Supporting Information

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SI Materials and Methods

Materials. Phospho-S6 (ser235/236) and S6 rabbit monoclonal antibodies, and rabbit anti-tuberous sclerosis complex 1 (TSC1) antibody were purchased from Cell Signaling Technology. Mouse anti-peroxisome proliferator-activated receptor-γ (PPARγ) monoclonal antibody, rabbit anti-GHSR1a, mouse anti-GAPDH and mouse anti–β-actin antibodies were purchased from Santa Cruz Biotechnology. Mouse anti-Sterol Regulatory Element-Binding Protein 1 (SREBP1) monoclonal antibody and rabbit anti-albumin were purchased from Abcam. Rabbit anti-raptor polyclonal antibody was from Proteintech. Ghrelin, rhodamin-ghrelin peptides, and D-Lys3-GHRP6 were purchased from Phoenix Pharmaceuticals. Rapamycin, leucine, GW9662, collagenase IV, and oleic acid (OA) were from Sigma Aldrich. Lipofectamine 2000 was purchased from Invitrogen. Alzet microosmotic pumps (1002) were from DURECT Corporation. Aprotinin was purchased from Amersham Biosciences. Triglyceride Colorimetric Assay Kits were from Cayman Chemical Company. IRDye-conjugated affinity purified anti-rabbit and anti-mouse IgGs were purchased from Rockland. TRIzol reagent and the reverse transcription (RT) system were purchased from Invitrogen.

Isolation and culture of hepatocytes. C57BL/6J mice were anesthetized with 1% Nembutal (7 μL/g body weight) and injected i.p. with 1,000 IU heparin. After laparotomy, the portal vein was cannulated. The liver was perfused with 20 mL of prewarmed 37 °C Hanks buffer, followed by 20 mL of 0.02% collagenase (Sigma-Aldrich) at a flow rate of 2 mL/min. After perfusion, liver tissues were removed and washed with 20 mL of Hanks buffer. The capsule of the liver was removed, and hepatic tissues dispersed and incubated in 20 mL of 0.01% collagenase in a shaking water bath at 37 °C for 15 min. Cell suspension was then filtered through two layers of 60- to 80-μm nylon mesh, centrifuged at 500 rpm [Sorvall RT7 Benchtop Centrifuge with RTH 250 rotor (Ramsey, MN)] and washed twice with DMEM to remove tissue dissociation enzymes, damaged cells, and nonparenchymal cells. Dispersed hepatocytes were counted and seeded at a concentration of 5–7 × 10^5 cells per 100-mm dish containing 10 mL of high glucose DMEM supplemented with 10% (vol/vol) FBS. Cells were cultured at 37 °C in a humidified atmosphere of 5% (vol/vol) CO_2. Culture medium was changed daily.

Metabolic profiles. Oxygen consumption (VO2), carbon dioxide production (VCO2), spontaneous motor activity, and food intake were measured by using the Comprehensive Laboratory Monitoring System (CLAMS; Columbus Instruments), an integrated open-circuit calorimeter equipped with an optical beam activity monitoring device. The study was carried out in an experimentation room set at 20–23 °C with 12 h-12 h (0600~1800) dark-light cycles. The measurements were carried out continuously for 48 h. VO2 and VCO2 in each chamber were sampled sequentially for 5 s in a 10-min interval. Respiratory quotient (RQ), also known as respiratory exchange ratio (RER), was calculated as VCO2/VO2.

Oil red staining. Hepatic tissue slices or cells were washed by 1× PBS three times, then fixed with 4% paraformaldehyde for 10 min. After washing, slices or cells were incubated in 0.3% oil-red staining solution for 1 h at room temperature. Samples were then counterstained with hematoxylin for 30 s, followed by wash in running water for more than 30 min. All slides were mounted with 90% glycerol and stored at 4 °C before observation.

Measurements of plasma and hepatic triglyceride. Blood samples from C57BL/6J mice were transcardially collected after anesthesia and immediately transferred to chilled polypropyrene tubes containing EDTA-2Na (12.5 mg/mL) and aprotinin (1,000 units/mL) and centrifuged at 4 °C. The plasma was separated and stored at −70 °C before use. Plasma triglyceride was measured according to the manufacturer's instructions. Liver tissues were homogenized according to manufacturer's instructions, and the supernatant was used for triglyceride detection. Values were normalized to protein concentrations by using the Pierce BCA protein quantitative assay kit (Thermo-Fisher Scientific).

Gene expression analysis. For gene expression analyses, RNA was isolated from mouse liver or hepatic primary cells by using TRIzol (Invitrogen) and reverse-transcribed into cDNAs using the First-Strand Synthesis System for RT-PCR kit (Invitrogen). SYBR Green-based quantitative RT-PCR was performed by using the Mx3000 multiplex quantitative PCR system (Stratagene). Triplicate samples were collected for each experimental condition to determine relative expression levels. Sequences for the primer pairs used in this study are listed in Table S1.

Western blot analysis. Liver tissue and primary hepatocytes isolated from C57BL/6J mice were homogenized in lysis buffer. Proteins were subjected to SDS/PAGE separation with a 10% running gel, then transferred to a polyvinylidene fluoride membrane. Membranes were incubated for 1 h at room temperature with 5% fat-free milk in Tris-buffered saline containing Tween 20, followed by incubation overnight at 4 °C with primary antibodies. Specific reaction was detected by using IRDye-conjugated second antibody and visualized using the Odyssey infrared imaging system (LI-COR Biosciences).

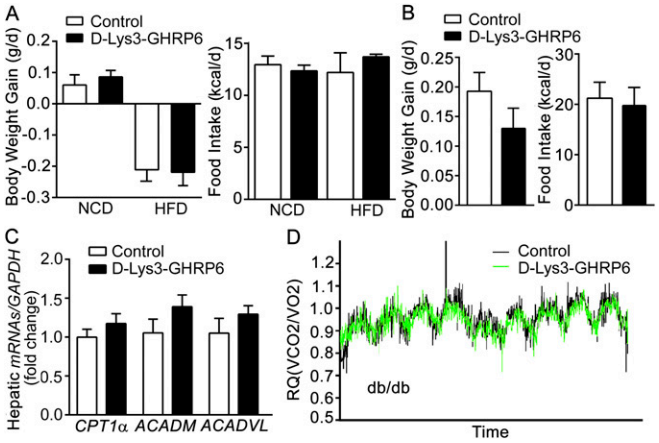

Fig. S1. No alteration on body weight gain, food intake, and β-oxidation in response to D-Lys3-GHRP6. Six-week-old C57BL/6J mice were divided into two groups and fed either normal chow diet (NCD) or 45% high fat diet (HFD) for 12 wk (*A*). Twelve-week-old db/db mice were purchased (*B*). D-Lys3-GHRP6 was administrated by i.p. injection daily at a dose of 10 μmol/kg for 1 wk. Body weight gain and food intake were recorded daily. Levels of β-oxidation–related gene transcription in db/db mice were examined by real-time RT-PCR (*C*). *Gapdh* was used as internal control. Respiratory quotient profiles of db/db mice with D-Lys3-GHRP6 treatment (*D*). Results were expressed as mean ± SEM, $n = 6$.

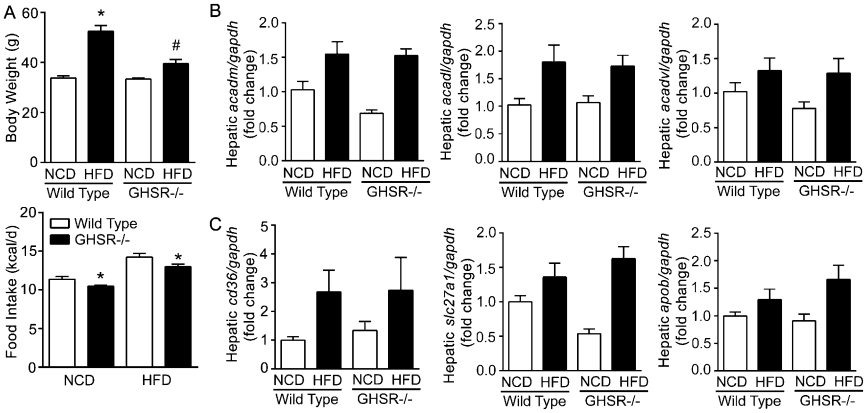

Fig. S2. Effects of GHSR1a gene deletion on body weight, food intake, expression of hepatic genes related to lipid β-oxidation, and transport. GHSR1a$^{-/-}$ mice and wild-type littermates were fed either NCD or 45% HFD for 16 wk. *A*, *Upper* shows body weight before sacrifice. *$P < 0.05$ vs. NCD control; #$P < 0.05$ vs. HFD control. Daily food intake is shown in *A*, *Lower*. *$P < 0.05$ vs. wild-type control. Alterations in mRNA levels of hepatic lipid β-oxidation (*B*) and transport-related genes (*C*) were analyzed by real-time RT-PCR. *gapdh* was used as an internal control. Results were expressed as mean ± SEM. At least six mice were included in each group.

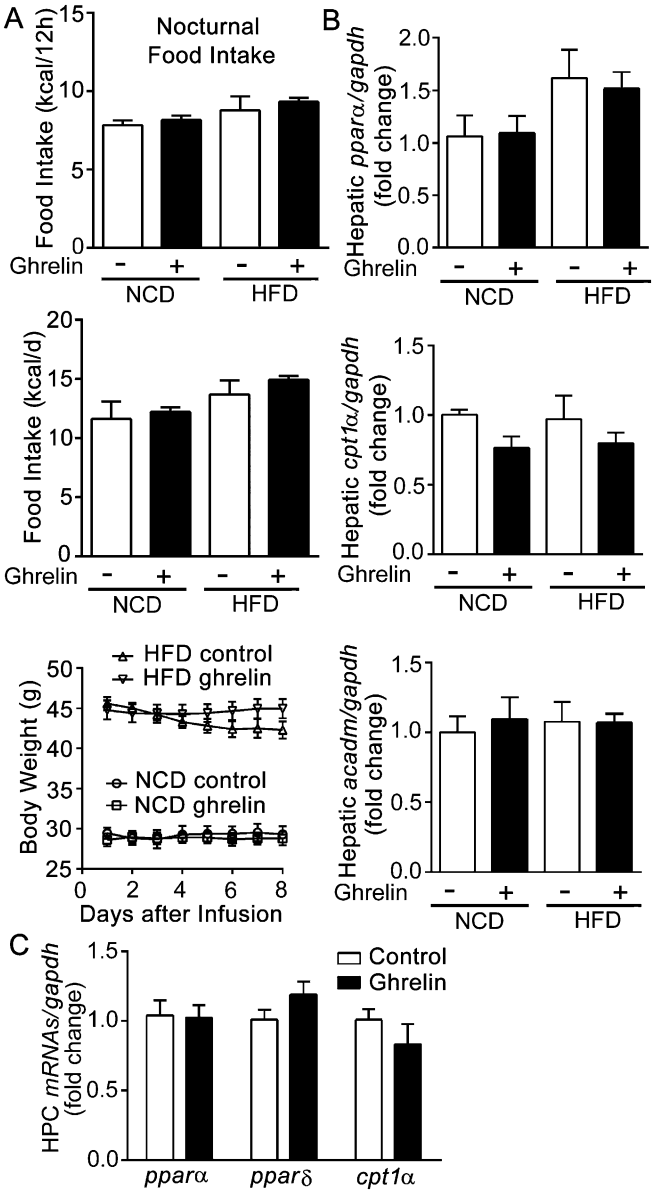

Fig. S3. Effects of exogenous ghrelin on food intake, body weight, and expression of hepatic lipid β-oxidation–related genes. Six-week-old C57BL/6J mice were divided into two groups and fed either NCD or 45% HFD for 12 wk. Acyl-ghrelin was continuously infused at a dose of 11 nmol/kg per day by a s.c. osmotic minipump for 2 wk. Food intake was recorded twice daily (*A, Top* and *Middle*) and body weight measured daily (*A*, *Bottom*). Hepatic mRNA levels of lipid β-oxidation–related transcriptional factors *pparα* and enzymes *cpt1α* and *acadm* were analyzed by RT-PCR (*B*). Results were expressed as mean ± SEM. At least five mice were included in each group. Hepatic primary cells (HPCs) were isolated from C57BL/6J mice and treated with ghrelin (10^{-8} M) or vehicle for 3 h. *pparα, pparδ,* and *cpt1α* were analyzed by RT-PCR (*C*). Results were expressed as mean ± SEM. Each experiment was repeated at least three times.

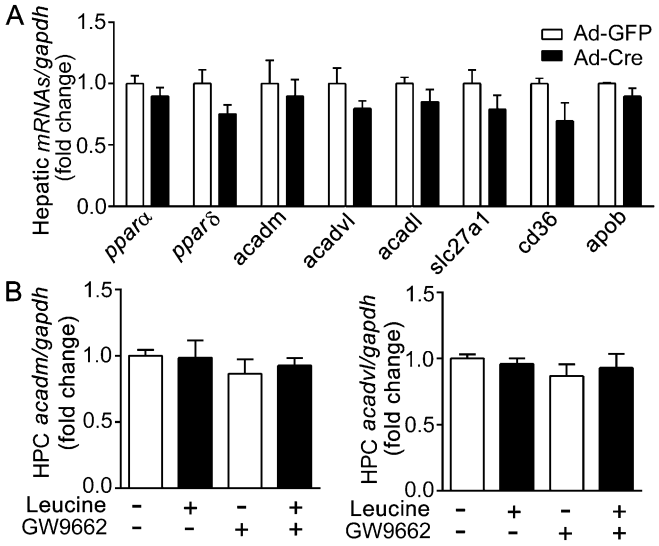

Fig. S4. No effect of mTOR and PPARγ on hepatic genes related to lipid β-oxidation and transport. Hepatic primary cells (HPCs) were isolated from $TSC1^{loxp/loxp}$ mice and infected with Ad-CMV-Cre (Ad-Cre) or Ad-CMV-GFP (Ad-GFP) adenovirus (10^6 pfu) for 36 h. Hepatocyte mRNA was analyzed for expression of genes related to hepatic lipid β-oxidation and transport (*A*). *Gapdh* was used as internal control. Results were expressed as mean ± SEM. Each experiment was repeated at least three times. Cultured hepatic primary cells (HPCs) isolated from C57BL/6J mice were treated with leucine (4 mM) or vehicle with or without GW9662 (20 μM) for 12 h. Hepatocyte mRNA levels of lipid β-oxidation–related enzymes *acadm* and *acadvl* were analyzed by RT-PCR (*B*). Results were expressed as mean ± SEM. Each experiment was repeated at least three times.

Table S1. Primers for quantitative RT-PCR

Mouse	NM	Forward (5′ to 3′)	Reverse (5′ to 3′)
gapdh (GAPDH)	NM_008084.2	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG
pparg (PPARγ)	NM_001127330.1	TCAGCTCTGTGGACCTCTCC	ACCCTTGCATCCTTCACAAG
pparg2 (PPARγ)	NM_011146.3	CTCTGGGAGATTCTCCTGTTGA	GGTGGGCCAGAATGGCATCT
srebf1 (SREBP1)	NM_011480.3	GGAGCCATGGATTGCACATT	GGAAGTCACTGTCTTGGTTGTTGA
acaca (ACC)	NM_133360.2	TGGTCGTGACTGCTCTGTGC	GTAGCCGAGGGTTCAGTTCC
fasn (FAS)	NM_007988.3	TGGGTTCTAGCCAGCAGAGT	ACCACCAGAGACCGTTATGC
gpam (GPAT)	NM_008149.3	CACACGAGCAGGAAAGATGA	GGACTGCATAGATGCTGCAA
dgat1 (DGAT1)	NM_010046.2	TTCCGCCTCTGGGCATT	AGAATCGGCCCACAATCCA
dgat2 (DGAT2)	NM_026384.3	CGTGACGTGCATTGGCTTC	TGGAGGGCTGAGAGGATGC
raptor (RAPTOR)	NM_028898.2	TGCTGCAGTCGCCTCTTATG	CCACACTCACCGTCTTCATC
ghsr1a (GHSR1a)	NM_177330.4	CTATCCAGCATGGCCTTCTC	AAGACGCTCGACACCCATAC
ppara (PPARα)	NM_011144.6	GAGAAGTTGCAGGAGGGGATTGTG	AAGACTACCTGCTACCGAAATGGG
ppard (PPARδ)	NM_011145.3	GTGTGGAAGCAGTTGGTGAA	TGCACGCCATACTTGAGAAG
cpt1a (CPT1α)	NM_013495.2	ATCGTGGTGGTGGGTGTGATAT	ACGCCACTCACGATGTTCTTC
acadm (MCADH)	NM_007382.5	TTACCGAAGAGTTGGCGTATGG	TGCGGAGGGCTCTGTCAC
acadl (LCADH)	NM_007381.4	CTCCCTGCGCGTCCTGAG	AAAATGTCATGCTCCGAGGAAAAG
acadvl (VLCADH)	NM_017366.3	GCCCAGACACACAACCTTTG	CCGAGCCGACTGCATCTC
cd36 (CD36)	NM_001159555.1	TGGTCAAGCCAGCTAGAAA	CCCAGTCTCATTTAGCCAC
slc27a1 (FATP1)	NM_011977.3	CCGTATCCTCACGCATGTGT	CTCCATCGTGTCCTCATTGAC
apob (apoB)	NM_009693.2	TCACCATTTGCCCTCAACCTAA	GAAGGCTCTTTGGAAGTGTAAAC