

***C. elegans* strains and RNAi**

C. elegans were maintained on NGM plates under standard conditions (1). N2 was used as the wild-type strain. For RNAi, *rrf-3(pk1426)II*; *xdIs26(Punc-54-PLIN1::GFP)* worms were grown from hatching on HT115 *E. coli* containing an empty vector control or expressing dsRNA.

Mice

In brief, the *Atgl* targeting vector was prepared by inserting the Frt-flanked neomycin cassette into intron 1 of the *Atgl* sequence. Then, exon 1 (containing the mutation F2A: TTC→GCG), intron 1, a *Neo* cassette, and exon 2 were cloned into a mouse bacterial artificial chromosome (BAC). The *Atgl(F2A)* sequence, amplified by PCR, was cloned into pDTA-C vector (provided by Biocytogen). For the 5' homologous arm, a 7.7kb fragment containing the *Atgl* promoter was amplified by PCR, which introduced a *Hind* III restriction site at the 5' end. For the 3' homologous arm, a 4.4kb fragment containing exon 2 through exon 9 was amplified by PCR, which introduced a *Ssp* I restriction site at the 3' end. The targeting vector was linearized and electroporated into ESCs. The correct ESC clones were selected by Southern blotting, then microinjected into 3.5-day blastocysts derived from BALB/c females, and transferred to pseudopregnant C57BL/6 mice. Chimeric mice were mated with C57BL/6 mice for germline transmission to F1 progeny. The *Neo* cassette was removed by mating F1 mice with mice expressing FLP recombinase. The heterozygous mice were crossed to obtain homozygous mice. For genotyping, genomic DNA extracted from the tail was subjected to PCR using the following primers: 5'-CCATCACCTTTGCCCTGAGGAG-3', and 5'-CCTGTCATTGTTCTAGCTGCTC-3'. The PCR product size from the wild-type allele is 255 bp, and the product size of the mutant allele is 337 bp.

Molecular biology

For C-terminal Flag-tagged human ATGL, ATGL(F2V) and ATGL(F2A) constructs,

the amino acid mutations were introduced by PCR with the following forward primers: *hATGL*-forward 5'- GGATCCATGTTTCCCCGCGAGAAGAC -3', *hATGL(F2V)*-forward 5'- GGATCCATGGTTCCCCGCGAGAAGAC -3', *hATGL(F2A)*-forward 5'- GGATCCATGGCTCCCCGCGAGAAGAC -3', and the common reverse primer *hATGL*-reverse 5'- GGTACCCAGCCCCAGGGCCCCGATCAC -3'. The coding regions of these three forms of human ATGL were amplified and inserted into the pFlag-CMV-5.1 vector (Sigma-Aldrich, St Louis, USA) through the *Bam*H I and *Kpn* I restriction sites.

For alignment of ATGL N-terminal sequences, the N-terminal sequences of various ATGL proteins were obtained from the NCBI database and aligned. Accession numbers were: *Homo sapiens*, NP_065109.1; *Rattus norvegicus*, NP_001101979.2; *Mus musculus*, NP_001157161.1; *Bos taurus*, NP_001039470.1; *Gallus gallus*, NP_001106762.2.

For qRT-PCR, the reactions were performed with Trans Start Green qPCR superMix (TransGen, Beijing, China) on an Agilent Technologies MX3000P system. All data were normalized to *Rplp0* (36B4, NM_007475).

Cells

3T3L1 preadipocytes were cultured in Dulbecco's Modified Eagle Medium/high glucose (DMEM/High glucose) (Hyclone, Logan, USA), supplemented with 10% newborn calf serum (NBCS) (ThermoFisher, Waltham, USA). For differentiation, 3T3L1 preadipocytes were maintained in the above medium until completely confluent. Then cells were changed into differentiation medium 1 for two days (2), which consists of DMEM/high glucose, 10% fetal bovine serum (FBS) (ThermoFisher #10099141c), 5 µg/mL insulin (Sigma-Aldrich, I9278), 0.4 µg/mL dexamethasone (Sigma-Aldrich, D2915), 0.5 mM IBMX (Sigma-Aldrich, I5879), and 2 µM rosiglitazone (Merck, 122320-73-4). On day 3, cells were cultured in DMEM/high glucose containing 10% FBS and 5 µg/mL insulin for another two days. On day 5, cells were cultured in DMEM/high glucose containing 10% FBS. The cells should be fully differentiated by day 8-10. For transfection, a total of 100 pmol

siRNA oligonucleotides were transfected into cells in 6-well plates using Lipofectamine 3000 (Invitrogen, Waltham, USA). The sequences for siRNAs are shown in Supplementary Table S4.

Lipolysis assay for cells and mouse tissues

The lipolysis assay was performed in 3T3L1 adipocytes as previously described (3). In brief, 3T3L1 fibroblasts were induced to differentiate into adipocytes for 4 days. On day 5 of differentiation, the cells were infected with Ad-ATGL(WT) or Ad-ATGL(F2A) with a multiplicity of infection (MOI) of 2000 pfu/cell. Cells were incubated with viruses for 24 hours, then the medium was removed and incubation was continued for a further 24 hours with DMEM containing 10% FBS. On day 7 of differentiation, 3T3L1 adipocytes were incubated in PBS containing 2% fatty acid-free BSA with or without 10 μ M isoproterenol at 37°C. Aliquots of medium were collected and the free fatty acid (FFA) and glycerol content were determined using commercially available kits. In OA-loaded HeLa cells, lipolysis was induced by ISO (0.25 mM IBMX/1 μ M isoproterenol) for 8 hours. For the lipolysis assay in mouse adipose tissue, gonadal fat pads from overnight-fasted mice were cut into 50 mg samples and incubated at 37°C in 500 μ l KRB buffer (12 mM HEPES, 121 mM NaCl, 4.9 mM KCl, 12 mM MgSO₄ and 0.33 mM CaCl₂, pH7.4), supplemented with 2% fatty acid-free BSA and 0.1% glucose with or without 10 μ M isoproterenol. 3 hours after isoproterenol stimulation, aliquots of medium were collected for FFA and glycerol detection.

Immunoprecipitation

For immunoprecipitation, proteins from HeLa cells were extracted using RIPA lysis buffer, containing 100 mM Tris Base, 1% deoxycholate, 0.1% SDS, 5 mM EDTA, 1% Triton-X-100, 150 mM NaCl, pH 8.0, which was supplemented with 100 mM PMSF, protease inhibitor cocktail (Roche, P8340) and 100 mM sodium vanadate. Lysates were incubated for 15 min on ice, then centrifuged at 12,000 rpm for 15 min at 4°C. The supernatants were incubated with anti-Flag M2 affinity gel (Sigma-Aldrich,

A2220) at 4°C overnight. After four washes with lysis buffer, the beads were denatured with 2X SDS sample buffer for 10 min at 100°C for western blotting.

Western blotting

Proteins were extracted from cells or tissues using mammalian protein extraction buffer (ThermoFisher, 78501). The primary antibodies used in this study were anti-Flag (Sigma-Aldrich, SAB4200071), anti-ATGL (Cell Signaling Technology, CST #2138), anti- α -Tub (Abcam ab7291), anti-Ubiquitin (Abcam ab19247; CST#3933), anti-K48-linked specific polyubiquitin (CST #4289), anti-UBR1 (Proteintech, 26069-1-AP), anti-UBR2 (Abcam, ab217069), anti-PNPLA4 (Santa Cruz sc393988), anti-PNPLA3 (Proteintech, 11442-1-AP), anti-phospho-Akt (Ser473) (CST#9271), anti-phospho-Akt(Thr308) (CST#9275), and anti-Akt (CST#9272).

Lipid analysis

Lipids were extracted based on the chloroform/methanol (2:1, v/v) lipid extraction method (4). In brief, cells or 50 mg liver tissue were extracted with chloroform/methanol (2:1, v/v), then vortexed and refrigerated overnight. 3 mL of 0.043% magnesium chloride was then added to separate the aqueous phase from the chloroform phase which contains the lipids. After separation of the two phases, the upper water/methanol phase was removed. A 0.5-mL aliquot of the bottom phase was added to 1 mL of 0.5% Triton X-100 in chloroform and dried down. Triglyceride and total cholesterol levels were tested using commercially available kits (BioSino, Beijing, China). Free fatty acid level was tested using a kit (Merck, MAK044).

Dye staining and confocal imaging

The procedures for BODIPY 493/503 (Invitrogen, D3922) and Oil Red O staining of *C. elegans* were performed as described previously (5). Images were acquired by a confocal microscope (Leica SP8). The size of lipid droplets was calculated using image J. The number of lipid droplets was determined by visual inspection.

Fatty acid oxidation

The fatty acid oxidation (FAO) assay was performed using liver tissue as described with minor modifications (6). In brief, 200 mg of liver tissue was homogenized in 1 mL of STE buffer (0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA pH7.4) using a Dounce homogenizer. After centrifugation at 420g for 10 min at 4°C, the supernatant was saved for further testing. The fatty acid oxidation assay was performed by adding 200 µL tissue homogenate and 800 µL reaction mix (50 mM sucrose, 50 mM Tris-HCl, 20 mM KH₂PO₄, 10 mM MgCl₂, 1 mM L-carnitine, 2 mM EDTA, 0.7% BSA/500 µM palmitate/0.5 µCi [³H] palmitate (Perkin Elmer, NET043001mc)). The reaction was incubated at 37°C for 30 min and terminated by the addition of 0.5 mL of 3 M perchloric acid. After incubation for 1 hour at room temperature, the mixture was centrifuged at 13,200 rpm for 15 min at 4°C. 200 µL supernatant was mixed with 1.6 mL of scintillation liquid for radioactivity measurement. The results were normalized to protein concentration.

Triglyceride hydrolase (TGH) activity assay

TGH activity was measured according to previous work (3). In brief, 200 µg protein were extracted from adipose tissue in lysis buffer (50 mM Tris, 0.1 M sucrose, 1 mM EDTA pH7.4 and protease inhibitor cocktail), and added into 100 µL of substrate (0.015 µmol cold triolein/0.032 µmol [³H]-triolein). The substrate was prepared as follows. 0.015 µmol of cold triolein, 0.032 µmol of [³H]-triolein and 1 µmol of egg yolk lecithin were added into a glass tube and evaporated under a stream of N₂ gas. 100 µM of sodium taurocholate, 0.1 M of DTT and 2% fatty acid-free BSA were added and the volume was brought to 100 µL using 50 mM potassium phosphate buffer (pH 7.4). After sonication to form micelles, the substrate should appear clear. 100 µL of adipose protein lysate (200 µg total protein) was added into 100 µL of substrate, then incubated for 1 hour. The reaction was stopped by adding 3.25 mL of methanol:chloroform:heptane (10:9:7) and vortexed. After adding 1 mL of 0.1 M potassium carbonate and 0.1 M boric acid, each tube was vortexed and centrifuged at

800g for 20 min. 1 mL of the top phase from the mixture was removed and added into a scintillation vial containing scintillation cocktail. Samples were analyzed in a scintillation counter. 100 μ L of substrate was also counted to quantify the [3 H]-triolein. The FFA level was calculated using the following equation: 1 nmol FFA = CPM of 100 μ L substrate/90.

Glucose tolerance test (GTT), insulin sensitivity test (ITT) and energy expenditure measurement

For GTT, mice were fasted overnight and injected intra-peritoneally with glucose (2 g/kg body weight). Blood glucose was measured using a One Touch UltraVue Blood Meter (LifeScan Inc.). For ITT, mice were fasted for 5 hours and injected intra-peritoneally with insulin (0.8 U/kg body weight). For GTT and ITT data, the integrated area under the curve (iAUC) was calculated using GraphPad. For energy expenditure measurement, mice were monitored with a TSE PhenoMaster/LabMaster system for 3 days. The oxygen (O₂) consumption (mL/min) and carbon dioxide (CO₂) production (mL/min) were measured. Measurements were taken at 6-min intervals for the whole monitoring period. Energy expenditure (EE) was calculated as previously described (7).

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