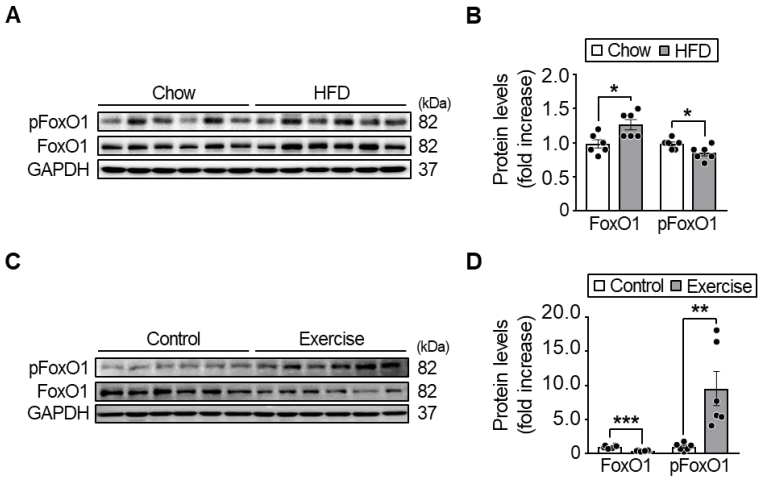
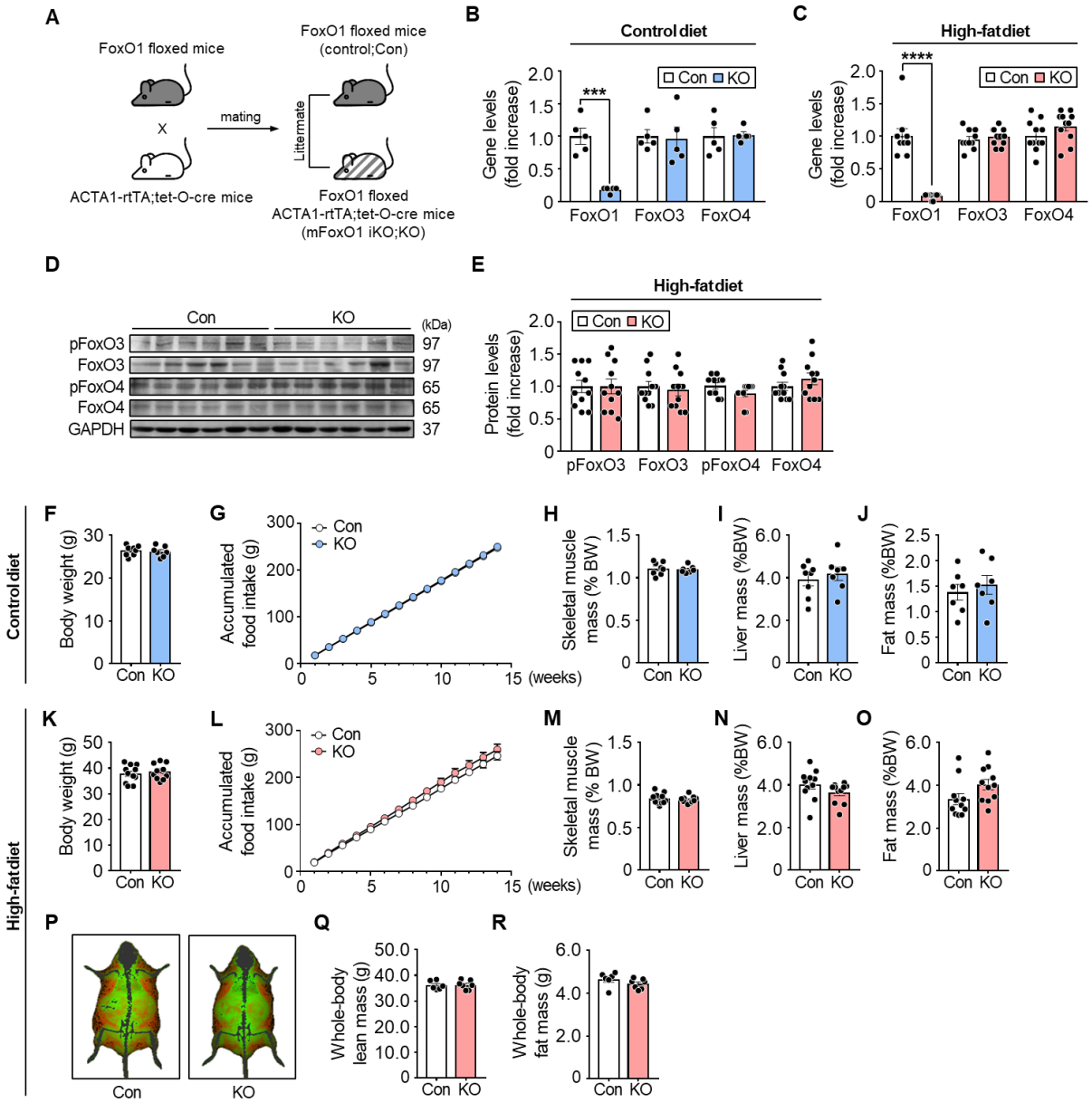
Supplementary Figures and Legends

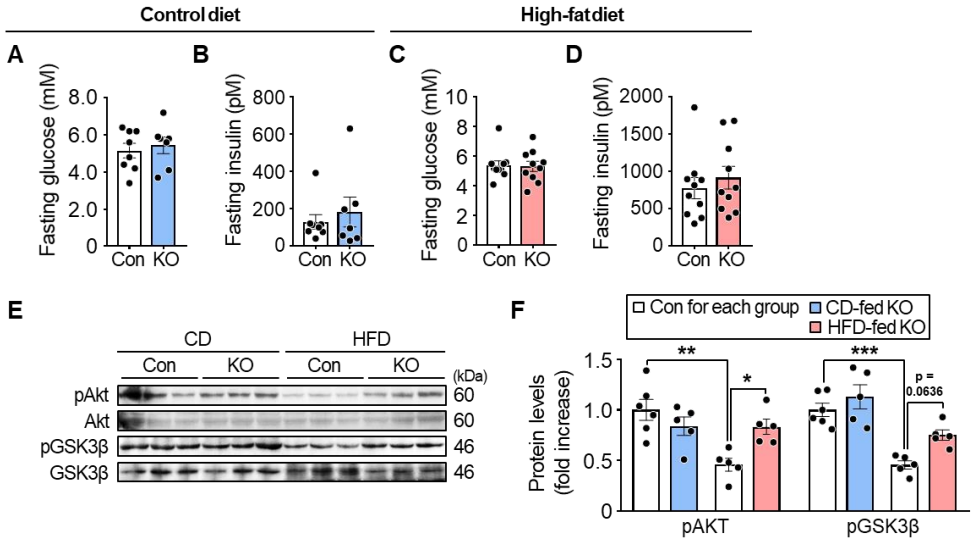


Supplementary Figure 1. Protein expression and phosphorylation of FoxO1. The results of A–D were measured in HFD-fed male mice. (A and B) Protein expression and phosphorylation of FoxO1 in the skeletal muscle. n = 6 samples per group. (C and D) Protein expression and phosphorylation of FoxO1 following 3 weeks of exercise training in the skeletal muscle. n = 6 samples per group. GAPDH levels were used as a loading control and all phosphorylated forms were normalized to their respective protein forms. Data are displayed as the means ± SE. Statistical analyses with two groups were performed using two-tailed Student's t-test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. HFD, high-fat diet; pFoxO1, phosphorylated forkhead box protein O1.

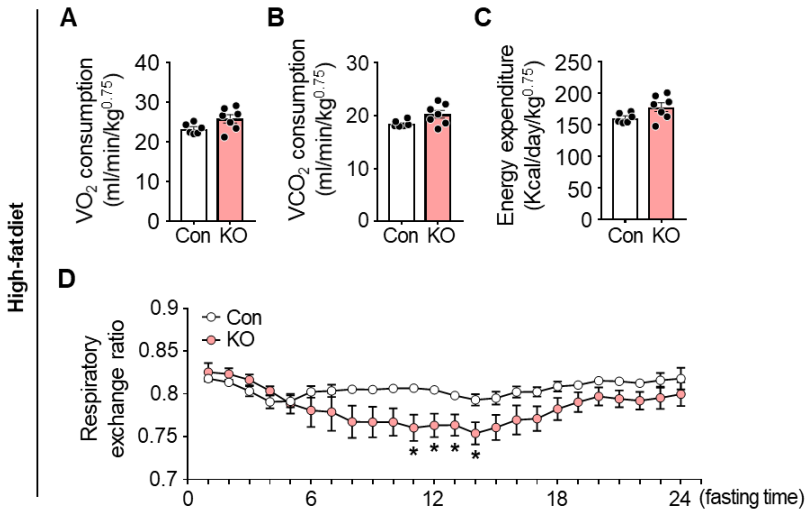


Supplementary Figure 2. Characteristics of mFoxO1 iKO mice. (A) A schematic design of production of mFoxO1 iKO mouse model. (B-R) The experiments were performed in mFoxO1 iKO male mice. (B) mRNA level of FoxO isoforms in the quadriceps muscle of CD-fed mice. n = 5 samples per group. (C) mRNA levels of FoxO isoforms in the quadriceps muscle of HFD-fed mice. n = 10-11 samples per group. (D and E) The expression of total protein and phosphorylated forms of FoxO3/4 in HFD-fed mice. n = 11 samples per group. GAPDH levels were used as a loading control and all phosphorylated forms were normalized to their respective

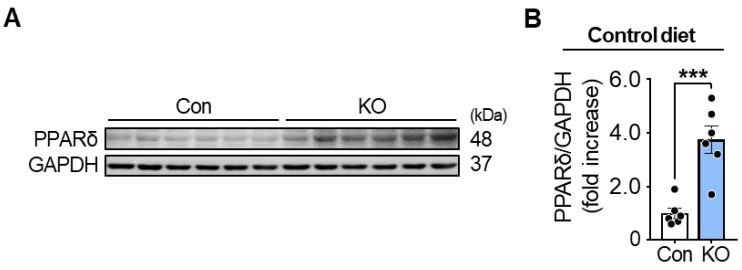
total protein. (F and G) Body weight and accumulated food intake in CD-fed mice. n = 7-8 (F) or 3 (G) samples per group. (H-J) Gastrocnemius, liver, and epididymal fat mass in CD-fed mice. n = 7-8 samples per group. (K and L) Body weight and accumulated food intake in HFD-fed mice. n = 11 (K) or 6-8 (L) samples per group. (M-O) Tissue weight in gastrocnemius, liver, and epididymal fat mass in HFD-fed mice. n = 11 samples per group. (P-R) Whole-body lean mass and fat mass in HFD-fed mice. The results of P-R were measured by dual-energy X-ray absorptiometry. n = 6-7 samples per group. Data are displayed as the means ± SE. Statistical analyses with two groups were performed using two-tailed Student's t-test. ***$p < 0.001$ and ****$p < 0.0001$. BW, body weight. Con, control; CD, control diet; HFD, high-fat diet; FoxO, forkhead box protein O; KO, skeletal muscle-specific inducible FoxO1 knockout.



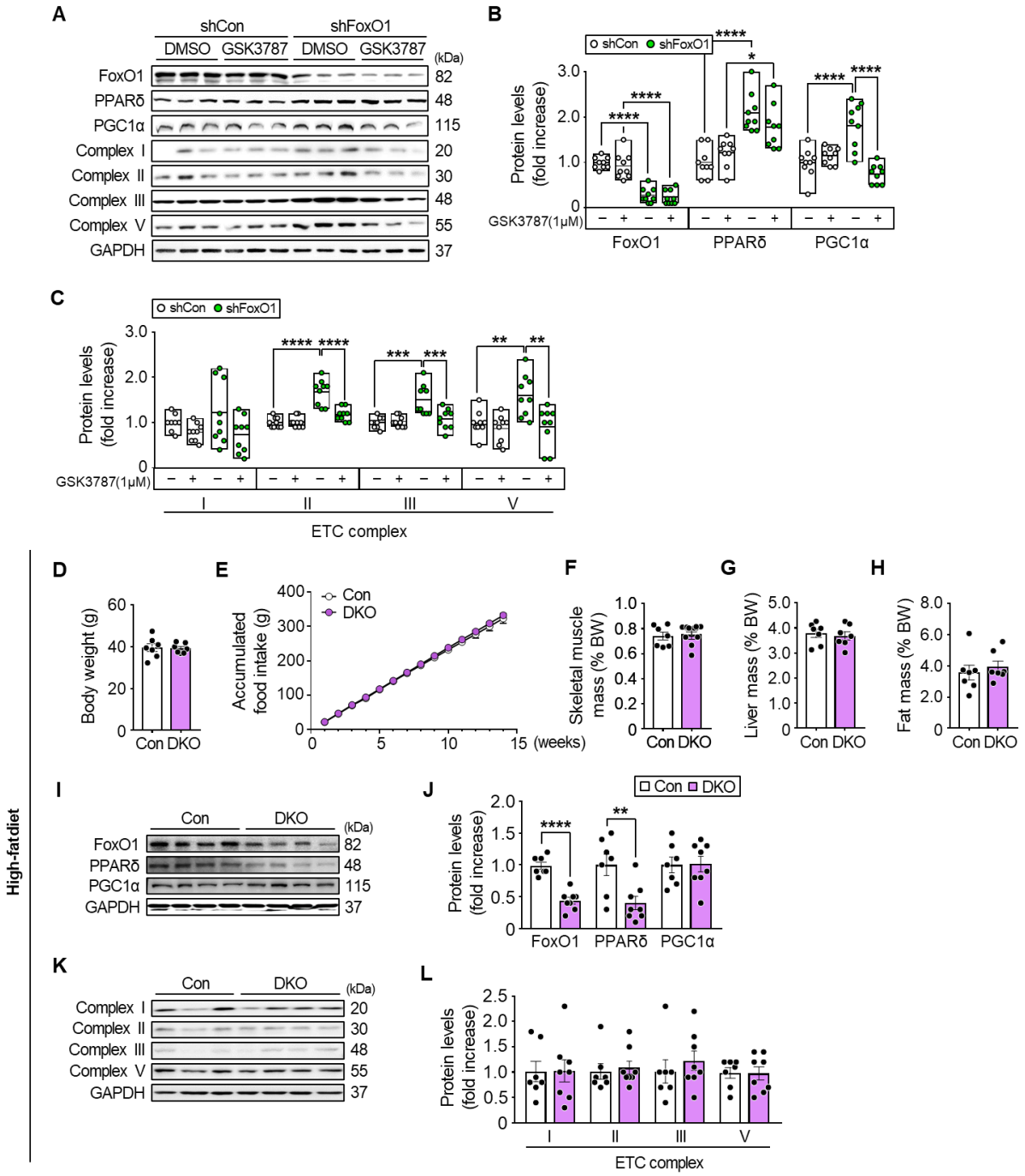
Supplementary Figure 3. Fasting plasma glucose and insulin levels and protein levels of insulin signaling molecules. (A and B) Plasma glucose and insulin levels after overnight fasting in CD-fed male mice. n = 7-8 samples per group. (C and D) Plasma glucose and insulin levels after overnight fasting in HFD-fed male mice. n = 10 samples per group. (E and F) The levels of pAkt and pGSK3β after insulin stimulation in the skeletal muscle of male mice. n = 5-6 samples per group. Data are displayed as the means ± SE. Statistical analyses were performed using a two-tailed Student's t-test with two groups and using two-way ANOVA with four groups. Con, control; KO, skeletal muscle-specific inducible FoxO1 knockout; CD, control diet; HFD, high-fat diet; pAkt, phosphorylated Akt; pGSK3β, phosphorylated glycogen synthase kinase 3β.



Supplementary Figure 4. Measurement of metabolic rate during the 24 h fasting period. (A) VO_2 consumption, (B) VCO_2 consumption, (C) energy expenditure, and (D) respiratory exchange ratio in HFD-fed male mice. Respiratory exchange ratio is expressed as the ratio VCO_2/VO_2. n = 6-7 samples per group. Data are displayed as the means ± SE. Statistical analyses with two groups were performed using two-tailed Student's t-test. *$p < 0.05$. Con, control; KO, skeletal muscle-specific inducible FoxO1 knockout; HFD, high-fat diet.



Supplementary Figure 5. PPARδ protein expression in CD-fed mFoxO1 iKO mice. (A and B) Protein levels of PPARδ in the skeletal muscle of CD-fed male mice. Mice were fed CD for 14 weeks. n = 6 samples per group. GAPDH levels were used as a loading control. Data are displayed as the means ± SE. Statistical analyses with two groups were performed using two-tailed Student's t-test. PPARδ, peroxisome proliferators-activated receptor δ; Con, control; KO, skeletal muscle-specific inducible FoxO1 knockout; FoxO1, forkhead box protein O1.



Supplementary Figure 6. The protein levels of mitochondrial ETC in shFoxO1-transfected C2C12 cells and metabolic characteristics of mFoxO1/PPARδ iDKO mice. (A-C) Protein levels of FoxO1, PPARδ, PGC1α, and ETC in the shCon- or shFoxO1-transfected C2C12 cells treated with DMSO and PPARδ inhibitor (GSK3787). The C2C12 cells were transfected with shCon or shFoxO1 as described in the Materials and Methods section. n = 8-

9 samples per group. The results of D–L were measured in HFD-fed control and mFoxO1/PPARδ iDKO male mice. (D and E) Body weight and accumulated food intake. n = 7 samples per group. (F-H) The weight of gastrocnemius, liver, and epididymal fat mass. n = 7 samples per group. (I-L) The protein levels of FoxO1, PPARδ, PGC1α, and ETC in skeletal muscle. n = 7-8 samples per group. All samples within the group were biologically independent. GAPDH levels were used as a loading control. Data are displayed as the means ± SE. Statistical analyses were performed using a two-tailed Student's t-test with two groups and using two-way ANOVA with four groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. shCon, control shRNA-transfected cell; shFoxO1, FoxO1 shRNA-transfected cell; BW, body weight; Con, Control; DKO, skeletal muscle-specific inducible FoxO1 and PPARδ double-knockout (mFoxO1/PPARδ iDKO); ETC, electron transport chain; FoxO1, forkhead box protein O1; HFD, high-fat diet; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1α; PPARδ, peroxisome proliferators-activated receptor δ.

Supplementary materials and methods

Animals

FoxO1 floxed mice were purchased from the Korean Research Institute of Bioscience and Biotechnology (Daejeon, South Korea). ACTA1-rtTA;tet-O-Cre mice, in which the skeletal muscle-specific *Cre* gene was activated by doxycycline, and PPARδ floxed mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). FoxO1 floxed and ACTA-rtTA;tet-O-Cre mice were mated to generate the mFoxO1 iKO mice. FoxO1 floxed mice were used as controls. FoxO1 floxed, PPARδ floxed, and ACTA-rtTA;tet-O-Cre mice were mated to generate the mFoxO1/PPARδ iDKO mice. FoxO1 floxed and PPARδ floxed mice were used as controls. Backcrossing with the C57BL/6N line was performed at least 5 times in all mouse lines before being used in the experiments. Male mice were fed a 60% HFD (Research Diet, New Brunswick, NJ, USA) and an AIN93G diet (CD, Research Diet) containing 0.625 g/kg doxycycline from 8 weeks of age for approximately 14 to 20 weeks. The body weight and food intake of the mice were measured on the same day of the week, and the measurement order and location of the cages in the ventilated racks were randomly assigned. The mice were housed in a room with a 12 h light/dark cycle and had *ad libitum* access to water. During the experiment, if the animal exhibits a rapid weight loss of 20% or more of the body weight due to a decrease in food intake or other reasons, or if the wound is severe, the experiment of the individual is terminated and euthanasia is performed. After the experiments, the mice were anesthetized with an intraperitoneal injection of avertin (> 375 mg/kg), plasma was collected from the retro-orbital plexus using capillary tubes coated with heparin and the tissues were excised, weighed, and used fresh or stored at -80 °C.

Body composition measurement

Body composition (whole-body lean mass and fat mass) was measured using dual-energy X-

ray absorptiometry (iNSiGHT VET DXA, Osteosys, Seoul, South Korea). Mice were anesthetized using an intraperitoneal injection of avertin (250 mg/kg) before body composition measurements. The body composition value was calculated with software provided by the manufacturer. Lean and fat tissue were shown in green and red, respectively, in the color-body composition image.

Exercise training

Mice were fed a 60% HFD for 8 weeks and then acclimated to a treadmill machine (Ugo Basile, Comerio VA, Italy) at 15 m/min daily for 3 days: 10, 15, and 20 min on the first, second, and last day, respectively. The mice were trained at 15 m/min using a two-step progressive training protocol, involving 30 min for 6 days and 40 min for the next 12 days. This was followed 5 days per week for 3 weeks. The tissues from mice were harvested 3 h after the last training and stored at -80 °C.

Muscle function test

1. Endurance exercise test: Before the endurance exercise test, mice were adapted to a motorized treadmill for three consecutive days (10 m/min for 9 min followed by 20 m/min for 1 min at 0 ° incline). During the test, mice were warmed up at 10 m/min for 1h, followed by increasing the speed by 3 m/min every 15 min until the mice were completely exhausted; the incline was similarly increased by 5 ° until it reached 15 °. The exhaustion of mice was judged by staying on the electric shock grid for five consecutive seconds.

2. Inverted-hanging grip test: The inverted-hanging grip test was performed as previously described [1]. The mice performed three trials with a 1 h inter-trial rest interval, and the performance was evaluated as an average value.

3. Forelimb grip strength measurement: Forelimb grip strength was measured using a BIO-

GS3 grip strength meter (BIOSEB, Vitrolles, France). The mice were acclimatized on a grid (100 × 160 mm; 30 ° angle) for 10 s and then placed horizontally from the grid by lifting the tail. Only the forelimbs of the mice were allowed to grip the grid and were then slowly pulled in a horizontal direction until the grip released. The mice performed five trials with a 30 min inter-trial rest interval, and the results were averaged.

Hyperinsulinemic-euglycemic clamp

Vein cannulation was performed 4 days before the hyperinsulinemia-euglycemic clamp. After overnight fasting, human insulin (Lilly, Indianapolis, IN, USA) was infused at a rate of 24 pmol/kg/min, and 20% glucose was infused through the Y-connector to maintain glucose levels at approximately 6.5 mM. Blood was withdrawn from the mouse tail vessel. Plasma glucose levels were measured using a glucose analyzer (Analox, Stourbridge, UK) and plasma insulin concentration was measured using enzyme-linked immunosorbent assay (Merck, Kenilworth, NJ, USA). Insulin-stimulated whole-body glucose turnover was assessed using infused radiolabeled [3-^{3}H] glucose (0.1 μCi/min; PerkinElmer, Waltham, MA, USA). Insulin-stimulated glucose uptake in the skeletal muscle was measured using a bolus injection of 2-deoxy-D-[1-^{14}C] glucose (10 μCi; PerkinElmer, MA, USA). The glucose uptake rate in the skeletal muscle was calculated from the plasma 2-[^{14}C] DG profile using MLAB (Civilized Software, Silver Spring, MD, USA). To measure glycogen synthesis rate and glycogen levels in skeletal muscle, skeletal muscle sample was incubated in 30% KOH at 100°C for 15 min and glycogen was precipitated with ethanol. Glycogen synthesis rate was determined with the radioactivity of ^{3}H and the glycogen levels were determined with 0.2% anthrone reagent at 620 nm.

Palmitate oxidation rate in isolated mitochondria

Mitochondrial isolation and the measurement of palmitate oxidation rate were performed as previously described [2, 3]. Fresh quadriceps were immediately placed in 20 μl of ice-cold SETH buffer (260 mM sucrose, 1.25 mM EDTA, and 10 mM Tris-HCl, pH 7.4) per mg muscle and homogenized in a glass tube with a motor-driven teflon pestle. The homogenate was centrifuged at 420 g for 10 min at 4 ℃ and the supernatant was collected to obtain the mitochondrial. A portion of the obtained supernatant was used for protein quantification using the Bradford assay (Bio-Rad, Hercules, CA, USA). Two hundred μl of 1 M NaOH was added to a 48-well plate and 50 μl of the mitochondrial homogenate was transferred to another well of the 48-well plate connected by a very small hole to the NaOH-loaded well. After adding 200 μl of the reaction mixture (100 mM sucrose, 10 mM Tris HCl, 5 mM potassium phosphate, 80mM potassium chloride, 1mM magnesium chloride, 0.06 mM coenzyme A, 1 mM L-carnitine, 0.1 mM malate, 2 mM ATP, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.5% bovine serum albumin, pH 7.4) to sample loaded wells, the 48-well plate was sealed. [^{14}C]-palmitate (0.5 μCi) was added to the sample-loaded wells using a syringe and then the mixture was incubated for 30 min at 37 ℃. After incubation, the $^{14}CO_2$-captured NaOH was transferred to a vial and analyzed for radioactivity.

Lipid kinetics (FAO and lipolysis analysis using stable isotope tracers)

In vivo mice infusion experiment and metabolic flux analysis were performed by Myocare (Myocare Inc., Seoul, South Korea).

1. Stable isotope tracer infusion: After 6 h of fasting, a primed constant infusion was conducted, comprised of [1-^{13}C]bicarbonate (prime: 12.75 nM/g, rate: 0.15 nM/g/min) for the first 40 min followed by [U-$^{13}C_{16}$]palmitate (rate: 1.85 nM/g/min) and [1,1,2,3,3-D_5]glycerol (rate: 2.5 nM/g/min) for the subsequent 110 min using an infusion pump (Pump 11 Elite, #70-4500, Harvard Apparatus, Holliston, MA, USA). Expired air and blood samples were collected

to determine CO_2 enrichment ($^{13}C/^{12}C$ ratio) and plasma tracer enrichment, respectively. At the end of the tracer infusion, tissues were quickly clamped using liquid nitrogen and stored at -80 °C until further analyzed.

2. *Measurement of stable isotopic enrichment*

2.1 Tracer enrichment of metabolites: Supernatant from homogenized tissues and plasma were dried and derivatized. Metabolite enrichment was analyzed using gas chromatography (GC) mass spectrometry (#5977B, #8890, Agilent, Santa Clara. CA, USA), as previously described [4].

2.2 CO_2 Enrichment: Expired CO_2 samples collected in evacuated tubes were analyzed to determine ^{13}C enrichment using trace gas analyzer isotope ratio mass spectrometry (TG-IRMS, Isoprime Ltd., Manchester, UK) with a trace gas pre-concentration unit, as previously described [5]. Briefly, CO_2 gas injected using a gas-tight syringe was cryogenically concentrated in glass-lined cryofocusing traps immersed in liquid nitrogen and separated on a 30 m GC capillary column filled with Poraplot Q (ChromPack, Varian, Palo Alto, CA, USA). The sample analytes and reference gases were then introduced into the IRMS instrument to measure the abundance of ions with mass-to-charge ratios of 44, 45, and 46 for CO_2 [6].

3. *Calculations of metabolite kinetics*

3.1 Rate of appearance of tracee: Respective tracer infusion rates (F) were divided by isotopic enrichment at the plateau to determine the rate of appearance (Ra) of the tracee (i.e., palmitate and glycerol) [7, 8]. The tracer enrichment of palmitate, and glycerol was expressed as mole percent excess, which was calculated as TTR /(1+TTR), where TTR is the tracer-to-tracee ratio.

3.2 Rate of palmitate acid oxidation: As previously described [7], the rate of palmitate oxidation is calculated as the product of the rate of palmitate uptake (i.e., R_d palmitate) and the fraction of oxidized R_d palmitate. In the present study, the palmitate oxidation rate was calculated using a modified equation, in which VCO_2 was replaced with the product of R_a CO_2

and C (recovery factor for $^{13}CO_2$ retention) as VCO_2 equates with R_a CO_2 • C. R_a CO_2 was calculated by dividing F by ECO_2 of [1-^{13}C]bicarbonate and multiplying ECO_2 of [U-$^{13}C_{16}$]palmitate. The value was then divided by the enrichment of plasma palmitate (Ep). Since multiple labelled palmitates were used, the complete oxidation of 1 mole of [U-$^{13}C_{16}$] palmitate produces 16 mole of $^{13}CO_2$; thus, $^{13}CO_2$ enrichment was divided by the number of labels (i.e., n = 16).

3.3 Fractional contribution of palmitate flux to the tricarboxylic acid cycle: The contribution of palmitate flux to the tricarboxylic acid (TCA) cycle in the designated tissues was quantified as citrate enrichment (M+2) in the tissue normalized by plasma palmitate enrichment. Fully transferred palmitate increased citrate enrichment.

3.4 Rates of triacylglycerol-fatty acid futile cycling: Recycling occurs between triacylglycerol and FFA in intra- and extra-adipocytes [7]. The intracellular cycling rate, extracellular futile cycling rate, and total futile cycling rate were calculated, as previously described [9].

Indirect Calorimetry

Indirect calorimetry of each mouse was performed for 24 h in an individual sealed metabolic chamber (Oxylet; Panlab, Cornellà, Spain). All mice were fasted during indirect calorimetry measurements and had free access to water. The system monitored VO_2 (mL/min) and VCO_2 (mL/min) for 3 min per mouse, then calibrated for body weight. The respiratory exchange ratio and energy expenditure were analyzed using the software supplied with the instrument (Metabolism; Pan Lab).

Quantitative real-time polymerase chain reaction

TRIzol (Invitrogen, Waltham, MA, USA) and TRI-Solution (Bio Science Technology,

Ventura, CA, USA) were used to isolate RNA from tissue and cell samples, respectively. cDNA was synthesized from equal amounts of RNA using a reverse transcription kit (Applied Biosystems, Waltham, MA, USA). Quantitative real-time polymerase chain reaction was performed on a Real-Time PCR 7500 System using Power SYBR Green PCR Master Mix (Applied Biosystems). The ribosomal protein lateral stalk subunit P0 (Rplp0) was used as a reference gene for sample normalization. The primer sequences are listed in Supplementary Table 2.

Western blotting

Muscle samples were homogenized in lysis buffer containing 150 mM NaCl, 50 mM HEPES, 50 mM sodium fluoride, 1 mM benzamide, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 1 mM sodium ortho-vanadate, 1 mM phenylmethylsulfonyl fluoride, 1% NP40, 10% glycerol, 0.22% β-glycerophosphate, and protease inhibitor cocktail (Santa Cruz Biotechnology, Dallas, TX, USA). Protein concentrations were determined using the Bradford assay (Bio-Rad). Equivalent amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to 0.45 μm polyvinylidene fluoride membranes (Millipore, Burlington, MA, USA). The membranes were blocked with 1X double blocker (Tech & Innovation, Gangwon, South Korea) for 10 min or 5% skim milk in tris buffered saline with 0.1% Tween 20 for 1 h, followed by overnight incubation with primary antibodies at 4 °C. Details of the primary antibodies used are presented in Supplementary Table 3. Sheep anti-rabbit immunoglobulin (Ig)G and goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad) were used as secondary antibodies and incubated at approximately 25 °C for 1 h. Proteins were detected after treatment with a chemiluminescence detection reagent (Millipore), using a LAS-4000 image analyzer (Fujifilm, Tokyo, Japan). The intensity of the

protein bands was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels.

Lipid assay

Skeletal muscle samples were homogenized with a 20-fold volume of chloroform-methanol (2:1) solution containing 0.01% butylated hydroxyl toluene in a glass tube to extract TG. The homogenized samples were carefully vortexed for 1 min and further incubated overnight at 4 °C. Distilled water (0.25 mL) was added, and the mixture was centrifuged at 3000 rpm for 30 min. For all the samples, an equal volume of lower chloroform layer was carefully separated using a glass syringe and was replaced with an equal volume of a chloroform solution containing 1% triton X-100 and dried overnight. Completely dried samples were dissolved in distilled water. TG levels were measured using free glycerol reagent (Sigma-Aldrich, Saint Louis, MO, USA) and TG reagent (Sigma-Aldrich). FFA levels in plasma were determined using a non-esterified fatty acid assay kit (FUJIFILM Wako Diagnostics, Mountain View, CA, USA).

Measurement of mitochondrial respiratory capacity in vivo

Mitochondria were isolated from skeletal muscle samples and mitochondrial oxygen consumption was analyzed using an Oxytherm System (Hansatech Instruments Ltd, Norfolk, UK), as previously described [10]. Briefly, fresh tissues were homogenized in buffer A containing 1.8 mM ATP, 1 mM EDTA, 100 mM KCl, 50 mM Tris base, 5 mM $MgCl_2 \cdot 6H_2O$ and a protease, and further centrifuged at 720 ×g at 4 °C for 5 min. The supernatant was transferred to a fresh tube and centrifuged at 1000 ×g for 5 min, and the resulting supernatant was discarded. The precipitated mitochondria were resuspended in buffer A and further centrifuged at 9000 ×g for 5 min. After discarding the supernatant, the mitochondrial pellets

were resuspended in respiratory buffer (2 mM $MgCl_2$, 20 mM HEPES, 2.5 mM KH_2PO_4, and 125 mM KCl, pH adjusted to 7.2). The isolated mitochondria were added to the chamber of an Oxytherm System along with 950 µL of respiratory buffer and warmed to 37 °C under constant agitation. Mitochondrial respiration was assessed in the absence of substrate (State 1), in the presence of 5 mM pyruvate/2.5 mM malate or 5 mM palmitoyl-carnitine/2.5 mM malate (State 2), and in the presence of 2.5 mM adenosine diphosphate (ADP) (State 3). ATP synthase activity was inhibited by the addition of 2.5 µM oligomycin, leading to State 4 respiration. Oxygen consumption rates were normalized to the concentration of isolated mitochondria and were expressed as nanomoles/mL/µg. The respiratory control ratio (RCR), which represents mitochondrial coupling efficiency, was calculated as the ratio of state 3 to state 4 respiration.

Electron microscopy

Muscle samples were prepared for electron microscopy, as previously described [11] and mitochondrial morphology was observed using H-7000 transmission electron microscope (Hitachi, Tokyo, Japan) at 75 kV. The area of cristae and mitochondria was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA) in six randomly selected intact mitochondria for each sample and presented as the average of the measured values. The number of mitochondria per unit area was determined as the average values measured after randomly selecting at least three locations per sample on a 15000x magnification screen.

Microarray

RNA was extracted from the quadriceps muscle and synthesized into fluorescent cDNA using a Low RNA Input Linear Amplification Kit (Agilent Technologies), following the manufacturer's instructions. The transcriptome analysis data were analyzed using the KEGG pathway enrichment analyses.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using an EZ-ChIP kit (Millipore) according to the manufacturer's instructions. Briefly, chopped muscle samples were incubated in 1% formaldehyde for 15 min at room temperature with gentle agitation to cross-link protein/DNA complexes. The reaction was terminated by adding glycine to the final concentration of 125 mM for 5 min at room temperature, and the samples were washed twice with cold PBS. Washed samples were ground using a glass tissue grinder (Wheaton, Millville, NJ, USA) in cold PBS containing a protease inhibitor cocktail, and the pellets collected by centrifugation were resuspended in SDS lysis buffer containing a protease inhibitor cocktail. Chromatin was prepared by sonicating the pellet (Bandelin Sonopuls GM70, Bandelin, Berlin, Germany) on ice at 10 cycles (pulsed on-time 10 s and off-time 30 s; 70% power). The chromatin was precleared with protein G agarose beads under orbital shaking for 1 h at 4°C and then immunoprecipitated with antibodies against FoxO1 (Cell signaling technology, #2880, 1.3 μg per 10 μg chromatin DNA) or an equal amount of rabbit IgG (Cell signaling technology, #2729) under orbital shaking at 4°C overnight. The obtained protein/DNA complexes were reverse crosslinked with NaCl, and the purified DNA was eluted using spin columns. To assess the enrichment of FoxO1 on the PPARδ promoter, standard polymerase chain reaction (PCR) and quantitative PCR were performed using the eluted DNA according to the protocol recommended by the EZ-ChIP kit. The standard PCR was performed under the following conditions: initial denaturation at 94°C for 3 min, denaturation at 94°C for 20 s, annealing at 59°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 2 min. The denaturation, annealing, and extension steps were repeated 32 times. The quantitative PCR was performed under the following conditions: initial denaturation at 94°C for 10 min, denaturation at 94°C for 20 s, and annealing and extension at 60°C for 1 min. The denaturation and annealing

and extension steps were repeated 50 times. The primer sequences are listed in Supplementary Table 4. The quantitative PCR results were calculated as previously described [12, 13] and expressed as Fold Enrichment.

Cell culture

C2C12 mouse myoblasts (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; WELGENE, Daegu, Korea) supplemented with 10% fetal bovine serum (Gibco, Amarillo, TX, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (WELGENE). Myotubes were differentiated in DMEM supplemented with 2% horse serum (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were grown in an incubator with 5% CO_2 at 37 °C. Three small hairpin (sh) RNA plasmids (shCon, shFoxO1, and shPPARδ, Santa Cruz Biotechnology) were transfected into C2C12 cells using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. A stably transfected cell clone was selected 48 h after transfection using 2 μg/mL puromycin. Transfected cells were treated with GSK3787 (a PPARδ antagonist) for 24 h at 100 μM on the second day of differentiation. The cells in the control group were treated with an equal volume of dimethylsulfoxide (DMSO).

Mitochondrial imaging in C2C12 cells

For the fluorescence imaging of mitochondria, C2C12 cells stably expressing shCon or shFoxO1 (1.5×10^4/well) were plated on a black, clear bottom 96 well plate (Greiner Bio-One™, Monroe, NC, USA). Twenty-four hours after plating, the cells were stained with NucBlue (Thermo Fisher Scientific) and MitoTracker Green (Thermo Fisher Scientific) for 30 min, washed, and treated with DMSO or 2.5 μM rotenone for 1 h. Images were captured using ImageXpress Micro confocal (Molecular Devices, San Jose, CA, USA), and mitochondrial

length (long: > 8 μm; short: < 8 μm) was analyzed using MetaXpress software (Molecular Devices). The criterion for classifying mitochondrial length was determined by referring to a previous study [14].

Luciferase assay

PPARδ promoter activity was measured using a Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia, Rockville, MD, USA). The PPARδ promoter plasmid (GeneCopoeia) was transfected into shCon- and shFoxO1-transient cells using Lipofectamine 3000. After 24 h of transfection, the differentiation medium was treated with PPARδ promoter plasmid-transfected cells, and PPARδ promoter activity was measured 72 h after differentiation.

Mitochondrial oxygen consumption in C2C12 cells

C2C12 cells on day 4 of differentiation were plated at 2.3×10^4 cells per well in Seahorse XFe96/XF Pro cell culture microplates (Seahorse Bioscience, North Billerica, MA, USA). The next day, oxygen consumption rates were measured using the Seahorse XF Pro Analyzer (Seahorse Bioscience). Before measurements, cells were equilibrated for 1 hour at 37℃ in XF Assay Medium (Seahorse Bioscience) supplemented with 25 mM glucose and 4 mM glutamine. Oligomycin (1 μM), FCCP (2 μM), and a mixture of rotenone (0.5 μM) and antimycin A (0.5 μM) were sequentially injected into each well. OCR values were normalized to protein concentration.

Supplementary Table 1. The information of human participants

	Non-diabetes	Diabetes	*P* value
Number of cases (F)*	14 (7)	14 (7)	
Age (yr)	65.5 ± 1.10	68.6 ± 1.42	0.0997
Duration of diabetes (mo)	0	26.3 ± 6.80**	0.0007
Fasting blood glucose (mM)	5.4 ± 0.15	7.5 ± 0.63	0.0030
Hb A1c (%)	5.4 ± 0.07	7.8 ± 0.47	0.00004
Hb A1c (mmol/mol)	35.2 ± 0.83	61.4 ± 5.14	0.00004
Weight (kg)	62.3 ± 2.26	63.8 ± 2.91	0.6737
Height (cm)	161.8 ± 2.75	160.1 ± 2.52	0.6628
BMI (kg/m^{-2})	23.7 ± 0.48	24.8 ± 0.83	0.2645
TG (mg/dL)	107.9 ± 12.15	109.1 ± 10.16	0.9430
Total cholesterol (mg/dL)	166.6 ± 9.82	141.9 ± 10.00	0.0888
Systolic blood pressure (mmHg)	118.6 ± 1.79	119.3 ± 1.47	0.7837
Diastolic blood pressure (mmHg)	73.1 ± 1.70	72.4 ± 1.53	0.7572

*The number of female participants (F) in brackets.
**Two people were diagnosed with diabetes when they visited Gumi Cha Hospital. Therefore, the duration of diabetes is not accurate.

Supplementary Table 2. RT-qPCR primers used in the experiment

Gene Name	Primer sequence (5'→3')
Rplp0	Forward, CACTGGTCTAGGACCCGAGAA Reverse, GGTGCCTCTGGAGATTTTCG
Foxo1	Forward, TCGAGTGCAGAATGAAGGAA Reverse, CATGGACGCAGCTCTTCTC
Foxo3	Forward, GTCCCAGATCTACGAGTGGAT Reverse, GTTGTGCCGGATGGAGTT
Foxo4	Forward, GCTCTCTAGGAGCGGTCTCT Reverse, CCCATCTATTGGGCCAAA
Ppard	Forward, GTCTCCGCAAGCCCTTCAG Reverse, ATGAAGAGCGCCAGGTCACT
Ppargc1a	Forward, CCCTGCCATTGTTAAGACC Reverse, TGCTGCTGTTCCTGTTTTC
Cpt1a	Forward, TACGGAGTCCTGCAACTTTG Reverse, GTGCTGGTGCTTTTCACAAG
Acadm	Forward, TGGATCTGTGCAGCGGATT Reverse, GGGTCACCATAGAGCTGAAGACA
Fabp3	Forward, ACTCGGTGTGGGCTTTGC Reverse, ATGATGGTAGTAGGCTTGGTCATG

Supplementary Table 3. Primary antibodies used in the experiment

Antibodies	Technology	Catalog number
FoxO1	Cell Signaling Technology	#2880
pFoxO1	Cell Signaling Technology	#9461
pFoxO3	Cell Signaling Technology	#13129
FoxO3	Cell Signaling Technology	#12829
pFoxO4	Invitrogen	#PA5-104944
FoxO4	Invitrogen	#PA5-20974
PPARδ	Invitrogen	#PA5-29678
PGC1α	Abcam and Sigma-Aldrich	#ab54481 and #516557
pAkt	Cell Signaling Technology	#9271
Akt	Cell Signaling Technology	#9272
pGSK3β	Cell Signaling Technology	#8566
GSK3β	Cell Signaling Technology	#5676
CD36	Proteintech	#18836-1-AP
CPT1	Santa Cruz Biotechnology	#sc-393070
CACT	Proteintech	#19363-1-AP
LCAD	Abcam	#ab196655
MCAD	Abcam	#ab92461
Complex I	Abcam	#ab110413
Complex II	Abcam	#ab110413
Complex III	Abcam	#ab110413
Complex IV	Cell Signaling Technology	#8674
Complex V	Abcam	#ab110413
Cytochrome C	Cell Signaling Technology	#8674
DRP1	Santa Cruz Biotechnology	#sc-271583
pS616-DRP1	Cell Signaling Technology	#3455
pS637-DRP1	Cell Signaling Technology	#4867
MFN1	Abcam	#ab104274
GAPDH for mice	Santa Cruz Biotechnology	#sc-32233
GAPDH for human	Santa Cruz Biotechnology	#sc-47724

Supplementary Table 4. ChIP assay primers of PPARδ used in the experiment

Location	Sequence (5'→3')
- 1110 to – 968	Forward, TGAGTTGGGGTGACAGGC Reverse, CCTGTGCTTATAGGCCTTGTGA

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