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Supplemental information

Chronic cold exposure induces autophagy to promote fatty acid oxidation, mitochondrial turnover, and thermogenesis in brown adipose tissue

Winifred W. Yau, Kiraely Adam Wong, Jin Zhou, Nivetha Kanakaram Thimmukonda, Yajun Wu, Boon-Huat Bay, Brijesh Kumar Singh, and Paul Michael Yen

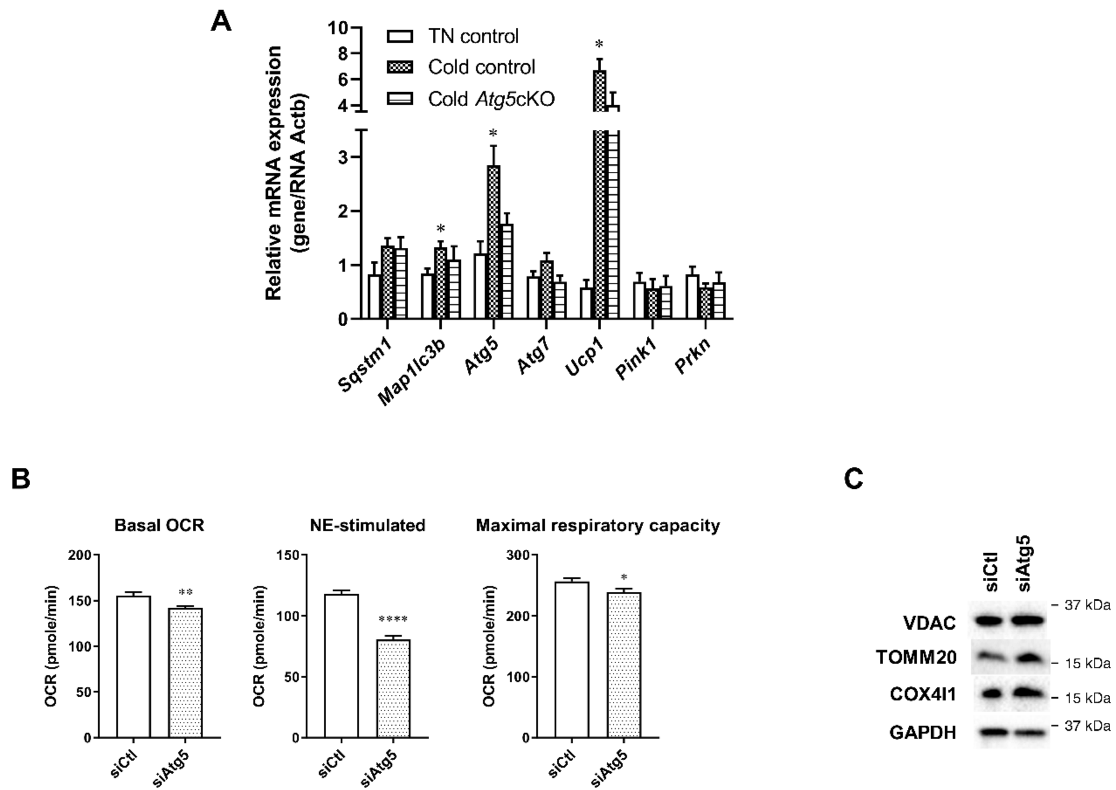

Figure S1. Autophagy in BAT is required for thermogenesis in chronic cold exposure, Related to Figure 4

(A) mRNA expression of autophagy gene in BAT of control and *Atg5*cKO mice housed at thermoneutral condition or in cold for 72 hours. Values are means±SEM for 5 mice in each group. (B) Basal, NE-stimulated and maximal OCR in primary brown adipocytes transfected with control or *Atg5* siRNA for 48 hr before analysis (C) Representative immunoblots and densitometry showing mitochondrial proteins in primary brown adipocytes transfected with control or *Atg5* siRNA for 48 hr. Statistical significance shows as *p<0.05, **p<0.01 and ****p<0.0001.

Transparent Methods

Study approval

All mice were maintained according to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 1.0.0. Revised 2011), and experiments were approved by the IACUC at Duke-NUS Graduate Medical School.

Statistics

All cell culture related experiments were performed in triplicate and independently repeated to ensure data reproducibility. Data were pooled and represented as either mean ± SD for cell culture experiments or mean ± SEM for animal experiments.

Densitometry data were calculated for all Fig.s using ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA) were pooled and represented as mean ± SD. Statistical significance of differences ($p < 0.05$) was examined by two-tailed Student's t-test or one-way analysis of variance followed by Tukey post-hoc test using GraphPad PRISM v8.0 (GraphPad Software, Inc.).

Reagents

Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich and all culture media were from Gibco (Thermo Fisher Scientific). Transfection reagents and siRNA (Ambion, 4390771) were purchased from Thermo Fisher Scientific. The tandem RFP/GFP-tagged LC3 plasmid and mito-RFP-EGFP plasmids were kind gifts from Prof. T. Yoshimori (Osaka University, Osaka, Japan) and Dr Andreas Till (Institute of Clinical Molecular Biology Christian-Albrechts-University of Kiel; Kiel, Germany), respectively. pMitoTimer was a gift from Zhen Yan (Addgene, 52659). Norepinephrine (NE) was purchased from Sigma-Aldrich (A0937).

Animals and experimental protocol

Male C57BL/6 mice (8 to 10 wk old) were housed in hanging polycarbonate cages and kept on a 12 h light-dark cycle in a temperature-controlled room at 24°C. They were allowed *ad libitum* access to water and a standard laboratory chow. Mice are house at thermoneutral condition (30°C) or in cold (4°C) for 72 hr before sacrifice. Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals and experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and Duke-NUS Graduate Medical School. Body temperature was measured using a rectal probe after 72 hr exposure to thermoneutral condition (30°C) or cold (4°C).

Adenoviral infection

For tissue-specific knockout study, male homozygous *Atg5*$^{Flox/Flox}$ mice (C57BL/6/129) were obtained from the Riken BioResource Center, Japan, courtesy of Dr. Noboru Mizushima. A total of 5×10^{11} genome copies of control adenovirus or Ucp1-Cre-expressing adeno-associated virus (Vector BioLabs, AAV8-UCP1-eGFP and AAV8-UCP1-iCre) was injected into tail vein of 8-week-old male *Atg5*$^{Flox/Flox}$ mice for four weeks before initiating the cold exposure experiments. Core body temperature was measured using a rectal temperature probe on the last day of cold challenge.

Primary culture of brown preadipocytes

Primary brown adipocytes were derived from the interscapular BAT of 2 to 3 weeks old C57BL/6 mice. The precursor cells were isolated according to the protocols described by Rehnmark et al. and Klein et al. Briefly, BAT from 6 to 8 mice was cut into small pieces and digested with 0.2% (w:v) collagenase (Sigma-Aldrich, C6885) for 30 min in a shaking water

bath. After filtering through a 150-μm nylon screen (Fisher Scientific, 22363549), the pellet containing preadipocytes were collected by centrifugation at 200 g for 5 min. Preadipocytes were maintained in DMEM (Gibco, 11995–065) supplemented with 15% heat inactivated FBS (Gibco, 10500–064), non-essential amino acids (Gibco, 11140–076), 5 ng/ml human basic FGF (Thermo Fisher Scientific, PHG0021), 100 U/ml penicillin and 50 µg/ml streptomycin (Sigma-Aldrich, F7524). Differentiation was initiated with DMEM containing 10% fetal bovine serum (FBS; Gibco, 15140122), dexamethasone (Sigma-Aldrich, D1756), 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, I5879) and insulin (Sigma-Aldrich, I9278). Cells were allowed to differentiate for 6 d before treatment. For NE treatment, cells were treated with 1 µM NE for 0, 6, 24 or 72 hr. For autophagic flux assay, cells were treated with 50 nM bafilomycin A1/Baf (Sigma-Aldrich, B1793) 6 hr before harvest. For RNA interference studies, Lipofectamine® RNAiMAX Reagent (Invitrogen, 13778075) was used to transfect the cells 48 hr before NE treatment.

Cell line transfection
Plasmid transfection were performed in the brown adipocyte cell line mBAP-9 (Wu and Smas, 2008). Cells were seeded on cell culture slide (SPL Life Sciences, 30104) and maintained in DMEM containing 10% FBS and differentiated by DMEM containing 10% FBS, insulin, T_3, dexamethasone and IBMX for 6 d before treatment. Transfection was carried out 2 d before treatment using Lipofectamine® 3000 Reagent (Invitrogen, L3000008) in accordance to the manufacturer's protocols. After transfection, cells were treated with 1 µM of NE for 72 h before fixation with 4% paraformaldehyde (Sigma-Aldrich, P6148). Cell imaging was performed using a Zeiss LSM 710 Confocal Microscope.

Protein carbonylation
The amount of protein carbonyls was measured with an OxyBlot™ Oxidized Protein Detection Kit (EMD Millipore, S7150) according to the manufacturer's protocol.

RNA isolation and quantitative reverse transcription polymerase chain reaction
Total RNA was extracted from the BAT with TRIzol reagent (Invitrogen) followed by InviTrap spin cell RNA Minikit (Stratec Molecular) according to the manufacturer's instructions. RNA concentration was measured by NanoDrop 8000 (Thermo Scientific), and cDNA was reverse transcribed from 1000 ng of total RNA using high-capacity cDNA reverse transcription kits (BioRad). The quantitative reverse transcription polymerase chain reactions (qRT-PCRs) were performed using the QuantiFast SYBR Green PCR Kit (QIAGEN) on the 7900HT Fast Real-Time PCR system (Applied Biosystems). Relative mRNA levels were calculated using the 2–ΔΔCt method and normalized to suitable reference genes following cold exposure. Data are expressed as the fold change relative to the mean value of non-treated mice.

Electron microscopy
Cells were seeded in a 4-chambered coverglass (Thermo Scientific Nunc, NNU 155383-PK) and allowed to differentiate for 7 d before addition of NE or bafilomycin A1 (Baf; Sigma-Aldrich, B1793). After treatment, cells were fixed with 2.5% glutaraldehyde (nacalai tesque, 17025–25) in sodium phosphate buffer (0.1 M, pH 7.4) and washed 3 times with phosphate buffered saline (PBS; Axil Scientific, BUF-2040-1X1L). The samples were then post-fixed with 1% osmium tetroxide and dehydrated with a series of alcohol with increasing concentration. After embedding samples in Araldite (Pelco, 18060), ultra-thin sections were cut and double-stained with uranyl acetate and lead citrate. Images were taken using the JEOL JEM-1010 transmission electron microscope (Japan).

Western blot analysis

Cells and tissues were dissociated in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1% Triton X-100 (Bio-Rad, 1610407), 0.5% sodium deoxycholate (Sigma-Aldrich, D6750), 0.1% sodium dodecyl sulfate, 2 mM EGTA, 2 mM EDTA, protease inhibitors (Sigma-Aldrich, P8340) and phosphatase inhibitors (Sigma-Aldrich, P5726, P0044). Proteins were denatured by boiling in Laemmli sample buffer (250 mM Tris-HCl, pH 7.4, 2% w:v sodium dodecyl sulfate, 25% v:v glycerol, 50 mM DTT, 0.01% w:v bromophenol blue). Equal amount of proteins was resolved on sodium dodecyl sulfate-polyacrylamide gels using the Mini-PROTEAN 3 Electrophoresis unit and transferred to polyvinylidene difluoride membranes (Bio-Rad) using the TransBlot® Turbo™ Transfer System (Bio-Rad). The following antibodies were used to detect the target proteins. Cell Signaling Technology: MAP1LC3B (2775; RRID:AB_915950), SQSTM1 (5114; RRID:AB_10624872), COX4I1 (4850; RRID:AB_2085424), ATG5 (2630; RRID:AB_2062340), PRKAA (5831; RRID:AB_10622186), phospho-PRKAA (Thr172) (2535; RRID:AB_331250), ULK1 (8054; RRID:AB_11178668), phospho-ULK1 (S555) (5869; RRID:AB_10707365), MTOR (2983; RRID:AB_2105622), phospho-MTOR (Ser2448) (5536; RRID:AB_10691552), RPS6KB1 (9202; RRID:AB_331676), phospho-RPS6KB1 (T389) (9205; RRID:AB_330944), EIF4EBP1 (9452; RRID:AB_331692), phospho-EIF4EBP1 (Thr37/46) (2855; RRID:AB_560835), LIPE (4126; RRID:AB_490997), VDAC (4661; RRID:AB_10557420), DNM1L (8570; RRID:AB_10950498) and GAPDH (2118; RRID:AB_561053). Santa Cruz Biotechnology: TOMM20 (sc-11415; RRID:AB_2207533) and Abcam: UCP1 (ab10983; RRID:AB_2241462), phospho-PNPLA2 (S406) (ab135093; RRID:AB_2888660), PPARGC1A (ab191838; RRID:AB_2721267) and CPT1A (ab128568; RRID:AB_1114163).

Seahorse XF analyzer measurement for mitochondrial oxygen consumption rate (OCR)

Preadipocytes were seeded on XF-24-well culture microplates and allowed to differentiate for 7 d. After treatment, oxygen consumption was measured using a microplate (type XF24) extracellular analyzer (Seahorse Bioscience, Billerica, MA, USA). Reagents were optimized using the Mito stress kit from Seahorse Bioscience (Agilent, 100850–001) using the protocol and algorithm program in the analyzer. Oligomycin (1 µM), which inhibits the F0 proton channel of the F0F1-ATP synthase, was employed to determine the oligomycin-independent leak of the OCR. The mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 2 µM) was added to determine the total respiratory capacity of the mitochondrial electron transport chain. Rotenone (1 µM) and antimycin A (1 µM) (R+A) was added to block complex I and complex III of electron transport chain. The following mitochondrial functional parameters were calculated as follows: (i) basal O2 consumption = baseline OCR reading per well, before compounds are injected subtracting non-mitochondrial respiration (after R+A injection); (ii) NE stimulated = OCR reading per well after NE injection subtracting baseline OCR reading per well (before compounds are injected); (iii) maximum respiratory capacity = oxygen consumption reading per well (after FCCP injection) subtracting non-mitochondrial respiration (after R+A injection). Computed data were plotted as bar graphs.

Metabolic profiling of acylcarnitines

Acylcarnitines in BAT were measure in the Duke-NUS Metabolomics Facility according to previously established mass spectrometry (MS)-based methods. Briefly, brown adipose tissue was homogenized in 50% acetonitrile and 0.3% formic acid. For acylcarnitine extraction, 100 µl of tissue homogenate was extracted using methanol. The acylcarnitine extracts were derivatized with 3 M hydrochloric acid in methanol, dried, and reconstituted in methanol for analysis in liquid chromatography/mass spectrometry (LC/MS). Acylcarnitine measurements were made using flow injection–tandem mass spectrometry on the Agilent 6430 Triple

Quadrupole LC/MS system (Agilent Technologies, CA, USA). The sample analysis was carried out at 0.4 ml/min of 80:20 methanol:water as mobile phase and injection of 2 µl of sample. Data acquisition and analysis were performed on Agilent MassHunter Workstation B.06.00 software.