SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids and viruses

Mutagenesis was carried out using QuikChange II kit (Agilent) according to the manufacturer's instructions. Entry clones were shuttled with LR Clonase (Thermo Fisher Scientific) into pAd/CMV/V5-DEST (Thermo Fisher Scientific) for adenovirus production or an in-house generated gateway compatible variant of pCLHCX-DEST, modified from pCLNCX (Novus), for mammalian expression. PM20D1- or LacZ-expressing adenoviruses were generating according to manufacturer's instructions (Thermo Fisher Scientific) and purified using Vivapure AdenoPACK 100 (Sartorius). PM20D1- or GFP-expressing retroviruses were generating according to manufacturer's instructions (Novus) and used directly. The Pm20d1-6xHis-Flag construct was cloned into pENN.AAV8.CB7.CI.WPRE.rBG (Penn Vector Core) using the PstI/HindIII sites. AAV-GFP virus (AAV8.CB7.CI.eGFP.WPRE.rBG) was purchased from Penn Vector Core. AAV-PM20D1 virus was generated by Penn Vector Core using the pENN.AAV8-Pm20d1 plasmid.

Global gene expression analysis and comparisons

The following publicly available datasets were used for the comparisons: UCP1-TRAP (GSE56248), brown versus white adipose tissues (GSE8044), and inguinal fat following 1 or 5 weeks cold exposure (GSE13432). For the UCP1-TRAP dataset (GSE56248), genes were considered expressed if the average signal intensity>1 FPKM and the FPKM>0.3 in all replicates (n=7230). For the brown versus white adipose tissues dataset (GSE8044), genes were considered enriched in UCP1+ cells if the BAT versus WAT fold change>4, and the adjusted p<0.05 (n=494). For the 1 or 5 week cold exposure of the inguinal fat (GSE13432), genes were considered cold-induced if the 4°C versus 30°C fold change>4, and the adjusted p<0.05 (n=200 and 96, respectively).

Chemicals

N-arachidonoyl dopamine, *N*-oleoyl ethanolamine, arachidonic acid, sodium oleate, phenylalanine, noladin ether, and R(+)-methanandamide were purchased from Sigma Aldrich; *N*-arachidonoyl glycine, *N*-arachidonoyl serine, *N*-arachidonoyl taurine, and *N*-methyl *N*-arachidonoyl amide were purchased from Cayman Chemical Company; *N*-arachidonoyl phenylalanine was purchased from Abcam; and *N*-arachidonoyl gamma amino butyric acid was purchased from Santa Cruz Biotechnology. The synthesis of the non-commercially available *N*-acyl amino acids is described below.

Synthesis of *N*-acyl amino acids

Method A: To a solution of amino acid (1 eq.) in acetone and water (1:1) was added with K_2CO_3 (2 eq.) and oleoyl chloride (1.5 eq.) at 0 ^oC. Then the mixture was stirred at room temperature overnight. The reaction mixture was acidified with HCl (1M) until pH 4.0 before extracted with ethyl acetate. The organic phase was washed with brine, dried over anhydrous Na_2SO_4. Then the solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel to give the desired amide.

Method B: To a solution of fatty acid (1 eq.) in DCM was added with oxalyl chloride (1.2 eq.) and one drop of DMF at 0 ^oC. Then the mixture was stirred at room temperature for 2 hours. The mixture was concentrated and dissolved in DCM, added to a suspension of amino acid (1.5 eq.) and DIPEA (2 eq.). The reaction mixture was stirred at room temperature overnight before acidified by HCl (1.0 M) to pH 4.0. The result mixture was extracted with DCM, washed with brine, dried over anhydrous Na_2SO_4. Then the solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel to give the desired amide.

Oleoyl-*L*-phenylalanine, Method A. White solid.
^{1}H NMR (400 MHz, DMSO-d^6) δ 0.85 (t, J = 5.0 Hz, 3H), 1.09-1.31 (m, 22H), 1.33-1.40 (m, 2H), 1.95-2.04 (m, 6H), 2.82 (dd, J = 10.0, 13.8 Hz, 1H), 3.04 (dd, J = 4.7, 13.4 Hz, 1H), 4.38-4.44 (m, 1H), 5.29-5.36 (m, 2H), 7.16-7.28 (m, 5H), 8.08 (d, J = 8.0 Hz, 1H), 12.61 (brs, 1H)
HRMS (ESI) m/z calcd for $C_{27}H_{44}NO_3$ $[M+H]^+$ 430.3316, found: 430.3317

Dodecanoyl-*L*-phenylalanine, Method A. White solid.
^{1}H NMR (400 MHz, DMSO-d^6) δ 0.86 (t, J = 6.6 Hz, 3H), 1.08-1.31 (m, 16H), 1.34-1.41 (m, 2H), 2.02 (t, J = 7.4 Hz, 2H), 2.83 (dd, J = 10.2, 13.8 Hz, 1H), 3.05 (dd, J = 4.6, 13.8 Hz, 1H), 4.38-4.44 (m, 1H), 7.17-7.28 (m, 5H), 8.08 (d, J = 7.0 Hz, 1H), 12.65 (brs, 1H)
HRMS (ESI) m/z calcd for $C_{21}H_{34}NO_3$ $[M+H]^+$ 348.2533, found: 348.2544

Pentadecanoyl-*L*-phenylalanine. White solid.
^{1}H NMR (400 MHz, DMSO-d^6) δ 0.85 (t, J = 7.0 Hz, 3H), 1.08-1.31 (m, 22H), 1.33-1.40 (m, 2H), 2.02 (t, J = 7.3 Hz, 2H), 2.82 (dd, J = 10.0, 13.8 Hz, 1H), 3.04 (dd, J = 4.7, 13.8 Hz, 1H), 4.38-4.44 (m, 1H), 7.16-7.28 (m, 5H), 8.09 (d, J = 8.2 Hz, 1H), 12.64 (brs, 1H)
HRMS (ESI) *m/z* calcd for $C_{24}H_{40}NO_3$ $[M+H]^+$ 390.3003, found: 390.2950

Palmitoyl-*L*-phenylalanine, Method A. White solid.
^{1}H NMR (400 MHz, DMSO-d^6) δ 0.85 (t, J = 6.7 Hz, 3H), 1.08-1.31 (m, 24H), 1.35-1.40 (m, 2H), 2.02 (t, J = 7.3 Hz, 2H), 2.83 (dd, J = 10.0, 13.8 Hz, 1H), 3.04 (dd, J = 4.7, 13.8 Hz, 1H), 4.38-4.44 (m, 1H), 7.17-7.28 (m, 5H), 8.08 (d, J = 8.2 Hz, 1H), 12.63 (brs, 1H)
HRMS (ESI) *m/z* calcd for $C_{25}H_{42}NO_3$ $[M+H]^+$ 404.3159, found: 404.3159

Stearoyl-*L*-phenylalanine, Method A. White solid.
^{1}H NMR (400 MHz, DMSO-d^6) δ 0.85 (t, J = 6.7 Hz, 3H), 1.08-1.31 (m, 28H), 1.33-1.40 (m, 2H), 2.01 (t, J = 7.3 Hz, 2H), 2.82 (dd, J = 10.0, 13.8 Hz, 1H), 3.04 (dd, J = 4.7, 13.8 Hz, 1H), 4.38-4.44 (m, 1H), 7.16-7.28 (m, 5H), 8.09 (d, J = 8.2 Hz, 1H), 12.63 (brs, 1H)
HRMS (ESI) *m/z* calcd for $C_{27}H_{46}NO_3$ $[M+H]^+$ 432.3472, found: 432.3477

Icosanoyl-*L*-phenylalanine, Method B. White solid.
^{1}H NMR (400 MHz, DMSO-d^6) δ 0.86 (t, J = 5.0 Hz, 3H), 1.09-1.31 (m, 32H), 1.33-1.40 (m, 2H), 2.02 (t, J = 7.3 Hz, 2H), 2.83 (dd, J = 10.2, 13.8 Hz, 1H), 3.05 (dd, J = 5.3, 14.2 Hz, 1H), 4.38-4.44 (m, 1H), 7.17-7.28 (m, 5H), 8.08 (d, J = 8.0 Hz, 1H), 12.97 (brs, 1H)
HRMS (ESI) *m/z* calcd for $C_{29}H_{50}NO_3$ $[M+H]^+$ 460.3785, found: 460.3796

Methyl oleoyl-*L*-phenylalaninate, Method A. White solid.
^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 7.0 Hz, 3H), 1.21-1.37 (m, 20H), 1.53-1.62 (m, 2H), 1.98-2.06 (m, 4H), 2.17 (t, J = 8.4 Hz, 2H), 3.12 (qd, J = 5.8, 13.8 Hz, 2H), 3.73 (s, 3H), 4.88-4.93 (m, 1H), 5.32-5.39 (m, 2H), 5.84 (d, J = 7.9 Hz, 1H), 7.07-7.10 (m, 2H), 7.22-7.31 (m, 3H)
HRMS (ESI) *m/z* calcd for $C_{28}H_{46}NO_3$ $[M+H]^+$ 444.3472, found: 444.3482

(*S*)-*N*-(1-amino-1-oxo-3-phenylpropan-2-yl)oleamide, Method A. White solid.
^{1}H NMR (400 MHz, DMSO-d^6) δ 0.85 (t, J = 6.7 Hz, 3H), 1.04-1.35 (m, 22H), 1.95-2.02 (m, 6H), 2.71 (dd, J = 10.0, 13.8 Hz, 1H), 2.98 (dd, J = 4.5, 13.8 Hz, 1H), 4.40-4.46 (m, 1H), 5.29-5.36 (m, 2H), 7.02 (brs, 1H), 7.13-7.19 (m, 1H), 7.21-7.26 (m, 4H), 7.39 (brs, 1H), 7.91 (d, J = 8.6 Hz, 1H)
HRMS (ESI) *m/z* calcd for $C_{27}H_{45}NO_3$ $[M+H]^+$ 429.3476, found: 429.3484

Oleoyl-*L*-leucine, Method A. White solid.
^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 7.0 Hz, 3H), 0.95 (d, J = 3.6 Hz, 3H), 0.97(d, J = 3.6 Hz, 3H), 1.27-1.37 (m, 20H), 1.56-1.77 (m, 5H), 1.98-2.06 (m, 4H), 2.22 (t, J = 7.3 Hz, 2H), 4.58-4.64 (m, 1H), 5.31-5.39 (m, 2H), 5.86 (d, J = 8.0 Hz, 1H)
HRMS (ESI) *m/z* calcd for $C_{24}H_{46}NO_3$ $[M+H]^+$ 396.3472, found: 396.3478

Oleoyl-*L*-isoleucine.
^{1}H NMR (400 MHz, DMSO-d^6) δ 0.81-0.88 (m, 6H),1.19-1.32 (m, 22H), 1.34-1.52 (m, 3H), 1.70-1.80 (m, 1H), 1.93-2.03 (m, 4H), 2.06-2.22 (m, 2H), 4.17 (dd, J = 6.2, 8.4 Hz, 1H), 5.28-5.36 (m, 2H), 7.92 (d, J = 8.4 Hz, 1H), 12.50 (brs, 1H)
HRMS (ESI) *m/z* calcd for $C_{24}H_{46}NO_3$ $[M+H]^+$ 396.3472, found: 396.3474

Oleoyl-*L*-glutamic acid.
^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 6.7 Hz, 3H), 1.19-1.38 (m, 20H), 1.58-1.68 (m, 2H), 1.93-2.07 (m, 4H), 2.08-2.18 (m, 1H), 2.20-2.30 (m, 3H), 2.42-2.60 (m, 2H), 4.65 (dd, J = 6.5, 13.6 Hz, 1H), 5.30-5.38 (m, 2H), 6.53 (d, J = 7.2 Hz, 1H), 8.23 (brs, 2H)
HRMS (ESI) *m/z* calcd for $C_{23}H_{42}NO_5$ $[M+H]^+$ 412.3057, found: 412.3064

Oleoyl-*L*-tyrosine.

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87 (t, *J* = 6.6 Hz, 3H), 1.19-1.38 (m, 22H), 1.47-1.58 (m, 2H), 1.94-2.04 (m, 4H), 2.07-2.17 (m, 3H), 2.96-3.10 (m, 2H), 4.75 (brs, 1H), 5.29-5.38 (m, 2H), 6.19 (brs, 1H), 6.69 (d, *J* = 6.7 Hz, 2H), 6.95 (d, *J* = 6.7 Hz, 2H)
HRMS (ESI) *m/z* calcd for $C_{27}H_{44}NO_4$ $[M+H]^+$ 446.3265, found: 446.3270

Oleoyl-*L*-tryptophan.
^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.19-1.38 (m, 22H), 1.47-1.58 (m, 2H), 1.94-2.06 (m, 4H), 2.07-2.17 (m, 2H), 3.30-3.42 (m, 2H), 4.91-4.96 (m, 1H), 5.29-5.38 (m, 2H), 6.00 (d, *J* = 7.4 Hz, 1H), 7.03 (d, *J* = 3.5 Hz, 1H), 7.12 (t, *J* = 8.0 Hz, 1H), 7.21 (t, *J* = 8.0 Hz, 1H), 7.36 (d, *J* = 8.1 Hz, 1H), 7.57 (d, *J* = 8.1 Hz, 1H), 8.22 (brs, 1H)
HRMS (ESI) *m/z* calcd for $C_{29}H_{45}N_2O_3$ $[M+H]^+$ 469.3425, found: 469.3433

Oleoyl-*L*-glutamine.
^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, *J* = 6.7 Hz, 3H), 1.19-1.38 (m, 20H), 1.58-1.68 (m, 2H), 1.93-2.07 (m, 5H), 2.25 (d, *J* = 7.6 Hz, 2H), 2.38-2.48 (m, 1H), 2.54-2.65 (m, 1H), 4.46 (dd, *J* = 6.2, 12.0 Hz, 1H), 5.30-5.38 (m, 2H), 6.17 (brs, 1H), 6.48 (brs, 1H), 7.18 (d, *J* = 6.2 Hz, 1H)
HRMS (ESI) *m/z* calcd for $C_{23}H_{43}N_2O_4$ $[M+H]^+$ 411.3217, found: 411.3224

Oleoyl-*L*-lysine
^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84 (t, *J* = 6.7 Hz, 3H), 1.17-1.34 (m, 22H), 1.36-1.44 (m, 2H), 1.50-1.58 (m, 2H), 1.63-1.75 (m, 2H), 1.93-1.99 (m, 4H), 2.13-2.24 (m, 2H), 2.84-2.96 (m, 2H), 4.07 (dd, *J* = 6.2, 12.0 Hz, 1H), 5.26-5.34 (m, 2H), 7.12 (d, *J* = 6.6 Hz, 1H)
HRMS (ESI) *m/z* calcd for $C_{24}H_{47}N_2O_3$ $[M+H]^+$ 411.3581, found: 411.3574

(*S*)-3-(3-methyl-3H-diazirin-3-yl)-2-(octadec-17-ynamido)propanoic acid "photo-probe", Method B. White solid.
^{1}H NMR (400 MHz, $CDCl_3$) δ 1.08 (s, 3H), 1.25-1.40 (m, 22H), 1.49-1.56 (m, 2H), 1.61-1.71 (m, 3H), 1.94 (t, *J* = 2.6 Hz, 1H), 2.10-2.15 (m, 1H), 2.17 (td, *J* = 2.6, 6.9 Hz, 2H), 2.17 (t, *J* = 7.3 Hz, 2H), 4.58-4.64 (m, 5H), 6.26 (d, *J* = 6.6 Hz, 1H), 7.52 (brs, 1H)
HRMS (ESI) *m/z* calcd for $C_{23}H_{40}N_3O_3$ $[M+H]^+$ 406.3064, found: 406.3059

General animal information
For AAV injection experiments, male mice (C57BL/6, Charles River) were gently warmed using a 250 W clear infrared heat lamp, immobilized, and injected via tail vein with 10^{10} virus/mouse diluted in saline in a total volume of 100 µl/mouse. Where indicated, mice treated with AAV were placed on high-fat diet (60% fat, Research Diets). For cold exposure experiments, mice were group housed and placed at 4°C for the indicated times. For the preparation of C18:1-Leu, C18:1-Phe, and C20:4-Gly for *in vivo* injections, compounds were dissolved in 18:1:1 (by volume) saline:Kolliphor EL (Sigma Aldrich):DMSO and sonicated until homogeneous. Mice were administered compounds at 5 µl/g body weight at the indicated doses. For all injection experiments, mice were mock injected with saline daily for 3 days prior to the start of the experiments. For glucose tolerance tests (GTTs), mice received their final dose of compound the day prior to the assay and were then fasted overnight. Glucose was administered at 1.5 g/kg.

Molecular studies
Quantatative PCR (qPCR) and western blotting were performed according to standard methods. For qPCR from whole tissues, all values were normalized by the ΔΔCt method to *Rps18* where indicated, or *Tbp* otherwise. The following antibodies were used: anti-Flag M2-HRP (Sigma Aldrich, A8592), anti-UCP1 (Abcam, ab10983), total OXPHOS rodent antibody cocktail (Abcam, ab110413). Coomassie staining was performed using SimplyBlue SafeStain (Thermo Fisher Scientific) according to the manufacturer's instructions. The following primers, written 5' to 3', were used for measuring the indicated genes: *Tbp*, ACCCTTCACCAATGACTCCTATG and TGACTGCAGCAAATCGCTTGG; *aP2*, AAGGTGAAGAGCATCATAACCCT and TCACGCCTTTCATAACACATTCC; *Ucp1*, ACTGCCACACCTCCAGTCATT and CTTTGCCTCACTCAGGATTGG; *Ppargc1a*, CCCTGCCATTGTTAAGACC and TGCTGCTGTTCCTGTTTTC; *Cox2*, GCCGACTAAATCAAGCAACA and CAATGGGCATAAAGCTATGG; *Cox4*, GCACATGGGAGTGTTGTGA and CCTTCTCCTTCTCCTTCAGC; *Erra*, CAAGAGCATCCCAGGCTT and GCACTTCCATCCACACACTC; *Ckmt1*, TGAGGAGACCTATGAGGTATTTGC and

TCATCAAAGTAGCCAGAACGGA; *Ckmt2*, GCATGGTGGCTGGTGATGAG and AAACTGCCCGTGAGTAATCTTG.
The following mutagenesis primers were used for the indicated PM20D1 mutants: H125A, GCAGGAACCACATCAATGGCAGCCATCAGCATGTAGGG and CCCTACATGCTGATGGCTGCCATTGATGTGGTTCCTGC; D127A, GGGGCAGGAACCACAGCAATGTGAGCCATCA and TGATGGCTCACATTGCTGTGGTTCCTGCCCC; H465A, AAACTTTCTCATTGATTCCAGCGACACCACTGAAGTCCTGAG and CTCAGGACTTCAGTGGTGTCGCTGGAATCAATGAGAAAGTTT.

Detection of endogenous, circulating PM20D1 by shotgun LC-MS/MS

Murine plasma specimens (50 µl) were depleted of albumin and IgG using Proteome Purify 2 Mouse Serum Protein Immunodepletion Resin (R&D Systems) and subsequently concentrated by 3 kDa molecular weight cut-off spin-filter columns (Millipore). 100 µg of plasma were deglycosylated using Protein Deglycosylation Mix (New England Biolabs) as per the manufacturer's denaturing protocol. Deglycosylated plasma samples were further reduced with 10 mM DTT prior to being resolved by SDS-PAGE using 4-12% NuPAGE Bis-Tris precast gels (Life Technologies). Gels were Coomassie stained and fragments were excised from the 50-80 kDa region. Gel pieces were destained with 40% ACN/0.5% formic acid, dehydrated with 100% ACN, vacuumed dried, and resuspended with 25 mM HEPES, pH 8.5 containing 1 µg of sequencing grade trypsin (Promega) for an overnight incubation at 37°C. Digests were quenched after 12 h with 1% formic acid and de-salted using homemade stage tips (Rappsilber et al., 2007).

Isobaric labeling of peptides was performed using a 10-plex tandem mass tag (TMT) reagents (Thermo Fisher Scientific). TMT reagents (5 mg) were dissolved in 250 µl dry acetonitrile and 3 µl was added to digested peptides dissolved in 25 µl of 200 mM HEPES, pH 8.5. After 1 hour (RT), the reaction was quenched by adding 2 µl of 5% hydroxylamine. Labeled peptides were combined, acidified with 40 µl of 10% FA (pH ~2) and de-salted using homemade stage tips.

All MS analysis was performed on an Oribtrap Fusion (Thermo Fisher Scientific) coupled to a Proxeon nLC-1200 ultra-high pressure liquid chromatography (UPLC) pump (Thermo Fisher Scientific). Peptides were re-suspended in 12 µl of 5 % formic acid and separated (2 µl) onto a packed 100 µM inner diameter column containing 0.5 cm of Magic C4 resin (5 µm, 100 Å, Michrom Bioresources) followed by 40 cm of Sepax Technologies GP-C18 resin (1.8 µm, 120 Å) and a gradient consisting of 6–27% (ACN, 0.125% formic acid) over 165 min at ~500 nl/min. The instrument was operated in data-dependent mode with a 60 s (±5 ppm window) expiration time, with FTMS1 spectra collected at 120,000 resolution with an AGC target of 500,000 and a max injection time of 100 ms. The ten most intense ions were selected for MS/MS and precursors were filtered according to charge state (required > 1 z). Monoisotopic precursor selection was enabled, isolation width was set at 0.7 m/z, ITMS2 spectra were collected at an AGC of 18,000, max injection time of 120 ms and CID collision energy of 35%. For the FTMS3 acquisition, the Orbitrap was operated at 60,000 resolution with an AGC target of 50,000 and a max injection time of 250 ms and an HCD collision energy of 55%. Synchronous-precursor-selection (SPS) was enabled to include 7 MS2 fragment ions in the FTMS3 spectrum.

A collection of in-house software was used to convert .raw files to mzXML format, as well as to correct monoisotopic m/z measurements. Assignment of MS/MS spectra was performed using the Sequest algorithm. A protein sequence database containing Mouse Uniprot database (downloaded 12/2015) as well as known contaminants such as human keratins and reverse protein sequences was used. Sequest searches were performed using a 10 ppm precursor ion tolerance, requiring trypsin protease specificity, while allowing up to two missed cleavages. TMT tags on peptide N termini/lysine residues (+229.162932 Da) were set as static modifications while methionine oxidation (+15.99492 Da) and deamidation of asparagine (0.984016) were set as variable modifications. An MS2 spectra assignment false discovery rate (FDR) of less than 1% was achieved by applying the target-decoy database search strategy and filtered using an in-house linear discrimination analysis algorithm with the following peptide ion and MS2 spectra metrics: XCorr, peptide ion mass accuracy, charge state, peptide length and missed-cleavages. Peptides were further filtered a 1% protein-level false discovery rate for the final dataset. Two tryptic peptides were identified for PM20D1: 39-48 R.IPSQFSEEER.V and 313-323 R.NLWLFHPIVSR.I.

For quantification, a 0.003 m/z (10-plex TMT) window centered on the theoretical m/z value of each reporter ion, with the maximum signal intensity from the theoretical m/z value was recorded. Reporter ion intensities were adjusted based on the overlap of isotopic envelopes of all reporter ions (manufacturer specifications). Total signal to noise values for all peptides were summed for each TMT channel (150 minimum) and all values were normalized to account for variance in sample preparation.

Purification of mammalian recombinant PM20D1

293A cells were infected with retrovirus expressing mouse PM20D1-6xHis-Flag or GFP-Flag in the presence of polybrene (8 μg/ml). After two days, cells were selected with hygromycin (150 μg/ml, Sigma Aldrich). The stable 293A cells were then grown in complete media. At confluence, the media (~500 ml) was changed, harvested 24 h later, and concentrated ~10-fold in 30 kDa MWCO filters (EMD Millipore) according to the manufacturer's instructions. The concentrated media was centrifuged to remove debris (600 x *g*, 10 min, 4°C) and the supernatant containing PM20D1-flag was decanted into a new tube. PM20D1-flag was immunoaffinity purified overnight at 4°C from the concentrated media using magnetic Flag-M2 beads (Sigma Aldrich). The beads were collected, washed three times in PBS, eluted with 3xFlag peptide (0.1 μg/ml in PBS, Sigma Aldrich), aliquoted, and stored at -80°C. For the purification of human PM20D1-flag, hPM20D1-flag plasmid (Origene) was transiently transfected into 293A cells. After two days, the media was changed, harvested 24 h later, concentrated, and purified exactly as described above.

Measurements of metabolites *in vivo* and enzyme activities *in vitro* by LC-MS

For separation of polar metabolites, normal-phase chromatography was performed with a Luna-5 mm NH_2 column (50 mm × 4.60 mm, Phenomenex). Mobile phases were as follows: Buffer A, acetonitrile; Buffer B, 95:5 water/ acetonitrile with 0.1% formic acid or 0.2% ammonium hydroxide with 50 mM ammonium acetate for positive and negative ionization mode, respectively. The flow rate for each run started at 0.2 ml/min for 2 min, followed by a gradient starting at 0% B and increasing linearly to 100% B over the course of 15 min with a flow rate of 0.7 ml/min, followed by an isocratic gradient of 100% B for 10 min at 0.7 ml/min before equilibrating for 5 min at 0% B with a flow rate of 0.7 ml/min. MS analysis was performed with an electrospray ionization (ESI) source on an Agilent 6430 QQQ LC−MS/MS. The capillary voltage was set to 3.5 kV, and the fragmentor voltage was set to 100 V. The drying gas temperature was 325 °C, the drying gas flow rate was 10 l/min, and the nebulizer pressure was 45 psi. For polar targeted and untargeted metabolomic analysis, representative metabolites were quantified by SRM of the transition from precursor to product ions (corresponding to amino acid fragment) at associated collision energies. Several representative fragmentation ions are as follows: C18:1-Ala, 352 > 88; C18:1-Gly, 338 > 74; C18:1-Phe, 428 > 164; C18:1-Leu/Ile, 394 > 130; C20:4-Phe, 450 > 164; C20:4-Leu/Ile, 416 > 130. Untargeted LC-MS was performed by scanning a mass range of m/z 50-1200, and data were exported as mzdata files and uploaded to XCMSOnline (xcmsserver.nutr.berkeley.edu) to identify metabolites that were differentially changed. For targeted metabolomics analysis, metabolites were quantified by integrating the area under the peak and were normalized to internal standard values corresponding to 1 nmol D_3,^{15}N-serine.

Cell culture

C2C12, U2OS, and HEK293A cells were cultured in DMEM with 10% FBS and pen/strep. The stromal-vascular fraction of inguinal (iWAT) pad from 4-12 week old male mice was dissected, minced, and digested for 45 min at 37°C in PBS containing 10 mM $CaCl_2$, 2.4 U/ml dispase II (Roche), and 1.5 U/ml collagenase D (Roche). The stromal-vascular fraction of brown fat (BAT) pads from newborn (P1-P14) pups was dissected, minced, and digested for 45 min at 37°C in PBS containing 1.3 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 5 mM glucose, 100 mM HEPES, 4% BSA, and 1.5 mg/ml collagenase B (Roche). Digested tissue was diluted with adipocyte culture media (DMEM/F-12, GlutaMAX supplement, Life Technologies, with 10% FBS and pen/strep) and centrifuged (600 x *g*, 10 min). The pellet was resuspended in 10 ml adipocyte culture media, strained through a 40 μm filter, and plated. Differentiation was induced by adipogenic cocktail containing 5 μg/ml insulin (Sigma), 5 μM dexamethasone (Sigma), 250 μM isobutylmethylxanthine (Sigma), and 1 μM rosiglitazone (Cayman) for 2 days. Two days after induction, cells were maintained in adipocyte culture media containing 5 μg/ml insulin and 1 μM rosiglitazone.

Cellular respiration measurements

Oligomycin was purchased from EMD Millipore, and FCCP and rotenone were purchased from Sigma. C2C12 or U2OS cells were seeded at 30,000 or 50,000 cells/well, respectively in an XF24 cell culture microplate (V7-PS, Seahorse Bioscience) and analyzed the following day. Primary iWAT or BAT adipocytes were seeded at 15,000 cells/well, differentiation was induced the following day as previously described, and the cells were analyzed on day 5. On the day of analysis, the cells were washed once with Seahorse respiration buffer (8.3 g/l DMEM, 1.8 g/l NaCl, 1 mM pyruvate, 20 mM glucose, pen/strep), placed in 0.5 ml Seahorse respiration buffer, and incubated in a CO_2-free incubator for 1 hr. Port injection solutions were prepared as follows (final concentrations in assay in parentheses): 10 μM oligomycin (1 μM final), 500 μM *N*-acyl amino acid (50 μM final), 2 μM FCCP (0.2 μM final), and 30 μM rotenone (3 μM final). The Seahorse program was run as follows: basal measurement, 3 cycles; inject port A (oligomycin), 3 cycles; inject port B (compounds), 8 cycles; inject port C (FCCP), 3 cycles; inject port D

(rotenone), 3 cycles. Each cycle consisted of mix 4 min, wait 0 min, and measure 2 min. For data expressed as a percentage of oligomycin-treated basal, the respiration at cycle 6 was normalized to 100%, and the maximum respiration at any time point between cycles 7 and 15 inclusive was used.

Isolation of crude mitochondria from BAT and respiration measurements

BAT pads from ten 6-12 week old male mice were harvested and minced on ice in isolation buffer (250 mM sucrose, 5 mM HEPES, 1 mM EGTA) supplemented with 2% fatty acid free BSA (Sigma). The tissue was then homogenized in a Teflon homogenizer and centrifuged (8,500 x *g*, 10 min, 4°C). The supernatant was discarded, the pellet was resuspended in 20 ml isolation buffer supplemented with 1% fatty acid free BSA, and centrifuged (700 x *g*, 10 min, 4°C). The supernatant was centrifuged one final time (8,500 x *g*, 10 min, 4°C). The pellet was resuspended in isolation buffer supplemented with 1% fatty acid free BSA (200 µl) to yield crude BAT mitochondria at a concentration of 50-80 mg/ml. For respiration measurements, BAT mitochondria were diluted to 0.3 mg/ml in mitochondria respiration buffer (5 mM KCl, 4 mM KH_2PO_4, 5 mM HEPES, 1 mM EGTA, 1% fatty acid free BSA, 10 mM pyruvate, 5 mM malate, 1 mM GDP) and plated at 15 µg/well (50 µl total volume) in an XF24 cell culture microplate. The plate was centrifuged (1000 x *g*, 20 min, 4°C). Additional pre-warmed mitochondrial respiration buffer was added (450 µl), and respiration was measured on a XF24 Extracellular Flux Analyzer. The Seahorse program was run as follows: basal measurement, 2 cycles; inject port A (compounds), 1 cycle, inject port B (FCCP), 1 cycle, inject port C (rotenone), 1 cycle. Each cycle consisted of mix 0.5 min, wait 0 min, measure 2 min. FCCP and rotenone were used at final concentrations of 2 µM and 3 µM, respectively.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured using tetramethylrhodamine methyl ester (TMRM, Life Technologies) fluorescence. C2C12 cells were plated in 6-well plates and grown to 80-90% confluence. Cells were then washed once with PBS and switched into Seahorse respiration buffer with the indicated compounds at the indicated concentrations: TMRM (100 nM), oligomycin (1 µM), FCCP (0.4 µM), C18:1-Phe (10 or 50 µM). After 20 min incubation at 37°C, the media was aspirated, cells were washed once with PBS and trypsinized. Trypsinized cells were directly resuspended in PBS (total volume 300 µl), kept on ice, and analyzed on a FACSCanto II (BD Biosciences) with 20,000 events per sample.

UV crosslinking and identification of *N*-acyl amino acid protein targets

Confluent 6-cm plates of C2C12 cells were washed twice with PBS and then treated with photo-probe (20 µM) with or without competitor (C20:4-Phe, 100 µM) in serum free media at 37°C for 20 min. The media was aspirated. Cells were then placed on ice and UV-irradiated (10 min, UV Stratalinker 2400). Control samples were left on ice under ambient light.

Following UV irradiation, cells were then scraped, centrifuged (5 min, 1,400 x *g*), washed once with cold PBS, and centrifuged again (5 min, 1,400 x *g*). The cell pellets were resuspended in 0.3 ml PBS and sonicated. Click chemistry was performed as follows: To 50 µl cell lysate at 1 mg/ml was added 3 µl TBTA (stock solution: 1.7 mM in 4:1 v/v DMSO:*t*-BuOH), 1 ul CuSO4 (stock solution: 50 mM in water), 1 ul TCEP (freshly prepared, stock solution: 50 mM), and 1 ul TAMRA-N_3 (stock solution: 1.25 mM in DMSO). Reactions were incubated at room temperature for 1h, and then quenched with 4x SDS loading buffer (17 ul). In-gel TAMRA fluorescence was visualized on a Typhoon FLA 9000 scanner (GE Healthcare Life Sciences).

For LC-MS/MS identification of photo-probe labeled proteins, click chemistry was performed using biotin-PEG3-azide (100 mM stock, Click Chemistry Tools). Samples were mixed and the reaction was allowed to proceed for 1 h at room temperature. After click reactions, proteomes were precipitated by centrifugation at 6,500 x *g*, washed twice in ice-cold methanol, then denatured and resolubilized by heating in 1.2% SDS/PBS to 80ºC for 5 minutes. Insoluble components were precipitated by centrifugation at 6,500 x *g* and soluble proteome was diluted in 5 ml 0.2% SDS/PBS. Labeled proteins were bound to avidin-agarose beads (170 ml resuspended beads/sample, Thermo Pierce) while rotating overnight at 4ºC. Bead-linked proteins were enriched by washing three times each in PBS and water, then resuspended in 6 M urea/PBS (Sigma-Aldrich) and reduced in dithiothreitol (1 mM, Sigma-Aldrich), alkylated with iodoacetamide (18 mM, Sigma-Aldrich), then washed and resuspended in 2 M urea and trypsinized overnight with 0.5 ug/ul sequencing grade trypsin (Promega). Tryptic peptides were diluted in PBS, acidified with formic acid (1.2 M, Spectrum) and prepared for MS analysis, as described previously (Nomura et al., 2010).

SUPPLEMENTAL REFERENCES

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