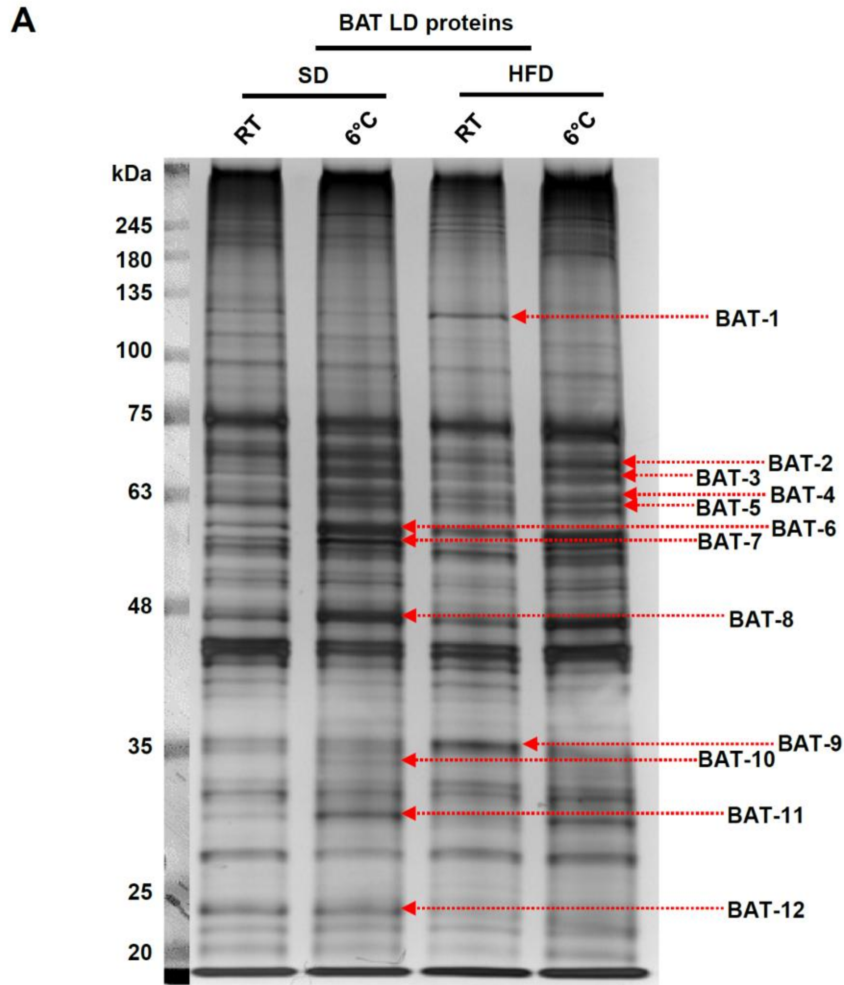
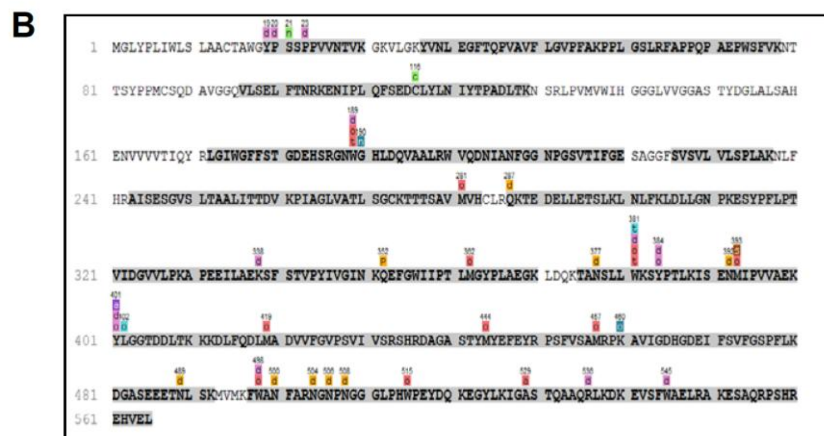
SUPPLEMENTARY DATA

Supplementary Figure S1. Identification of Ces3 as a major protein that targets lipid droplets (LDs) in response to cold exposure.

A. Silver stain of LD proteins isolated from BAT of SD or HFD fed mice with or without cold exposure at 6°C. (n = 3 ~ 6 / group). Arrows indicate the bands subjected for mass spectrometry analysis.

B. Identification of Ces3 (band BAT-7 in panel A) by LC-MS/MS. A coverage >85% of peptide fragments were identified that matched the sequence of Ces3. The labels at the top of the amino acids indicate different post-translational modifications





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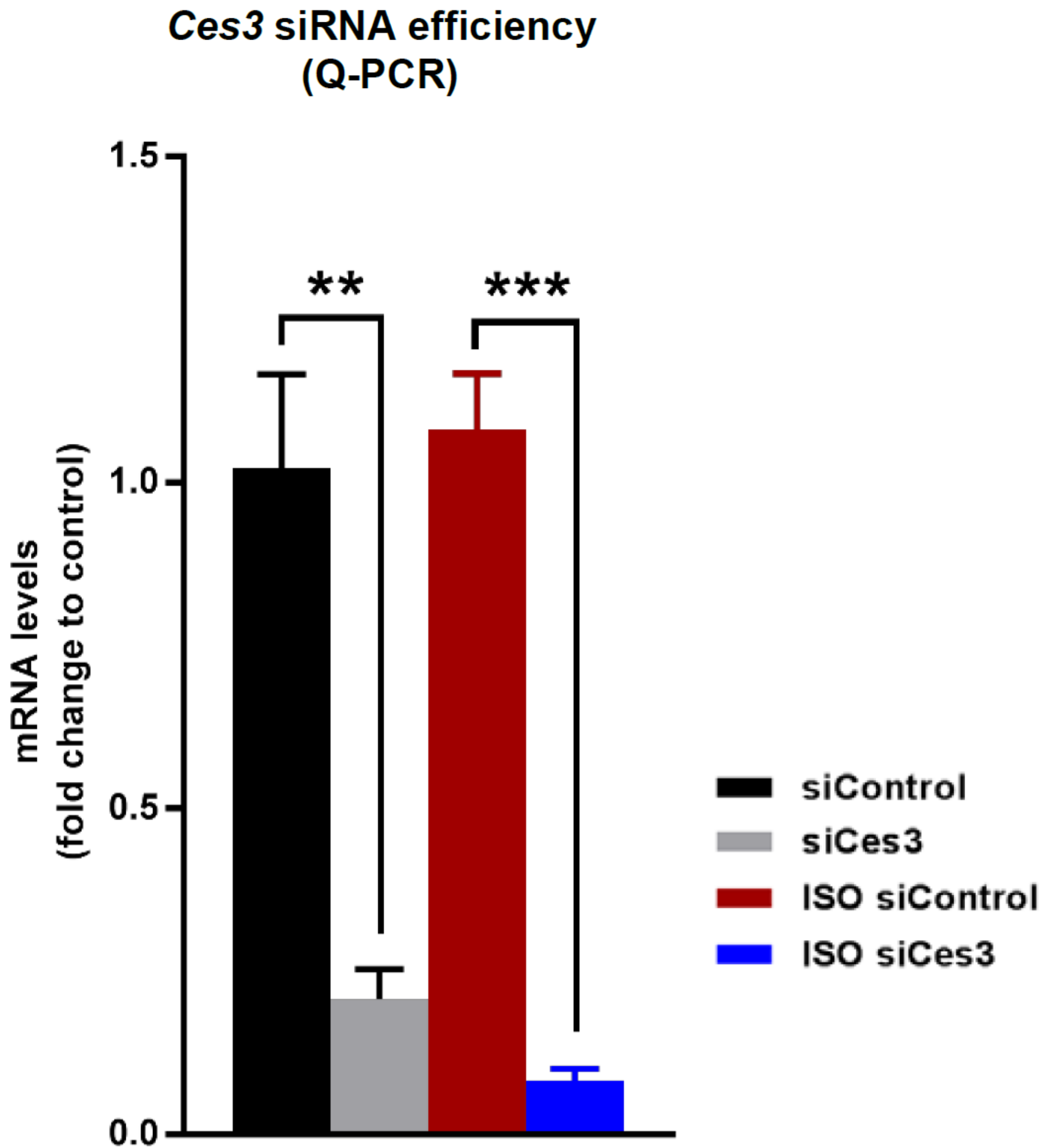
Supplementary Figure S2. Ces3 is efficiently knocked down by applying the siRNA on 3T3-L1 cells.

SiRNA was applied to the differentiated 3T3-L1 cells. The results show that with or without ISO treatment, siRNA significantly down-regulated mRNA levels of Ces3 in the cells.



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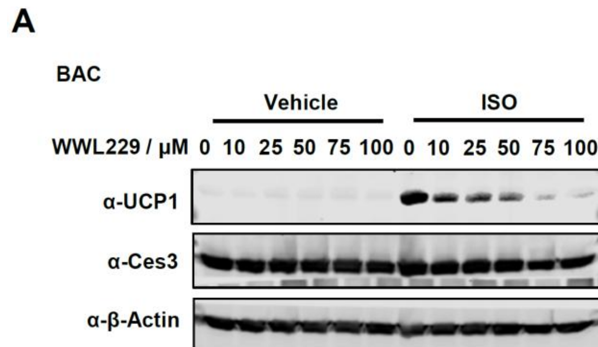
SUPPLEMENTARY DATA

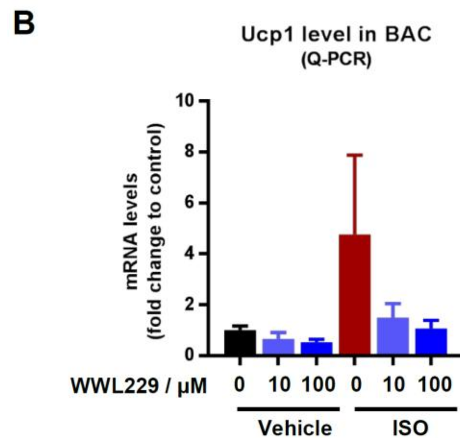
Supplementary Figure S3. The protein and mRNA levels of UCP1 in BAC cells when blunted Ces3 by WWL229.

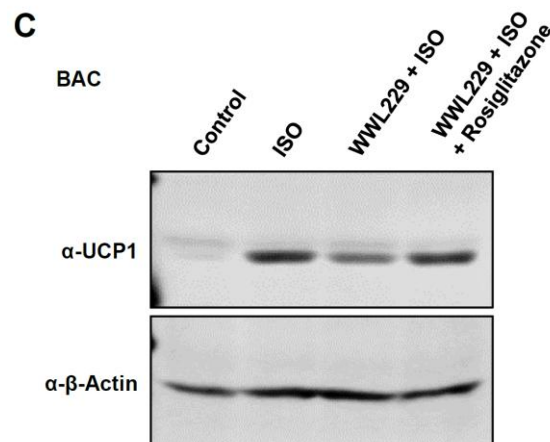
A. Western blotting analysis with α-UCP1, α-Ces3, and α-β-Actin antibodies on cell lysates of differentiated BAC cells treated by ISO together with or without indicated doses of WWL229 (10 μM, 25 μM, 50 μM, 75 μM, and 100 μM).

B. qPCR analysis of *Ucp1* gene in differentiated BAC cells treated by ISO together with or without indicated doses of WWL229 (10 μM and 100 μM) (n = 4 ~ 5).

C. Western blotting analysis with α-UCP1, and α-β-Actin antibodies on cell lysates of differentiated BAC cells treated by ISO together with or without WWL229 at the presence or absence of rosiglitazone (5 μM).







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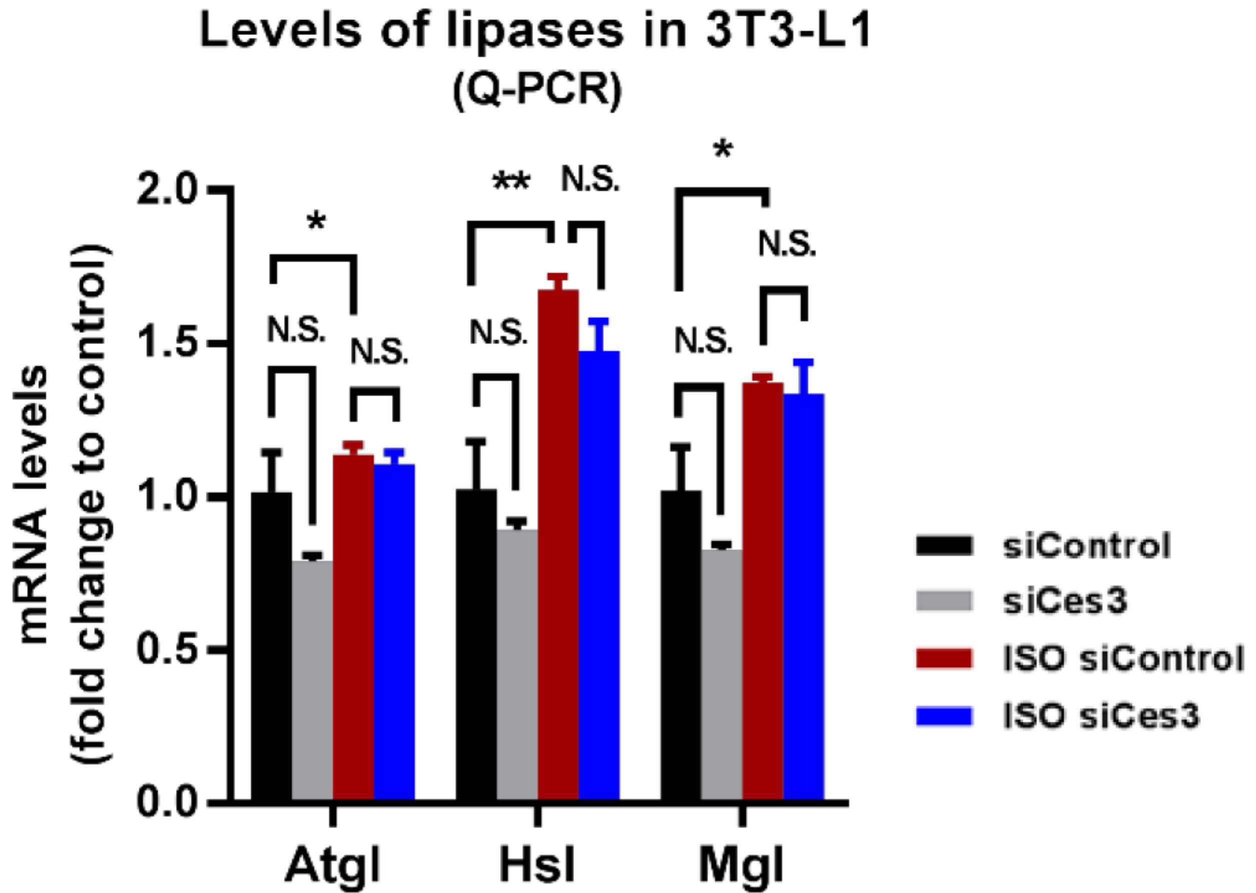
Supplementary Figure S4. Knockdown of Ces3 does not affect the expression of other lipases in 3T3-L1 cells.

qPCR analysis of mRNA levels of different lipases, including *Atgl*, *Hsl*, and *Mgl* in differentiated 3T3-L1 cells transfected with scramble or *ces3* siRNA. The cells were treated with ISO before harvesting (n = 3 ~ 6 / group; Student's *t* test, *, P < 0.05; **, P < 0.01).



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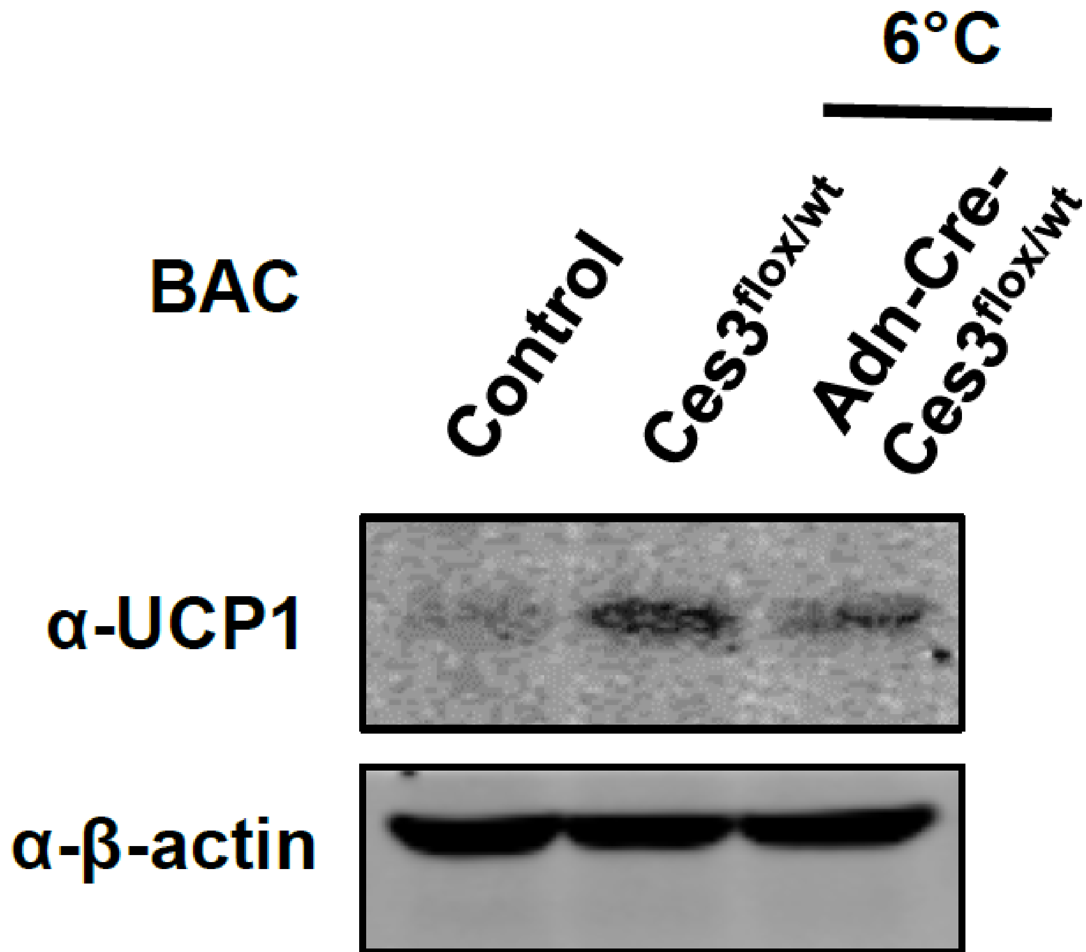
Supplementary Figure S5. The level of UCP1 in BAC cells is decreased by treatment of serum collected from cold exposed Adn-Cre-Ces3$^{flox/wt}$ mice when compared with the wild type mice. Western blotting analysis with α-UCP1 and α-β-Actin antibodies on cell lysates of differentiated BAC cells treated with fresh sera for 6 hrs. The sera were collected from Ces3$^{flox/wt}$ mice (control at room temperature), Ces3$^{flox/wt}$ and Adn-Cre-Ces3$^{flox/wt}$ mice upon cold exposure at 6°C (n = 2 ~ 6 / group).



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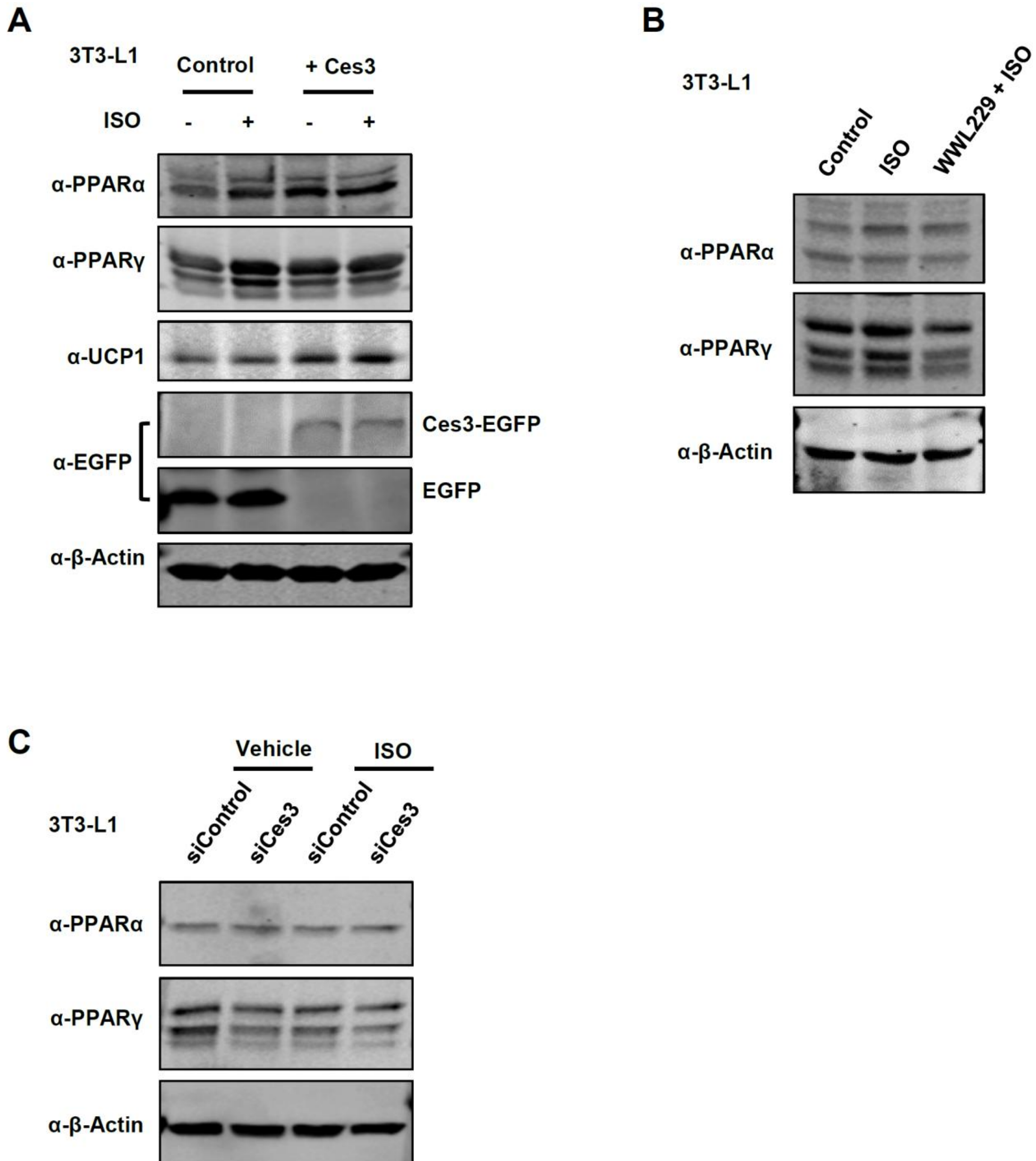
Supplementary Figure S6. Regulation of Ces3 does not significantly affect the levels of PPARα and PPARγ on protein levels in 3T3-L1 cells.

A. Western blotting analysis with α-PPARα, α-PPARγ, α-UCP1, α-EGFP and α-β-Actin antibodies respectively on cell lysates of differentiated 3T3-L1 cells transfected with or without Ces3 plasmid. The transfected cells were treated with or without ISO before harvesting.

B. Western blotting analysis with α-PPARα, α-PPARγ and α-β-Actin antibodies respectively on cell lysates of differentiated 3T3-L1 cells treated by ISO together with or without WWL229. **C.** Western blotting analysis with α-PPARα, α-PPARγ and α-β-Actin antibodies respectively on lysates of differentiated 3T3-L1 cells transfected with scramble or *Ces3* siRNA. The transfected cells were treated with ISO before harvesting.



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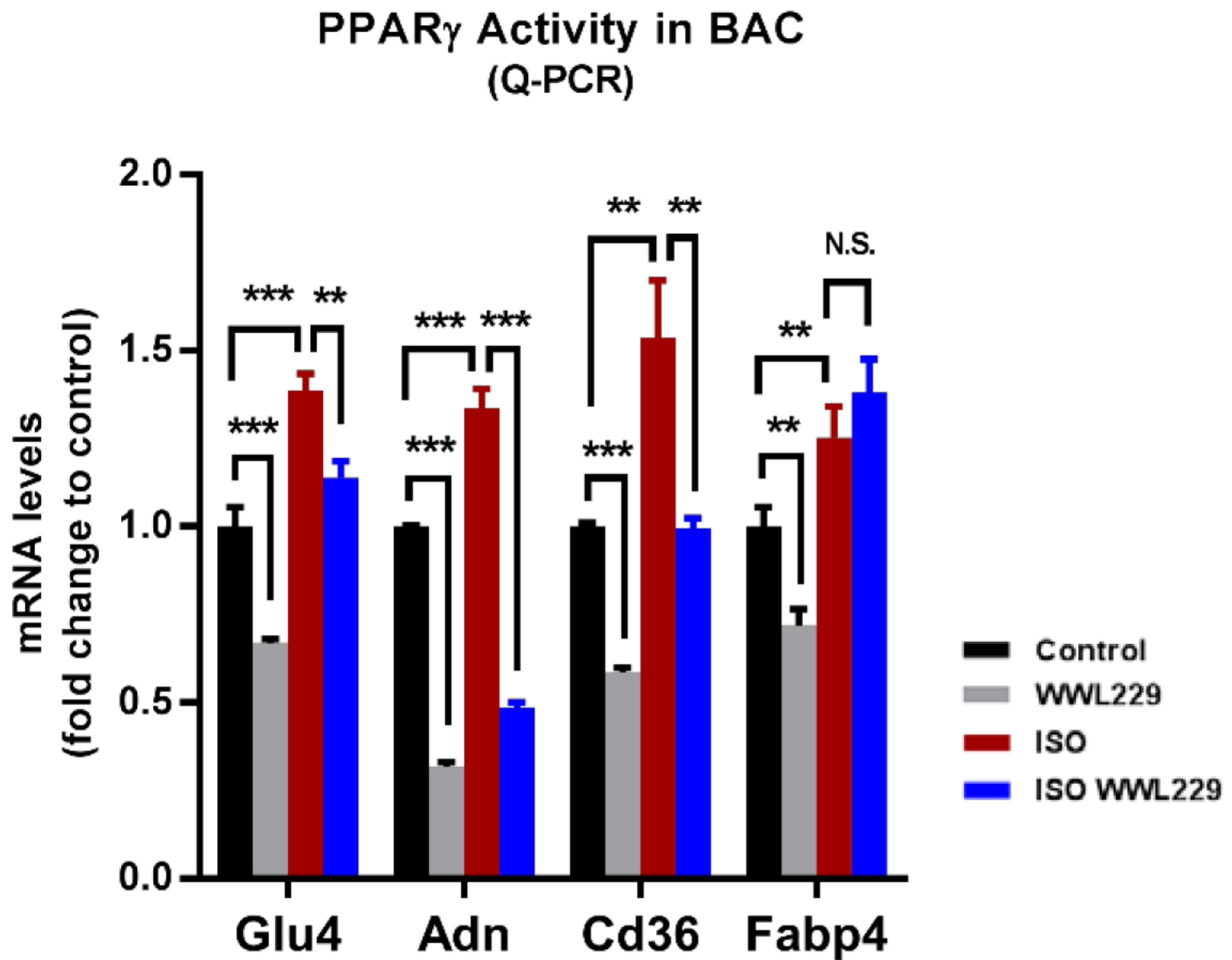
Supplementary Figure S7. WWL229 treatment significantly decreases downstream target genes of PPARγ in BAC cells.

qPCR analysis of levels of different downstream genes of PPARγ, including Glucose transporter type 4 (*Glu4)*, Adiponectin *(Adn)*, Cluster of differentiation 36 (*Cd36)* and Fatty acid binding protein 4 (*Fabp4)* in differentiated 3T3-L1 cells transfected with scramble or *ces3* siRNA. The cells were treated with ISO before harvesting (n = 3 ~ 6 / group; Student's *t* test, **, P < 0.01; ***, P < 0.001).



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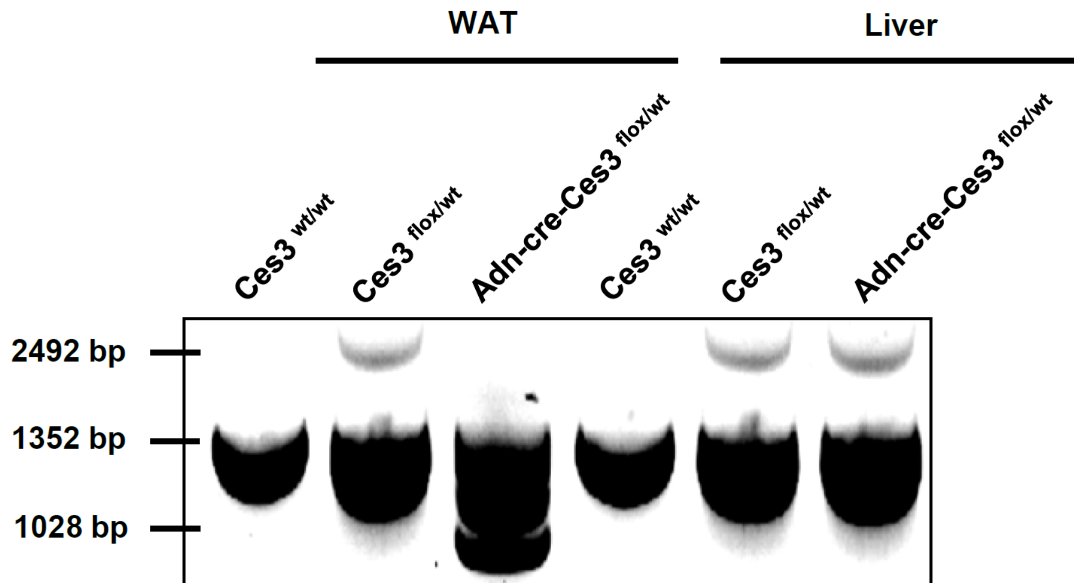
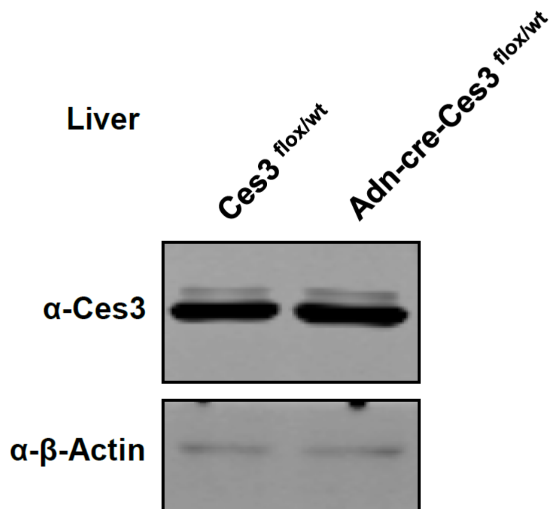
Supplementary Figure S8. Characterization of Adn-Cre-Ces3$^{flox/wt}$ mice.

A. Characterization of the knockout mice on genomic level by PCR using primers MTG12 and MTG20. Specifically, the primers of MTG12 and MTG20 locate outside of the two LoxP sites. This primer pair amplifies 1352 bp product in wild type allele, 2492 bp product in floxed allele without Cre and 1028 bp product in floxed allele with Cre. The 1028 bp band was detected exclusively in WAT of Adn-Cre-Ces3$^{flox/wt}$ mice, but not in liver and other tissues.

B. Western blotting analysis with α-Ces3, and α-β-Actin antibodies on lysates of liver of Ces3$^{flox/wt}$ or Adn-Cre-Ces3$^{flox/wt}$ mice.





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Supplementary Figure S9. WWL229 treatment slightly decreases body temperature at RT and mildly deceases energy expenditure in β3 agonist treated mice.

A. Body temperature of the wild type mice at room temperature upon WWL229 treatment (n = 3 / group; Student's *t* test, *, P < 0.05; **, P < 0.01).

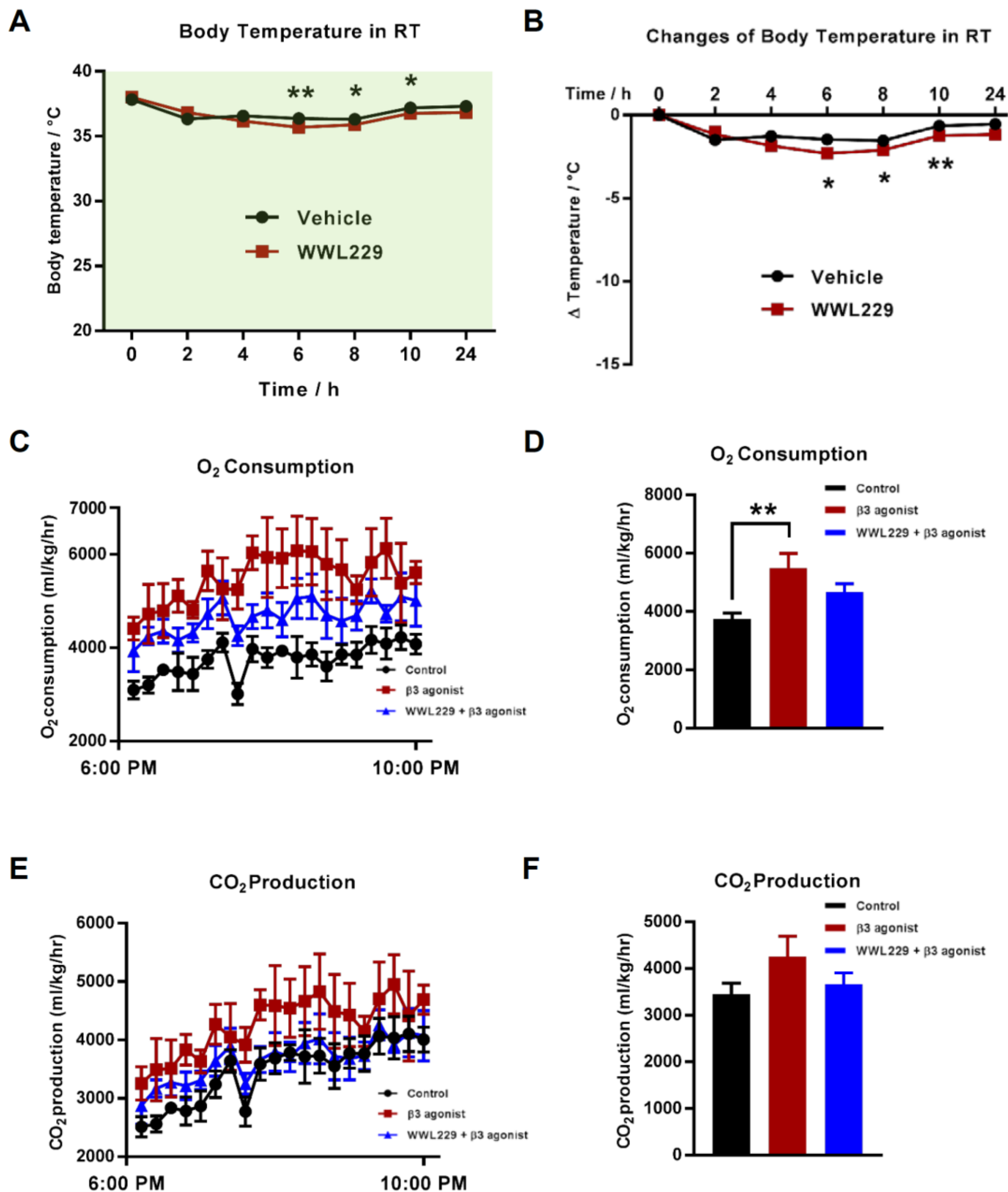
B. Changes of body temperature upon WWL229 treatment at room temperature (n = 3 / group; Student's *t* test, *, P < 0.05; **, P < 0.01).

C. O2 consumption in the wild type C57BL/6 mice treated with CL-316,243 (β3 agonist) at the presence or absence of WWL229 at room temperature (n = 3 ~ 4 / group).

D. Histogram representative of 4-hour periods of the results shown in panel (C) (n = 3 ~ 4 / group; Student's t test, **, P < 0.01).

E. CO2 production in the wild type C57BL/6 mice treated with CL-316,243 (β3 agonist) at the presence or absence of WWL229 at room temperature (n = 3 ~ 4 / group).

F. Histogram representative of 4-hour periods of the results shown in panel (E) (n = 3 ~ 4 / group).



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Animals

C57BL/6J (Stock 000664) and adiponectin-Cre (Stock 010803) were purchased from The Jackson Laboratory.

For the chronic cold exposure test, 6-week-old C57BL/6J male mice were fed on regular chow diet or high fat diet (HFD) (60% calories from fat, Research Diets) for 13 weeks. Then the mice were kept in chambers at 6°C or room temperature (RT) for 3 days. The mice were singly housed in the chambers without bedding and they had free access to water and diets. For the acute cold exposure test, 6-week-old male mice were kept in chambers at 6°C or RT for 12 hours. The mice were singly housed in the chambers without bedding and they had free access to water, but not diets. The mice rectal temperatures were measured before transferring the mice into chambers and every 2 hours after that. For Ces3 inhibitor treated mice, they were intraperitoneally injected with 50 mg/kg body weight of WWL229 or vehicle (1% dimethyl sulfoxide, 24% polyethylene glycol 400, and 6% Tween-80 in PBS) (1) and left at RT for 1 hour before the transferred into the chambers.

Metabolic cage study

For the indirect calorimetry study, 6-week-male mice were housed individually in TSE metabolic chambers (TSE Systems, Chesterfield, MO) and maintained on 12-hour light/dark cycle with *ad libitum* access to water and regular chow diet. After two days of acclimation, the mice were intraperitoneally injected with 50 mg/kg body weight of WWL229 or vehicle. 1 hour later,
the mice was injected with 1 mg/kg bodyweight of β3 agonist CL-316,243 or vehicle (PBS). Metabolic profiles were recorded continuously using TSE metabolic chamber documentation system. The data of the 4 hours period since β3 agonist injection were used for analysis.

Purification of lipid drop (LD) proteins and LC-MS/MS analysis

Isolation of LDs was carried out following the previous publication(2). Briefly, the tissues were cut into small pieces and suspended into the homogeneous buffer (25 mM tricine, pH7.6, 250 mM sucrose, and 0.5 mM PMSF) and differentiated 3T3-L1 cells were directly lysed in homogeneous buffer. Then the samples were transferred into a 2 mL loose-fitting Dunce Tissue Grinder (Kontes Glass Co.) and gently homogenized for 10 times. Afterward, the homogenates were collected and centrifuged at 2000 g at 4°C for 5 mins and the top LD fraction was carefully collected into a 1.5 ml tube. The supernatant of the remaining sample was further centrifuged at 20,000 g at 4°C for 20 mins and the top LD fraction was collected and combined with the previous collections. The LDs were then washed with 200 μL homogeneous buffer for 3 times. To extract LD proteins, 700 μL chloroform and 300 μL acetone were added to the washed LDs and mixed thoroughly by vortex followed by centrifuged at 20,000 g for 10 mins to precipitate the proteins. After being dried for 5 mins in the fume hood, the LD proteins were dissolved in 2 x SDS sample buffer and subjected to SDS-PAGE for further analysis.

Proteins of LDs were loaded and run on a standard 10% SDS-gel. For shotgun proteomics, protein samples were run into resolving gel about 1 cm and the gel squares containing sample proteins were cut off for analysis. For identifications of interest proteins, protein samples were fully separated in SDS-PAGE and then the gel was stained by Silver Staining Kit (Thermo Fischer Scientific) following the manufacturer's instructions. The gel bands of interest were then cut out
for analysis. The proteins in the cut gels were in-gel digested according to a previous publication (3) and then the tryptic-digested samples (in 2 % acetonitrile, 0.1% formic acid in water) were analyzed by LC-MS/MS on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Specifically, peptides were separated in Acclaim PepMap C18 column (75 µm ID x 15 cm, 2 µm, Thermo Scientific) at flow rate of 300 nL/min. The peptides were analyzed using data-dependent acquisition method (4). Orbitrap Fusion was operated by the measurement of FTMS1 at resolution of 120,000 FWHM, scan range of 350-1500 m/z, AGC target of 2E5 and maximum injection time of 50 ms. During a maximum 3 second cycle period of time, the ITMS2 spectra were collected under rapid scan rate mode, with CID NCE 35, isolation window of 1.6m/z, AGC target of 1E4 and maximum injection time of 35 ms. Dynamic exclusion was employed for 35 seconds. For data processing and analysis, the raw data were processed

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using Proteome Discoverer software v1.4 (Thermo Scientific). Spectra were searched against the Uniprot Mus musculus database using the Mascot search engine v2.3.02 (Matrix Science). Search results were trimmed to a 1% FDR for strict and 5% for relaxed condition using Percolator. Specifically, up to two missed cleavages were allowed for tryptic digestion. MS tolerance was set at 10 ppm and MS/MS tolerance were set at 0.8 Da. Heat maps of protein expression levels were generated by MATLAB software.

Cell culture and drug treatments

Mouse fibroblast 3T3-L1 cells were purchased from American Type Culture Collection. They were maintained in DMEM full medium (high glucose Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 10 μg/ml streptomycin) at 37°C in a humidified atmosphere containing 5% CO2. For adipocyte differentiation, 3T3-L1 cells were cultured until 100% confluence and then the medium was replaced with adipocyte differentiation medium I (high glucose DMEM supplemented with 10% fetal bovine serum (FBS),

100 units/ml penicillin, 10 μg/ml streptomycin, 1 μM dexamethasone, 5 μg/mL insulin, and 0.5 mM 3-isobutyl-1-methylxanthine) for 2 days followed by adipocyte differentiation medium II (high glucose DMEM supplemented with 10% FBS, 100 units/ml penicillin, 10 μg/ml streptomycin, and 5 μg/mL insulin) for 5 more days (5). The differentiated cells were subjected to further treatments.

Immortalized brown adipose cells (BAC) were a gift from Dr. Jiandie Lin's group at University of Michigan. BAC cells were maintained in the same medium as for 3T3-L1 cells as described above. For brown adipocyte differentiation, BAC cells were cultured until 100% confluence and then the medium was replaced with brown-adipocyte differentiation medium I (adipocyte differentiation medium I supplemented with 1 nM 3,5,3'-triiodothyronine (T3) and 5 μM rosiglitazone) for 2 days followed by brown-adipocyte differentiation medium II (white-adipocyte differentiation medium II supplemented with 1 nM T3) for 3 more days. The differentiated cells were subjected to further treatments.

Hela cells were a gift from Dr. Wenliang Li at University of Texas Health Science Center at Houston. The cells were maintained in DMEM medium containing 10% FBS, 100 units/ml penicillin and 10 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2.

Construction of plasmids

The constructs of pcDNA3-EGFP-Ces3 and pcDNA3-mRFP-Ces3 were generated by cloning the full-length cDNA of mouse Ces3 (NM_053200.2) into vectors of pcDNA3-EGFP (Addgene #13031) and pcDNA3-mRFP (Addgene #13032) with the restriction sites of EcoRI and NotI. The primers were as follows: F: 5'-ATGAATTC**ATG**GGCCTCTACCCTCTGATAT-3'; R: 5'-AAAATTT**TGC**GGCCGCTTGAGCTCAACATGTTCCCTG-3'.

Drug treatments and Ces3 knockdown by siRNA *in vitro*

Differentiated 3T3-L1 or BAC cells were pretreated with Ces3 inhibitor (WWL229, 10 μM or 100 μM), ATGL inhibitor (Atglistatin, 50 μM), PKA inhibitor (PKI 14-22 amide, 10 μM), adenylyl cyclase inhibitor (2', 5'-Dideoxyadenosine, 200 μM) or vehicle separately for 1 hour. Then the cells were maintained in the same treatment medium combined with or without 20 μM isoproterenol (ISO) for 6 more hours. Afterwards, the cells and cell culture media were harvested for further analysis.

For Ces3 knockdown experiments, differentiated 3T3-L1 or BAC cells were transfected with Ces3 siRNA (sc-72875, Santa Cruz Biotechonology) or scramble siRNA (sc-37007, Santa Cruz Biotechnology) with Lipofectamine RNAiMAX (Thermo Fisher Scientific). 2 days later, the cells were treated with or without 20 μM ISO for 6 hours. Then the cells and culture media were harvested for further analysis.

Hela cells were transfected with constructs of pcDNA3-EGFP-Ces3 or pcDNA3-EGFP. 24 hours later the cells were treated with 10 μM ISO, 10 nM CL316243 (β3-agonist), or 10 μM chloroquine for 6 hours before harvesting for co-immunoprecipitation analysis.

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Analysis of lipolysis in adipocytes

To analyze the lipolysis process, differentiated BAC cells were stained with 0.5 ng/µl BODIPY-C12 (Thermo Fisher Scientific) for 12 hours before treatment. Subsequently, the cells were pre-treated with or without WWL229 or atglistatin for 1 hour. Then the cells were maintained in the same treatments combined with or without ISO treatment followed by immediately transferred into the Cytation 5 Instrument (BioTek) for live imaging. The fluorescence density of
BODIPY-C12 was dynamically monitored during the process of live imaging. The level of lipolysis was assessed by a ratio of (D0 - D6)/D0%, in which D0 represents the density at time point of 0 hour and D6 at 6 hour.

Ex-vivo lipolysis assay

Adipose tissues (sWAT, eWAT and BAT), liver and muscle were surgically removed from 6-week-old wild type C57BL/6 male mice and washed with cold 1X PBS. Afterwards, the tissues were sliced into small pieces (0.5 mm x 0.5 mm x 0.5 mm) and 50 mg of tissues chucks were used for the *ex-vivo* lipolysis assay. The tissues were pretreated with 100 µM WWL229 or vehicle in 200 µl DMEM containing 2% fatty acid–free BSA at 37℃ for 1 hour with gentle shaking. Then, they were incubated with 20 µM ISO or vehicle together with or without WWL229 at 37℃ for 6 hours with gentle shaking. Glycerol level in the culture medium was determined using glycerol assay kit (BioAssay System).

Luciferase assay

Ces3-EGFP plasmid was co-transfected with the 3 x PPRE-luc (for PPARα and/or PPARγ) and β-Galactosidase plasmids into BAC cells with Lipofectamine 3000 followed the protocol provided by the manufacturer. 36 hours after transfection, cells were pre-treated by WWL229 for 1 hour and followed by ISO treatment for 12 hours. Luciferase activities in the lysed cells were determined by Dual-Light™ Luciferase & β-Galactosidase Reporter Gene Assay System (Thermo Fisher Scientific, USA), and normalized to β–galactosidase activity. 3 x PPRE-luc for PPARα was a gift from Dr. Yanqiao Zhang's lab in Northease Ohio University 3 x PPRE-luc for PPARγ plasmid was from Addgene (#1015, Addgene).

Western blotting

Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane followed by blocked in 5% fat free milk. Afterward, the PVDF membranes were incubated with primary antibodies at 4°C overnight followed by washed with 1 x PBST (0.1% Tween 20 in 1 x PBS, pH = 7.4) for 3 times (5 mins/each wash). Then the membranes were incubated with secondary antibodies at RT for 1 hour. After being washed with 1 x PBST for 3 times (5 mins/each wash) the blots were imaged by Odyssey software (LI-COR Biosciences) and the band densities were analyzed by ImageJ software. The primary and secondary antibodies used are listed as follows: anti-Ces3 (Thermo Fisher Scientific), anti-Perilipin-1, anti-Cide-C (Abcam Biotechnology), anti-HSL, anti-pHSL(Ser563), anti-pHSL(Ser565), anti-pHSL(Ser660), anti-UCP-1, anti-Adiponectin, anti-β-actin (Cell Signaling Technology), IRDye 680RD donkey anti-mouse IgG, IRDye 800CW donkey anti-rabbit IgG, and IRDye 800CW donkey anti-goat IgG (LI-COR Biosciences).

Measurement of Oxygen Consumption Rate (OCR) by Seahorse

Oxygen consumption rates (OCR) of differentiated adipocytes were measured at 37°C using a Seahorse XFe24 Analyzer (Agilent Technologies) as described previously (6). Briefly, 3T3-L1 cells were cultured and differentiated in XFe24 cell culture plates. After treatments of WWL229 or Ces3 siRNA as described above, the medium was replaced with pre-warmed assay medium (Seahorse base medium supplemented with 1 mM pyruvate, 10 mM glucose, and 2 mM glutamine, pH = 7.4) and the cells were incubated at 37°C in a non-CO2 incubator for 1 hour. Then the cells were transferred to Seahorse XFe24 to record the OCR at different time windows. The following compounds were subsequently injected into assay medium to assess cell mitochondrial function:
5 µM oligomycin, 1 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and a mixture of 5 µM rotenone and 15 µM antimycin A. The key parameters of mitochondrial respiration including

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basal respiration, proton leak, and maximal respiration were automatically calculated by the Seahorse Wave software. The OCR readings were normalized to the cell numbers in each well.

Histology

The adipose tissues were immediately collected after sacrificing the mice and fixed in 10% PBS-buffered formalin (pH = 7.4, Fisher Scientific) for 48 hours. The fixed tissues were paraffin embedded and sectioned at 5μm size. After deparaffinized, the sections were stained with hematoxylin and eosin (H & E, Sigma-Aldrich) using standard protocol as described previously (7). For immunofluorescence (IF) staining, the deparaffinized sections were permeabilized with 1 x PBS containing 0.2% Triton X-100 for 10 mins followed by incubated with sodium citrate buffer at 95°C for 30 mins for antigen retrieval. After being blocked in 5% bovine serum albumin, the sections were stained with primary antibodies at 4°C overnight followed by washed with 1 x PBST for 3 times (5 mins/wash). Then the sections were incubated with secondary antibodies at RT for 1 hour. After washed with 1 x PBST for 3 times (5 mins/wash), the sections were mounted and images were acquired with Leica TCS SP5 Confocal Laser Scanning Microscope. The primary antibodies used were described above. The secondary antibodies used are: Alexa Fluor 488-conjugated donkey anti-rabbit IgG and Alexa Fluor 647 conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories).

For the cell samples, cells were seeded and cultured on cover glass in 12-well cell culture plates. After treatments, the cells were fixed with 4% paraformaldehyde at RT for 20 mins

followed by permeabilized with 1 x PBS containing 0.2% Triton X-100. The IF staining was carried out as described above.

Total RNA extraction and quantitative PCR (Q-PCR)

Total RNAs of adipose tissue and cells were extracted by TRIzol (Invitrogen) following the manufacturer's instructions. cDNAs were obtained by reverse-transcribing 1 μg of total RNAs with RevertAid Reverse Transcription Kit (Thermo Fisher Scientific). Q-PCR reactions were carried out on Bio-Rad CFX96 system (Bio-Rad Laboratories). Results were normalized by 18s and calculated using the 2-ΔΔCt method (8). The primer sequences are listed as follows: mCes3: F: 5'-GGCATCAACAAGCAAGAGTTTGGC-3', R: 5'-CTTTTTGGTGAGGTGATCTGTCCC-3'; mCide-C: F: 5'-C ATTGTGCCATCTT CCTCCAG-3', R: 5'-ATCATGGCTCACAGCTTGG-3'. Other primer sequences were described previously (5; 7).

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