

# **OPA1 Regulates Lipid Metabolism and Cold-Induced Browning of White Adipose Tissue in Mice**

## **Supplemental Experimental Procedures and References**

### **Supplemental Figure Legends**

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## **Supplemental Experimental Materials:**

### **Animal Studies:**

*Opal*<sup>fl/fl</sup> (1) and *Fgf21*<sup>fl/fl</sup> mice (2) were generated, as previously described. Transgenic mice expressing cre recombinase under the control of the *Adipoq* gene promoter (B6.FVB-Tg(*Adipoq*-cre)1Evdr/J) (3), and transgenic mice expressing a tamoxifen-inducible cre under the control of the *Adipoq* gene promoter (C57BL/6-Tg(*Adipoq*-cre/ERT2)1Soff/J) (4) were acquired from the Jackson Laboratories (#028020 and #025124, respectively). Mice were weaned at 3 weeks of age and were either kept on standard chow (2029X Harlan Teklad, Indianapolis, IN, USA) or were fed special diets. For diet-induced obesity studies mice were divided into a control-diet group (Cont; 10% Kcal from fat—Research Diets, New Brunswick, NJ, USA, D12450J) or a high-fat diet group (HFD; 60% Kcal from fat—Research Diets D12492) and were kept on these respective diets for 12 weeks (special diets were initiated when mice were around 6 weeks, unless otherwise noted). To promote recombination and *Opal* deletion in our inducible model, 12-week-old mice were injected i.p. with 100 mg/kg tamoxifen (Sigma, St. Louis, MO, USA) dissolved in 10% ethanol in 90% peanut oil (Sigma, St. Louis, MO, USA) for 5 consecutive days. For the 12-hr fast studies, food was removed at 6 pm and experiments were performed at 6 am. Glucose tolerance tests (GTT), were performed, after a 6-h fast, as previously described in mice administered glucose intraperitoneally (2 g/kg body weight) (5). Insulin tolerance tests (6) were performed after a 2-h fast by injecting insulin intraperitoneally (0.75 U/kg body weight; Humulin, Eli Lilly, Indianapolis, IN, USA). For both assays, blood glucose was determined using a glucometer at regular time intervals (Glucometer Elite; Bayer, Tarrytown, NY, USA).

### **Cold exposure experiments:**

For the acute cold exposure experiments, 8-10-week-old mice were initially individually housed in the rodent environmental chamber (Power Scientific) at 30 °C for 7 days. Mice were then fasted for 12 hours the night prior to the cold exposure. The initial temperature ( $t_0$ ) was recorded using a rectal probe (Fisher Scientific, Lenexa, KS, USA) at 7 am of day 8, after which the temperature was switched to 4 °C. Once the temperature stabilized at 4°C, we recorded rectal temperatures hourly for up to 2 hours. For the 3-day cold exposure experiments, core body temperature telemeters (Respironics, G2 E-Mitter, Murrysville, PA, USA) were surgically implanted into the abdominal cavity of 12-14-week old male mice, who were allowed to recover for 6 days post-surgery, while being individually housed in a rodent environmental chamber (Power Scientific) at 30°C. Mice were then transferred to an OxyMax Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments International) at 30°C for 4 days, followed by 4°C for 3 days, as previously described (7). Core body temperature and metabolic parameters were recorded every 17 minutes throughout the experiment.

### **Quantitative RT-PCR**

Real-time PCR was performed using SYBR Green (Life Technologies, Carlsbad, CA, USA), in an ABI Prism 7900HT instrument (Applied Biosystems, Waltham, MA, USA) (8). RNA concentration was determined by measuring the absorbance at 260 and 280 nm using a spectrophotometer (NanoDrop 1000, NanoDrop products, Wilmington, DE, USA). Total RNA (1 µg) was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) (6). Data were normalized to *Gapdh* or *Tbp* expression. qPCR primers were designed using Primer-Blast or previously published sequences. The specific primers are listed in the Supplemental table 1.

<b>Gene name</b>	<b>Forward</b>	<b>Reverse</b>
<i>Opa1</i>	ATACTGGGATCTGCTGTTGG	AAGTCAGGCACAATCCACTT
<i>Ucp1</i>	GTGAAGGTCAGAATGCAAGC	AGGGCCCCCTTCATGAGGTC
<i>Prdm16</i>	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
<i>Gapdh</i>	AACGACCCCTTCATTGAC	TCCACGACATACTCAGCAC
<i>Ppargc1a</i>	GTAAATCTGCGGGATGATGG	AGCAGGGTCAAAATCGTCTG
<i>Dio2</i>	AATTATGCCTCGGAGAAGACCG	GGCAGTTGCCTAGTGAAAGGT
<i>Cpt1b</i>	TGCCTTTACATCGTCTCCAA	AGACCCCGTAGCCATCATC
<i>Evlov6</i>	TCAGCAAAGCACCCGAAC	AGCGACCATGTCTTTGTAGGAG
<i>Cox4i</i>	CGCTGAAGGAGAAGGAGAAG	GCAGTGAAGCCAATGAAGAA
<i>Ndufa9</i>	ATCCCTTACCCTTTGCCACT	CCGTAGCACCTCAATGGACT
<i>Gpat</i>	ACGCACACAAGGCACAGAG	TGCTGCTCAGTACATTCTCAGTA
<i>Ndufv1</i>	TGTGAGACCGTGCTAATGGA	CATCTCCCTTCACAAATCGG
<i>Dgat2</i>	TCATGGGTGTCTGTGGGTTA	CAGAGTGAAACCAGCCAACA
<i>Gpat</i>	ACGCACACAAGGCACAGAG	TGCTGCTCAGTACATTCTCAGTA
<i>Pnpla2</i>	AGGAATGGCCTACTGAACCA	AGGCTGCAATTGATCCTCCT
<i>Tbp</i>	TCTGGAATTGTACCGCAGCTT	CTGCAGCAAATCGCTTGGA
<i>Ppar<math>\gamma</math></i>	ATCCCTGGTTTCATTAACCT	GCTGCATAAAGTCACCAAAG
<i>Cebp<math>\beta</math></i>	AAGCTGAGCGACGAGTACAAGA	GTCAGCTCCAGCACCTTGTG
<i>Tnfa</i>	ACGGCATGGATCTCAAAGAC	AGATAGCAAATCGGCTGACG
<i>F4/80</i>	CTGGGATCCTACAGCTGCTC	AGGAGCCTGGTACATTGGTG

Gene name	Forward	Reverse
<i>P53</i>	AGAGACCGC CGTACAGAAGA	CTGTAGCATGGGCATCCT TT
<i>P16</i>	CCCAACGCCCCGAACT	GCAGAAGAGCTGCTACGTGAA

**Supplemental Table 1:** Primers utilized for the mRNA expression analyses.

### **Immunoblotting and antibody list:**

Approximately, 50 mg of frozen tissue was homogenized in 200 µl lysis buffer containing (in mmol/l) 50 HEPES, 150 NaCl, 10% glycerol, 1% Triton X-100, 1.5 MgCl<sub>2</sub>, 1 EGTA, 10 sodium pyrophosphate, 100 sodium fluoride, and 100 µmol/l sodium vanadate. Right before use, HALT protease/phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA) were added to the lysis buffer and samples were processed using the TissueLyser II (Qiagen Inc., Germantown, MD, USA). Tissue or cell lysates were resolved on SDS–PAGE and transferred to nitrocellulose membranes (Millipore Corp., Billerica, MA, USA). Membranes were incubated with primary antibodies overnight and with secondary antibodies for 1 h, at room temperature.

*List of Primary Antibodies:* OPA1 (1:1,000, BD Biosciences, San Jose, CA, USA, #612606), FGF21 (1:1,000, Abcam, Cambridge, UK, #ab171941), VDAC (1:1,000, Thermo Scientific, #PA1-954A), GAPDH (1:1,000, Cell Signaling Technology, Danvers, MA, USA, #2118), UCP1 (1:1,000, Abcam, #Ab10983), α-tubulin (1:1,000, Sigma, St. Lois, MO, #T9026), β-actin (1:1,000, Sigma, #A2066), HSL (1:1,000, Cell Signaling Technology, Danvers, MA, USA, #4107), phospho-HSL (1:1,000, Cell Signaling Technology, Danvers, MA, USA, #4139), ATGL (1:1,000, Cell Signaling Technology, Danvers, MA, USA, #2138), SCD1 (1:1,000, Cell Signaling Technology, Danvers, MA, USA, #2794), perilipin (1:1,000, Cell Signaling Technology, Danvers,

MA, USA, #9349), phospho-perilipin (1:1,000, Cell Signaling Technology, Danvers, MA, USA, #4855) and FASN (1:1,000, Cell Signaling Technology, Danvers, MA, USA, #3180).

*List of Secondary Antibodies:* IRDye 800CW anti-mouse (1:10,000, LI-COR, Lincoln, NE, USA, #925-32212) and Alexa Fluor anti-rabbit 680 (1:10,000, Invitrogen #A27042). Fluorescence was quantified using the LiCor Odyssey imager.

### **Mitochondrial Isolation:**

Interscapular brown adipose tissue (iBAT), inguinal (iWAT) and gonadal white adipose tissue (gWAT) were excised, rinsed in ice-cold PBS and maintained in ice-cold isolation buffer (500 mM EDTA, 215 mM D-mannitol, 75 mM sucrose, 0.1% free-fatty acid bovine serum albumin, 20 mM HEPES, pH 7.4 with KOH) until ready for homogenization (9).

### *Oxygen consumption:*

The respiratory rates of mitochondria were measured using the Oroboros O<sub>2</sub>K Oxygraph system (Oroboros Instruments, Innsbruck, Austria) in 2.5 mL of buffer Z containing (in mmol/l): 30 KCl, 105 K-MES, 5 MgCl<sub>2</sub>·6H<sub>2</sub>O, 1 EGTA, 10 KH<sub>2</sub>PO<sub>4</sub> and 2.5 mg/ml BSA (pH 7.4 adjusted). We added 30, 40 and 25 µg of mitochondrial protein to determine respirations in iWAT, gWAT and BAT, respectively. Substrates were added to the assay buffer at the following final concentrations- Pyruvate at 2mM, Malate at 0.8mM and Palmitoyl carnitine at 0.06mM. Oxygen consumption was determined following ADP stimulation (1 mM; V<sub>ADP</sub>).

### **Histology and histological analysis:**

Fragments of gWAT and BAT were embedded in paraffin, portioned into 5-µm-thick sections, and stained with hematoxylin-eosin (Fisher, Pittsburgh, PA, USA). Light microscopy was performed

using a Nikon Eclipse Ti-S microscope (Nikon, Melville, NY, USA). Adipocyte area and number/area were measured in inguinal white adipose tissue (iWAT) collected from 3 10-12-week-old WT and Ad-KO mice using the National Institutes of Health (NIH) *ImageJ* software. Three sections were analyzed per mouse.

### **Supplemental Figure Legends:**

**Supplemental Figure 1: Constitutive deletion of OPA1 in adipocytes impairs mitochondrial respiration in BAT and induces FGF21. A.** OPA1 protein levels in BAT of wild type (WT) and OPA1 Adipo KO mice (Ad-KO) normalized to GAPDH and the respective densitometric quantifications. **B.** ADP-stimulated (state 3) pyruvate/malate-supported oxygen consumption rates in mitochondria isolated from BAT. **C.** ADP-stimulated (state 3) palmitoyl-carnitine/malate-supported oxygen consumption rates in mitochondria isolated from BAT. **D.** Representative images of H&E-stained histological sections of BAT from WT and Ad-KO mice. **E.** BAT mass over body mass. **F-J.** Data collected in 40-week-old female mice. **F.** Body mass. **G.** gWAT mass normalized to body mass. **H.** iWAT mass normalized to body mass. **I.** BAT mass normalized to body mass. **J.** Liver mass normalized to body mass. **K.** BiP protein levels in BAT of wild type (WT) and OPA1 Adipo KO mice (Ad-KO) normalized to GAPDH and the respective densitometric quantifications. **L.** FGF21 protein levels in BAT of wild type (WT) and OPA1 Adipo KO mice (Ad-KO) normalized to GAPDH and the respective densitometric quantifications. **M.** Serum FGF21 levels. **N-V.** Data collected in WT and OPA1/FGF21 Adipo DKO mice (Ad-DKO). **N.** OPA1 and FGF21 protein levels in BAT of wild type (WT) and OPA1 Adipo KO mice (Ad-KO) normalized to VDAC and the respective densitometric quantifications. **O.** Serum FGF21 levels. **P.** Body mass. **Q.** gWAT mass normalized to body mass. **R.** iWAT

mass normalized to body mass. **S.** Liver triglyceride levels. **T.** Glucose tolerance test (GTT). **U.** Area under the curve for the GTT. **V.** Fasting glucose levels. Data are expressed as means  $\pm$  SEM. Significant differences were determined by Student's *t*-test using a significance level of  $P < 0.05$ . (\*) Significantly different vs. WT mice.

**Supplemental Figure 2: Lipid metabolism is impaired in BAT of Ad-KO mice.** **A.** Relative mRNA expression of lipid metabolism genes (*Pnpla*, *Gpat* and *Dgat*) in BAT normalized to *Gapdh* in WT and Ad-KO mice. **B.** Protein levels of pHSL in BAT normalized to total HSL. **C.** Protein levels of ATGL in BAT normalized to tubulin. **D.** mRNA expression of adipogenesis (*Ppar $\gamma$*  and *Cebp $\beta$* ), inflammation (*Tnf $\alpha$*  and *F4/80*) and senescence (*P53* and *P16*) genes in gWAT of 8-week-old mice normalized to *Tbp*. **E.** mRNA expression of adipogenesis (*Ppar $\gamma$*  and *Cebp $\beta$* ), inflammation (*Tnf $\alpha$*  and *F4/80*) and senescence (*P53* and *P16*) genes in gWAT of 8-week-old mice normalized to *Tbp*. **F.** mRNA expression of adipogenesis (*Ppar $\gamma$*  and *Cebp $\beta$* ), genes in iWAT of 20-week-old mice normalized to *Gapdh*. **G.** Protein levels of pPerilipin normalized to total Perilipin in gWAT. Data are expressed as means  $\pm$  SEM. Significant differences were determined by Student's *t*-test, using a significance level of  $P < 0.05$ . (\*) Significantly different vs. WT mice.

**Supplemental Figure 3: Mice lacking OPA1 in adipocytes are cold-intolerant.** **A.** Last temperature recorded in WT and Ad-KO mice during 3 days of cold exposure. **B.** Body mass. **C.** Food intake for 24-hr during cold exposure. **D.** Locomotor activity for 24-hr during cold exposure. Data are expressed as means  $\pm$  SEM. Significant differences were determined by Student's *t*-test, using a significance level of  $P < 0.05$ . (\*) Significantly different vs. WT mice.



**Supplemental Figure 4: OPA1 Ad-KO mice are resistant to diet-induced obesity. A-C.** Data from WT and Ad-KO mice fed either a control diet (Cont – 10% fat) or a high-fat diet (HFD – 60% fat) for 8 weeks starting approximately 5 weeks of age (animals were housed at thermoneutrality from weaning). **A.** Total body mass. **B.** Total fat mass. **C.** Total lean mass. **D-H.** Data from WT and OPA1/FGF21 Ad-DKO mice fed either a control diet (Cont – 10% fat) or a high-fat diet (HFD – 60% fat) for 12 weeks. **D.** Total body mass. **E.** Total fat mass. **F.** Total lean mass. **G.** Glucose tolerance test (GTT). **H.** Area under the curve for the GTT. Data are expressed as means  $\pm$  SEM. Significant differences were determined by Two-Way ANOVA, using a significance level of  $P < 0.05$ . (\*) Significantly different vs. WT control, (#) significantly different vs. WT HFD.

**Supplemental Figure 5: Inducible deletion of OPA1 in adipose tissue of adult mice largely recapitulates the phenotype of Ad-KO Mice. A-L.** Data collected from WT and Ind-KO mice 4 weeks after tamoxifen injections (tamoxifen treatment started around 8 weeks of age). **A.** OPA1 protein levels in BAT normalized to GAPDH and the respective densitometric quantifications. **B.** Body mass. **C.** Total fat mass. **D.** Total lean mass. **E.** gWAT mass normalized to body mass. **F.** iWAT mass normalized to body mass. **G.** BAT mass normalized to body mass. **H.** Oxygen consumption expressed as a function of body mass. **I.** Food intake. **J.** Locomotor activity. **K.** Relative mRNA expression of lipid metabolism genes in BAT normalized to *Gapdh* levels. **L.** Relative mRNA expression of thermogenic genes in BAT normalized to *Gapdh* levels. Data are expressed as means  $\pm$  SEM. Significant differences were

determined by Student's *t*-test, using a significance level of  $P < 0.05$ . (\*) Significantly different vs. WT mice.

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