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Supplemental Material to:

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SUPPLEMENTAL INFORMATION

Distinct functions of *Ulk1* and *Ulk2* in the regulation of lipid metabolism in adipocytes

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SUPPLEMENTARY FIGURES

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

SUPPLEMENTARY FIGURES

Supplemental Figure S1

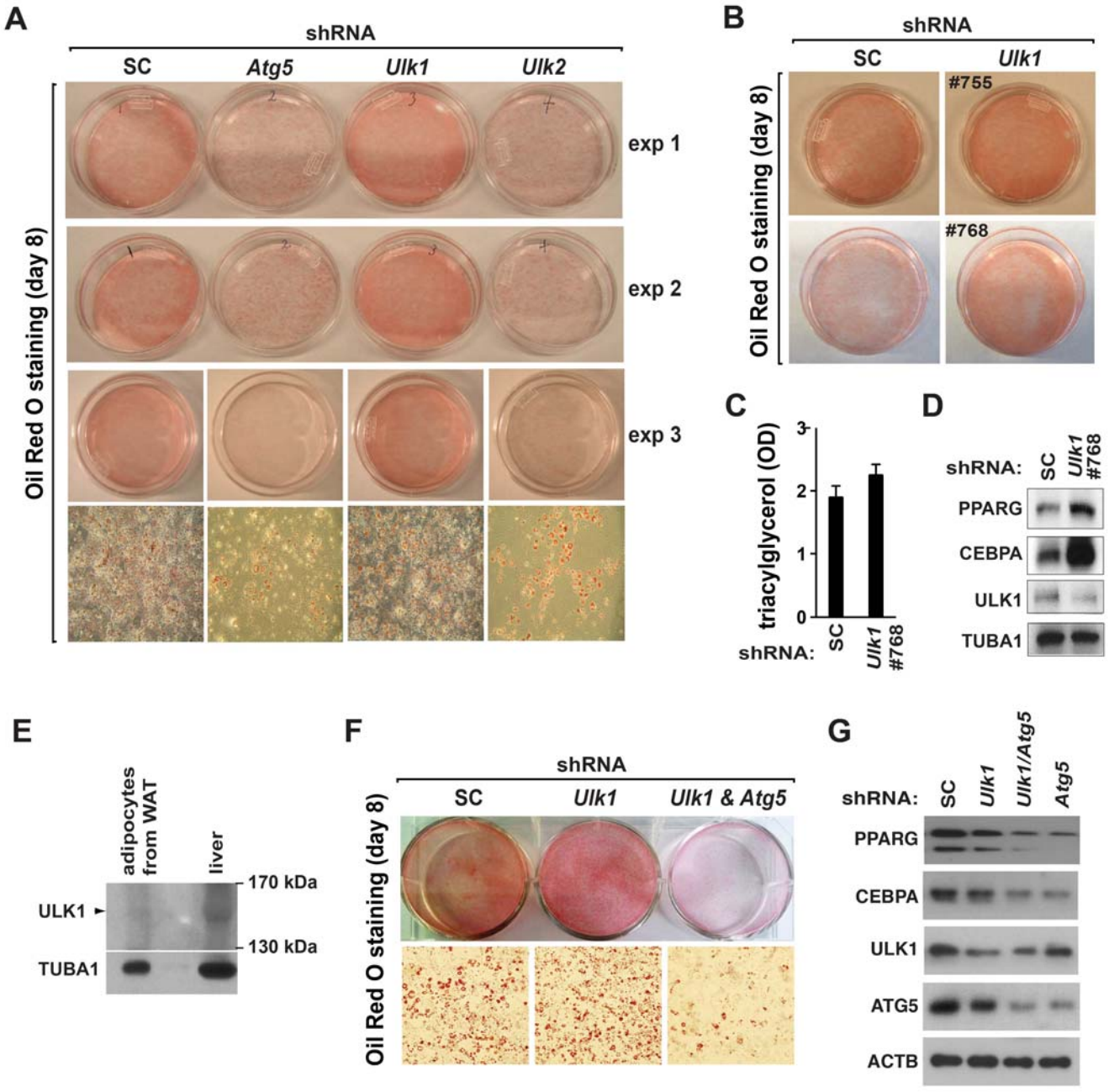

Figure S1. *Ulk1* knockdown does not suppress adipogenesis. (**A**) *Ulk1* knockdown does not suppress adipogenesis. The shRNA-transduced 3T3-L1 cells were differentiated as described in Materials and Methods. At day 8, cells were stained with Oil Red O. (**B**) Confirming the effect of *Ulk1* using two different shRNAs. 3T3-L1 cells were transduced by shRNA targeting a different sequence of *Ulk1* (#768, see Table S1). Differentiation was induced in the absence of troglitazone as described in Materials and Methods. Lipid droplets were stained using Oil red O. (**C**) Quantification of lipid droplet content. Lipid droplets from cells stained with Oil Red O were extracted using isopropanol. The optical density at a wavelength of 490 nm was measured. Error bars indicate SEM. (**D**) Proteins were extracted from the cells in (**B**) in RIPA buffer. Western blot analysis of protein expression. (**E**) The expression levels of *Ulk1* in adipocytes from C57BL/6 WAT and liver. (**F**) *Atg5* knockdown has a dominant effect over *Ulk1* knockdown in adipogenesis. (**G**) Western blot analysis of protein expression in shRNA-transduced 3T3-L1 cells at 8 day after induction of differentiation.

Supplemental Figure S2

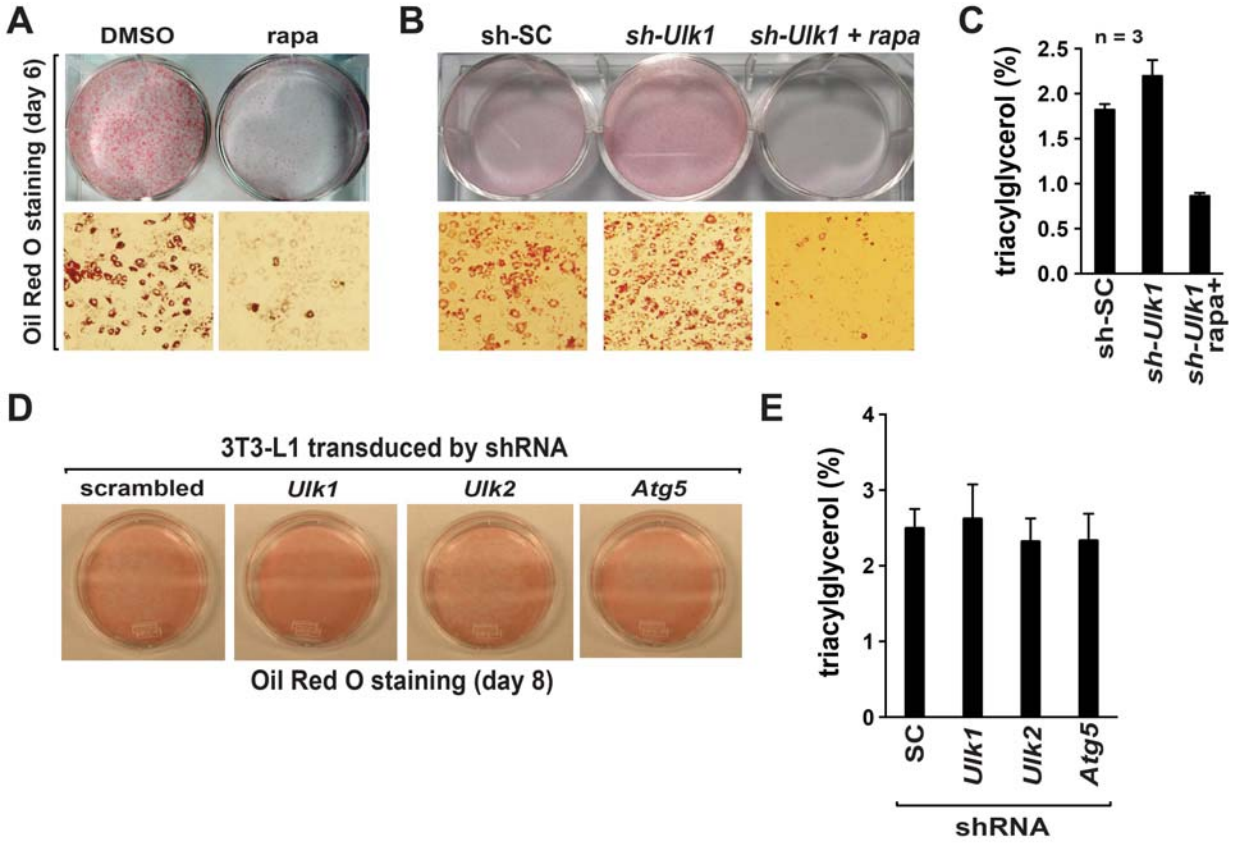

Figure S2. Rapamycin suppresses adipogenesis independently of *Ulk1*, and troglitazone makes the shRNA-transduced cells to be differentiated to similar extents. (**A**) 3T3-L1 cells were treated with rapamycin (50 nM) or vehicle (DMSO) for 6 days in the differentiation inducing culture condition (see Materials and Methods). Cells were stained with Oil Red O. (**B**) 3T3-L1 cells transduced by scrambled shRNA (sh-SC) or *Ulk1* shRNA were induced to be differentiated in the presence or absence of rapamycin for 6 days. Cells were stained with Oil Red O. (**C**) Quantification of lipid droplet content. Lipid droplets from cells stained with Oil Red O were extracted using isopropanol. The optical density at a wavelength of 490 nm was measured. Values are mean ± SD from three independent experiments. (**D**) shRNA-transduced 3T3-L1 cells were induced to differentiate in the presence of troglitazone as described in Materials and Methods. Cells were stained with Oil Red O at day 8 after the induction of differentiation. (**E**) Lipid droplet content was quantified as described in (**C**).

Supplemental Figure S3

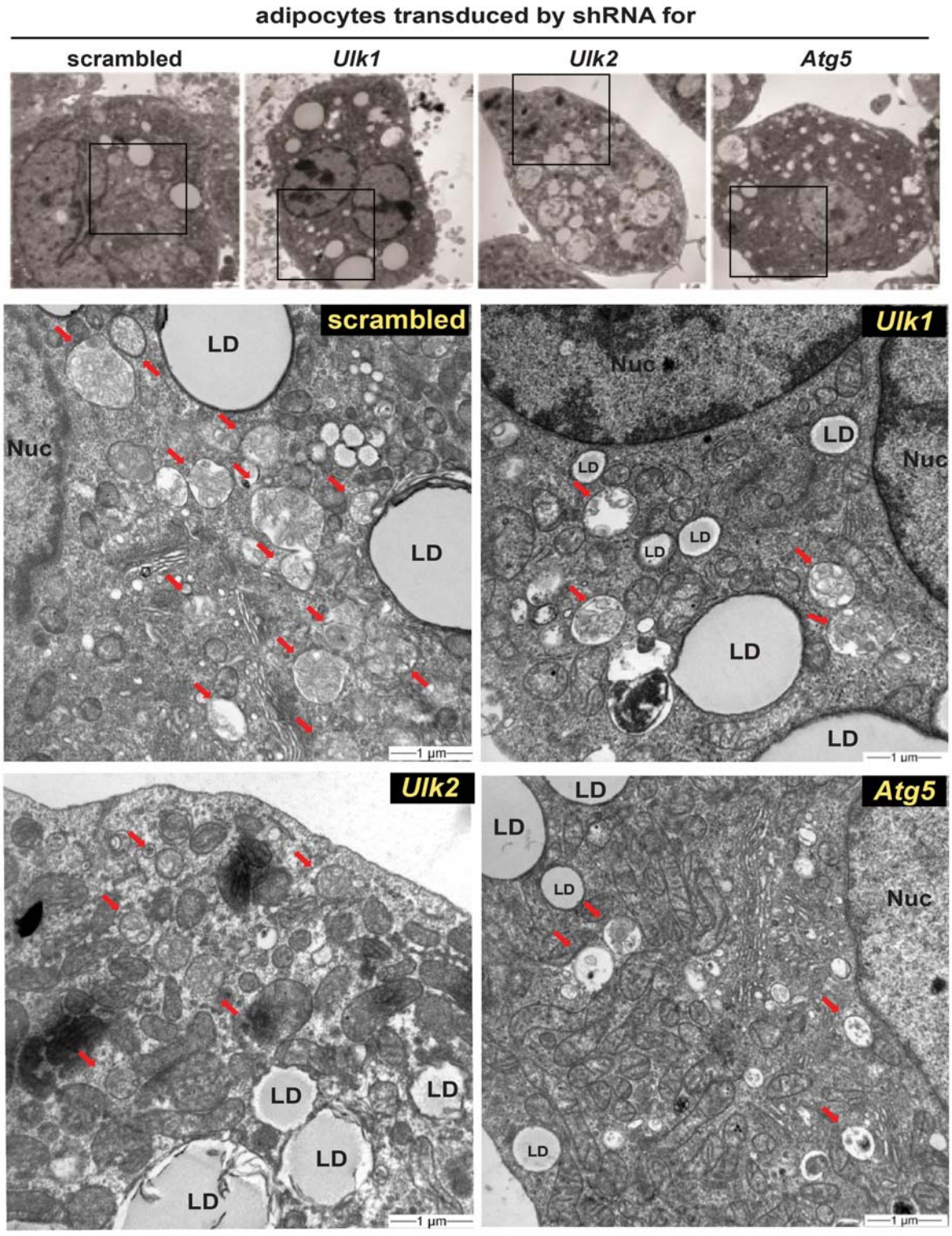

Figure S3. Knockdown of *Ulk1*, *Ulk2* or *Atg5* suppresses the formation of autophagosomes. EM analysis of autophagosomes in shRNA-transduced adipocytes. The red arrows point to autophagosomes.

Supplemental Figure S4

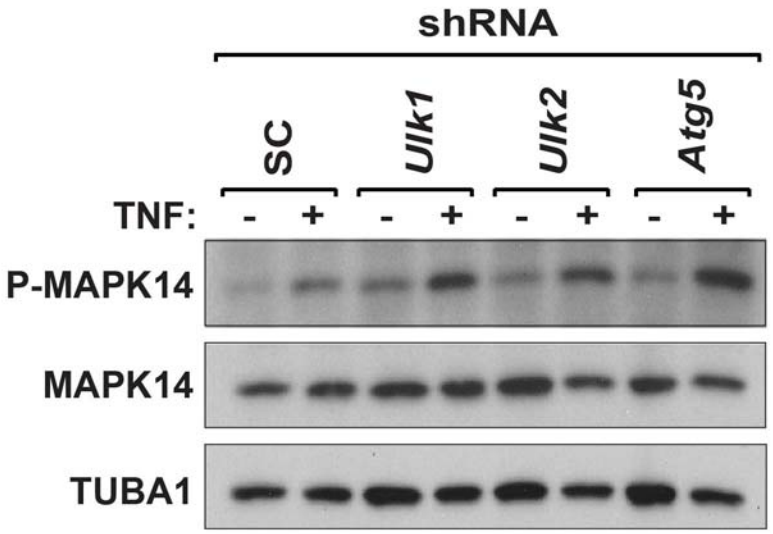

Figure S4. Knockdown of *Ulk1*, *Ulk2* or *Atg5* increased TNF-induced phosphorylation of MAPK14. Knockdown of *Ulk1*, *Ulk2* or *Atg5* in adipocytes increased MAPK14 phosphorylation. The shRNA-transduced 3T3-L1 adipocytes (day 8) were treated with or without 20 μM TNF for 24 h. The phosphorylation state of MAPK14 at Thr180/Tyr182 in cell lysate were analyzed by western blotting.

Supplemental Figure S5

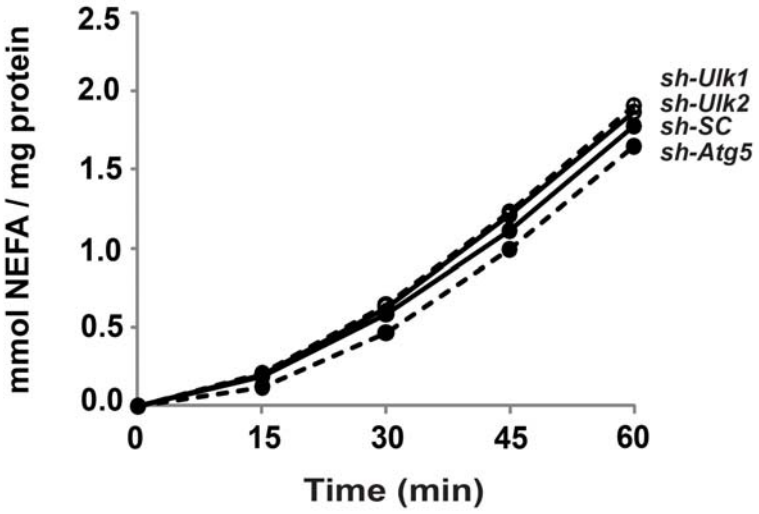

Figure S5. *Ulk1*, *Ulk2* and *Atg5* are not important for cAMP-stimulated lipolysis. The shRNA-transduced adipocytes were treated with 40 μM forskolin that stimulates cAMP-induced lipolysis (see details in Materials and Methods).

Table S1. Oligonucleotides for shRNA target sequences.

scrambled : 5'-AAC GTA CGC GGA ATA CTT CGA-3'
Ulk1 : 5'-CCT GGT CAT GGA GTA TTG TAA-3' (#755)
5'-CGC TTC TTT CTG GAC AAA CAA-3' (#768)
Ulk2 : 5'-CCA AAG ACT CTG CGA GTA ATA-3' (#695)
Atg5 : 5'-GGC ATT ATC CAA TTG GTT TA-3'

Table S2. Primers used for quantitative real-time RT-PCR to monitor the expression of mouse mRNAs and DNAs.

mRNA	**Accession #**	**Forward (F) and Reverse (R) Primers**
Actb	AK_145308	F: 5'- CCT AAG GCC AAC CGT GAA AA-3' R: 5'- GAG GCA TAC AGG GAC AGC ACA-3'
Ulk2	NM_013881	F: 5'- GTG CTG TGG AAA TGG TTC AAT CTG-3' R: 5'- CCT TGT GGT AGC GAT AAA CGA TGT C-3'
Gtf2e2	NM_026584	F: 5'- CAA GGC TTT AGG GGA CCA GAT AC-3' R: 5'- CAT CCA TTG ACT CCA CAG TGA CAC-3'
Acaca	AY_451393	F: 5'- CGG ACC TTT GAA GAT TTT GTC AGG-3' R: 5'- GCT TTA TTC TGC TGG GTG AAC TCT C-3'
Acacb	AY_451394	F: 5'- ATC ACC ACT CCT TCT GAC CCC ATC-3' R: 5'- TCT CCA CAG CAA TCA CTC CCA C-3'
Fasn	NM_007988	F: 5'-CT GGATAG CAT TCC GAA CCT G-3' R: 5'-TTC ACA GCC TGG GGT CAT CTT TGC-3'
Mt-co2	NC_005089	F: 5'- TTT TCA GGC TTC ACC CTA GAT GA-3' R: 5'- GAA GAA TGT TAT GTT TAC TCC TAC GAA TAT G-3'

Supplemental Experimental Procedures

Electron microscopy imaging. Cells on thermonox cover slips (Thermo Scientific, Rochester, NY, USA) were fixed in 1 ml of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer in 6 well plates overnight at 4°C. The cells on thermonox cover slips were washed three times with 0.1 M sodium cacodylate buffer and post fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Followed by incubation in 1% tannic acid, these samples were washed in distilled water and dehydrated using a 25-100% ethyl alcohol gradient. Samples were then infiltrated with 2:1 ethanol:Embed 812 resin for 1 h and subsequently transferred to a 1:2 ethanol:Embed 812 resin mixture for 1 h. Cells on thermonox cover slips were infiltrated with 100% resin and were embedded on top of 20 mm gelatin capsules and incubated at 58°C for 24 h to polymerize the resin. Embedded samples were trimmed and sectioned on a Leica UC6 Ultramicrotome (Leica Microsystems, Vienna, Austria). Thin sections (60 to 70 nm) were obtained and collected on a 200 mesh copper grid (Electron Microscopy Sciences, Hatfield, PA, USA) using a perfect loop. Grids were stained with 5% uranyl acetate for 20 min and Satos' lead citrate for 6 min. These sections were observed in the JEOL 1200 EX II transmission electron microscope (JEOL LTD, Tokyo, Japan). Images were obtained using a Veleta 2K x 2K camera with iTEM software (Olympus SIS, Munster, Germany). The total number of autophagosomes of 10 randomly selected cells was counted. In each cell, up to 10 autophagosomes were randomly selected and their perimeter and area were measured and recorded using the iTEM software (Olympus SIS).

Measurement of mitochondrial respiration and ATP production rates. Oxygen consumption rate (OCR) and ATP turnover rate were measured by XF24 bioenergetic assay (Seahorse Bioscience, Billerica, MA, USA). Assays have been previously described in detail.[1] Oligomycin A, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and antimycin A were purchased from Seahorse Bioscience (101706-110, XF cell mito-stress test kit, Seahorse Bioscience).

Measurement of intracellular ROS. Intracellular ROS levels were measured using the chloromethyl 2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA, Invitrogen), a cell permeable nonfluorescent precursor. This dye measures H2O2, ROO−, and ONOO−. Within the cells, CM- H2DCFHDA is hydrolyzed by nonspecific esterases to release CM-H2DCF, which is readily oxidized by intracellular ROS. The oxidized product emits green fluorescence with a wavelength of 516 nm after excitation with a wavelength of 492 nm. The protocol was modified from manufacture's instruction. Briefly, 3T3-L1 adipocytes (day 8) were loaded with 5 μM of fluorescent probe CM-H2DCFDA for 30 min at 37^{o}C. Cells without fluorescent probe were used as control. Cells were washed with PBS three times before measurement. The fluorescence intensity was measured by VICTOR3V Multilabel Counter 1420 (PerkinElmer, Waltham, MA). The measured values were normalized by protein concentration. All experiments were performed in triplicate.

Measurement of matrix superoxide using isolated mitochondria. 3T3-L1 adipocytes were homogenized in ice-cold KRH buffer, and lysed with 1:5 wt/vol of isolation buffer (20 mM Tris-HCl, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, pH 7.4 supplemented with protease inhibitors) using a dounce homogenizer. Homogenates were centrifuged at 700 g to remove nuclei, unbroken cells, and lipids. Mitochondria were recovered by centrifugation at 12,000 g. MitoSOX red (M36008; Invitrogen) was used to detect superoxide in isolated mitochondria as described previously.[2] Superoxide level was normalized to mitochondrial protein.

Measurement of protein carbonylation. 3T3-L1 adipocytes (day 8) were collected and sonicated in biotin hydrazide (BH) buffer (100 mM sodium acetate, pH 5.5, 20 mM NaCl, 0.1 mM EDTA). Lipid was removed by centrifugation, and SDS was added to a final concentration of 1%. Protein carbonylation was detected using EZ-link biotin hydrazide (Pierce, Rockford, IL) as described previously.[3] Polyvinylidene fluoride membranes (Millipore, Billerica, MA) were blocked in Odyssey Blocking Buffer (LI-COR, Lincoln, NE), and biotinylated proteins were detected with DyLight 800- conjugated streptavidin (Pierce) and visualized using an Odyssey Infrared Imager (LI-COR, Lincoln, NE).

Quantitation of mitochondrial DNA content. To quantitatively assess mitochondrial DNA (mtDNA) content, total DNA was isolated from adipocytes using DNAzol reagent (Invitrogen). mtDNA content was measured by quantitative PCR analysis of the copy numbers of the mitochondrial gene encoding cytochrome c oxidase II (*mt-Co2*/*CoXII*) using the primers listed in **Table S2**. The copy number of mtDNA was calculated as described previously.[4]

Quantitative real time RT-PCR. Total RNAs were prepared from 3T3-L1 adipocytes using TRIzol reagent. The first-strand cDNA was generated using iScript cDNA Synthesis kit and incubated with the iQ SYBR Green Supermix. PCR was performed using a ICycler Real Time PCR System (Hercules, CA). Primers specific for the examined genes are listed in Table S1. *Actb* (encoding β-actin) and transcription factor IIE (*Gtf2e2*/*TfIIe-beta*) were used as controls. Results were analyzed using iQ5 software supplied with the ICycler system. About 85% of knockdown compared to the level in control cells was confirmed for mouse *Ulk2* (NM_013881) by RT-PCR.

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