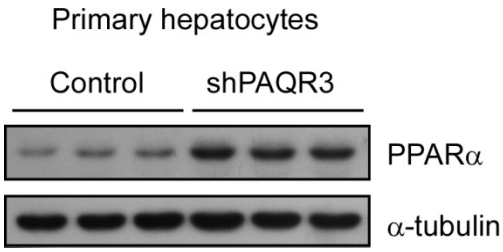

Figure S3 *Paqr3* knockdown elevates basal protein level of PPARα

Primary hepatocytes from male C57BL/6 mice were injected with adenovirus expressing control shRNA or the PAQR3-specific shRNA followed by immunoblotting with the antibodies as indicated.

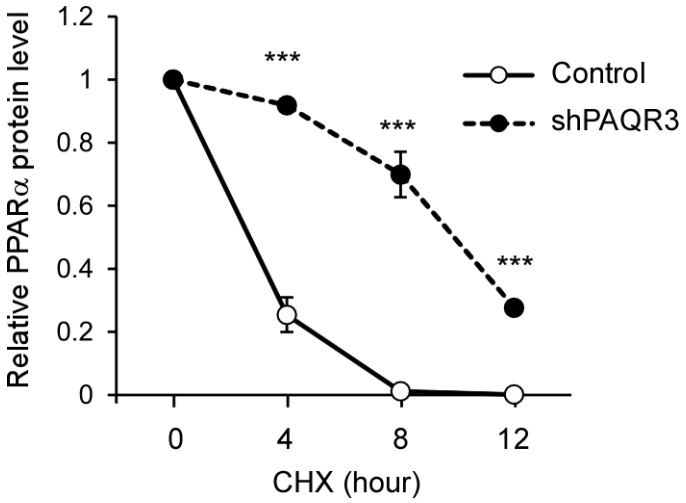

Figure S4 Degradation of PPARα protein is delayed by PAQR3 knockdown

Three independent experiments as Figure 4E were analyzed by ImageJ software to obtain the quantitative results of protein degradation rate. The data are presented as mean ± SD, *** for $p < 0.001$.

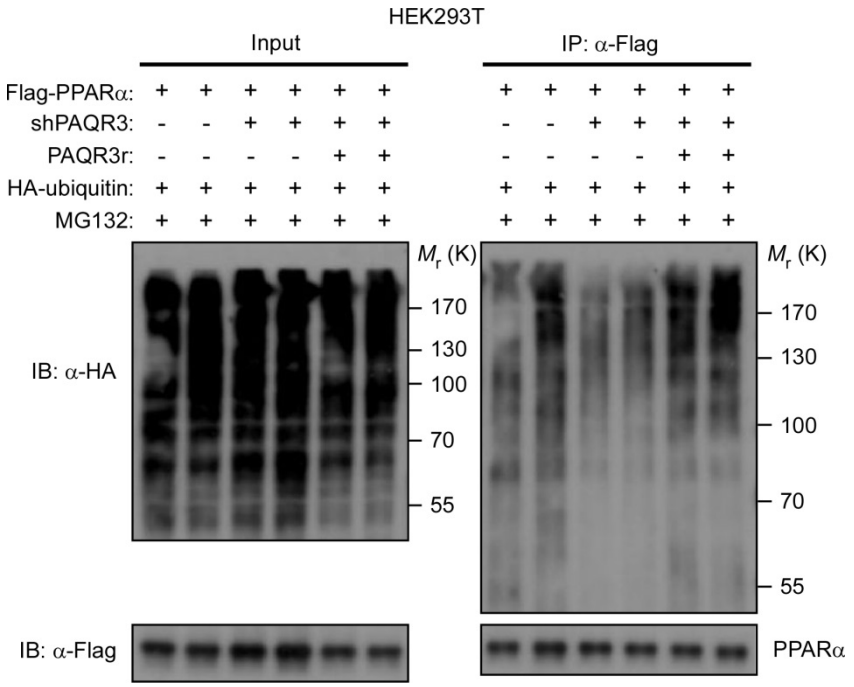

Figure S5 PAQR3 regulates polyubiquitination of PPARα in HEK293T cells

HEK293T cells stably expressing control or PAQR3-specific shRNA were transiently transfected with the plasmids as indicated. PAQR3r standing for shRNA-resistant PAQR3 expression plasmid. At 72 h after the transfection, the cells were treated with MG132 for 6 h. The cell lysate was used in immunoprecipitation (IP) and immunoblotting (IB) with the antibodies as indicated.

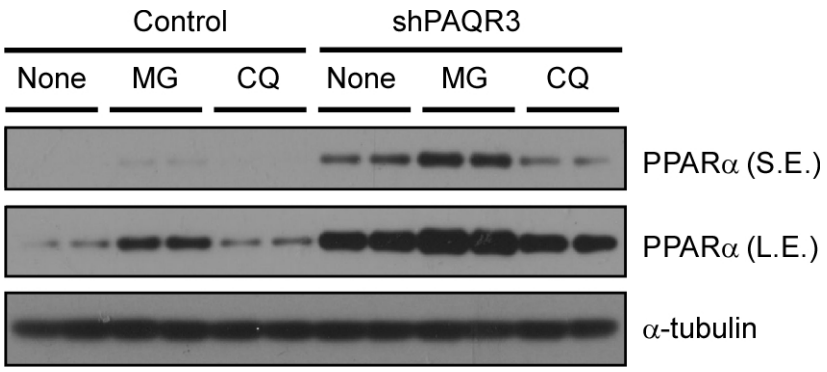

Figure S6 PPARα protein is mainly degraded by proteasome pathway

HEK293T stably transfected cells expressing control or PAQR3-specific shRNA were transiently transfected with PPARα expression plasmids. At 24 h after the transfection, cells were incubated with 20 μM chloroquine (CQ), a lysosomal inhibitor, for 4 h or 10 μM MG132 (MG), a proteasome inhibitor, for 6 h. The cells were used in immunoblotting with the antibodies as indicated. S.E. and L.E. for short or long exposure respectively.

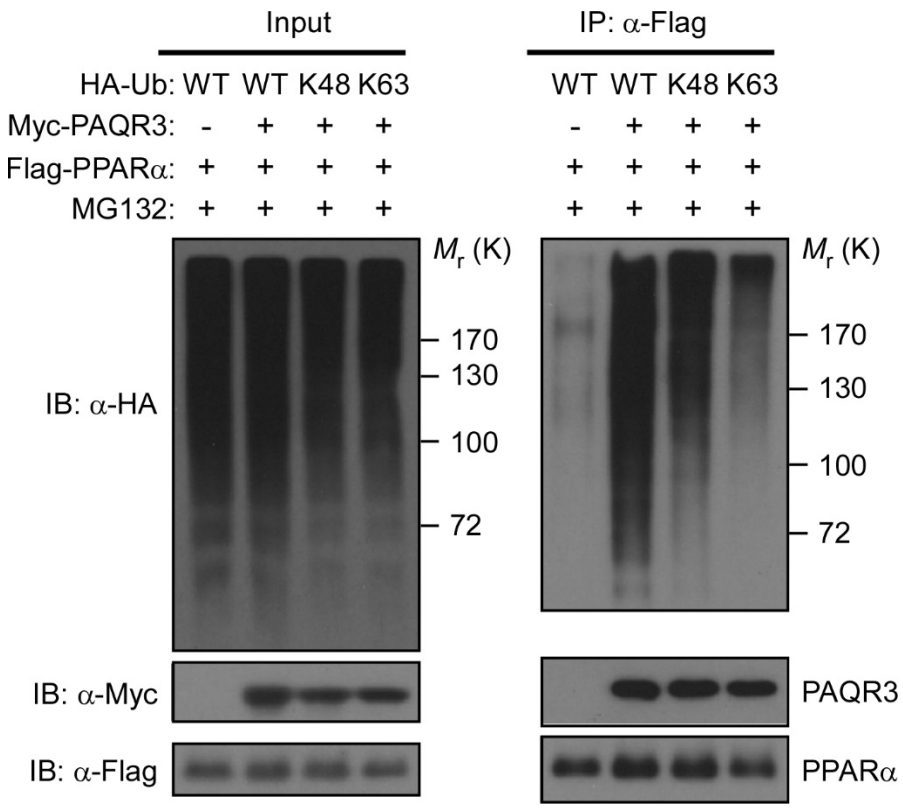

Figure S7 PPARα is mainly linked to lysine 48 of ubiquitin chain

HEK293T cells were transfected with plasmids encoding Myc-tagged PAQR3 and Flag-tagged PPARα, together with HA–tagged wild type ubiquitin, K48 ubiquitin (with the lysine residues of ubiquitin other than lycine-48 being mutated to arginine), or K63 ubiquitin (with the lysine residues of ubiquitin other than lycine-63 being mutated to arginine) as indicated. Twenty-four hours after transfection, the cells were treated with 10 μM MG132 for 6 additional hours before immunoprecipitation (IP) and immunoblotting (IB) with the antibodies as indicated.

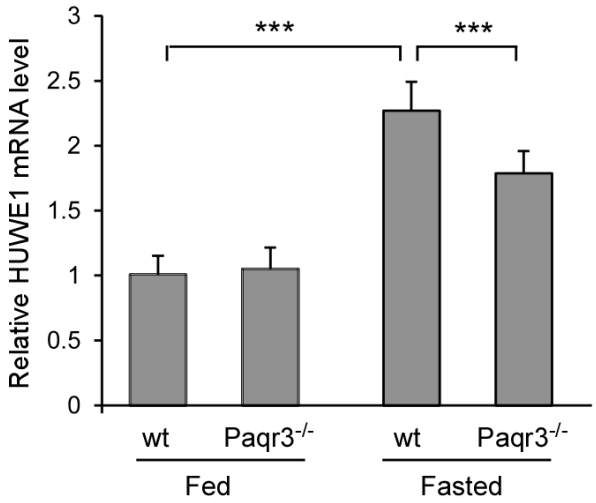

Figure S8 HUWE1 expression level in the mouse liver

Male mice with liver-specific deletion of PAQR3 and the age-matched flox/flox littermates (8-10 weeks old) were fed ad libitum or fasted for 18 h (n = 8 per group). Quantitative RT-PCR was used to measure the mRNA level of HUWE1. The data are presented as mean ± SD, *** for $p < 0.001$.

Table S1. Primers used in this study

	Forward primer	Reverse primer
mus-Paqr3	CTACCGGGCCTACCTTCCTT	TGACTCCAGATGTTTACCGTCTCA
mus-Acox1	TAACTTCCTCACTCGAAGCCA	AGTTCCATGACCCATCTCTGTC
mus-Fgf21	CTGCTGGGGGTCTACCAAG	CTGCGCCTACCACTGTTCC
mus-Ehhadh	ATGGCTGAGTATCTGAGGCTG	GGTCCAAACTAGCTTTCTGGAG
mus-Cd36	ATGGGCTGTGATCGGAACTG	GTCTTCCCAATAAGCATGTCTCC
mus-Hmgcs2	GAAGAGAGCGATGCAGGAAAC	GTCCACATATTGGGCTGGAAA
mus-Cpt1α	CTCCGCCTGAGCCATGAAG	CACCAGTGATGATGCCATTCT
mus-Mcad	CAAGTTTGCCAGAGAGGAGATTATC	AACGGGTACTCCCCGCTTT
mus-Scd1	GCGATACACTCTGGTGCTCA	CCCAGGGAAACCAGGATATT
mus-Acadl	TCTTTTCCTCGGAGCATGACA	GACCTCTCTACTCACTTCTCCAG
mus-Adipoq	TGTTCCTCTTAATCCTGCCCA	CCAACCTGCACAAGTTCCCTT
mus-Acaa1b	ATGCTTCCATGCTGAGATTGT	TCCATCCTTGAAGGCAGGCTT
mus-Acaa2	GATCTCAAGCTGGAAGATAC	ACCTCTGCTGAGACTGCAAG
mus-Ech1	AAGATAAGGACGCCATGCTGAA	TCCAGGTGGCCATGTAGTCA
mus-Hadha	TGCTCCTCGACCACGCTAAC	GCCTTGGTCTTTTTCCTGCTT
mus-Fas	AGGTGGTGATAGCCGGTATGT	TGGGTAATCCATAGAGCCCAG
mus-Acc1	TGACAGACTGATCGCAGAGAAAG	TGGAGAGCCCCACACACA
mus-Srebp1c	GGAGCCATGGATTGCACATT	GGCCCGGGAAGTCACTGT
mus-Me	GCCGGCTCTATCCTCCTTTG	TTTGTATGCATCTTGCACAATCTTT

mus-Gpat	AGCAAGTCCTGCGCTATCAT	CTCGTGTGGGTGATTGTGAC
mus-Pparγ	CTGGCCTCCCTGATGAATAA	CGCAGGTTTTTGAGGAACTC
mus-Chrebp	CCTCACTTCACTGTGCCTCA	ACAGGGGTTGTTGTCTCTGG
mus-Srebp2	CCGCTCTCGAATCCTCTTAT	CAGCACCTGACTCCAGTGAC
mus-Fabp	ATGAACTTCTCCGGCAAGTACC	GGTCCTCGGGCAGACCTAT
mus-ApoA1	GGCACGTATGGCAGCAAGAT	CCAAGGAGGAGGATTCAAACTG
mus-ApoB	CGTGGGCTCCAGCATTCTA	TCACCAGTCATTTCTGCCTTTG
mus-ApoE	GCTGGGTGCAGACGCTTT	TGCCGTCAGTTCTTGTGTGACT
mus-Huwe1	GAGGGCGTAAACATACAGAGAAG	CGCTGCTGTGTAAAGTGGC
mus-Actin	GATCATTGCTCCTCCTGAGC	ACTCCTGCTTGCTGAT CCAC
homo-Paqr3	CAATGGAGGAATTGGTGCTC	ATTTGGTGGCTTGATCCGAG
homo-Pparα	TTTGGCTCACTGTTTTCGTG	ACCACGGGTGCTAGCTTATG
homo-Cpt1α	TCCAGTTGGCTTATCGTGGTG	TCCAGAGTCCGATTGATTTTTGC
homo-Mcad	ACAGGGGTTCAGACTGCTATT	TCCTCCGTTGGTTATCCACAT
homo-Acox1	ACTCGCAGCCAGCGTTATG	AGGGTCAGCGATGCCAAAC
homo-Ehhadh	GGAGCATCGTGGAAAACAGC	ATGCCTCGGCCCATTGTTC
homo-Acaa1b	ATGTGGCTGAGCGGTTTGG	GGCGGATACCCTCATCCTG
homo-Ech1	ATAGTGGCTTCTCGCAGACTC	CAGTGAGGCGAAGGCTAATAC
homo-Fgf21	CTGTGGGTTTCTGTGCTGG	CCGGCTTCAAGGCTTTCAG
homo-Hadha	CTGCCCAAAATGGTGGGTGT	GGAGGTTTTAGTCCTGGTCCC

homo-Acaa2	AAGTCTCACCTGAAACAGTTGAC	CACGCAAACCAACATGCCT
homo-Actin	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA