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Supplemental Information

Role of Mitochondrial Complex IV in Age-Dependent Obesity

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SUPPLEMENTAL INFORMATION

Figure S1

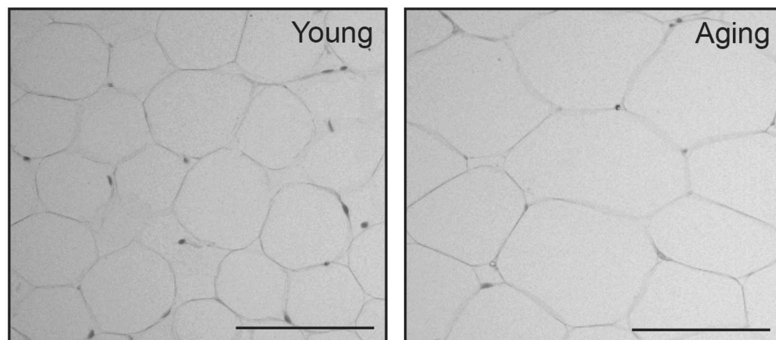

Figure S1 (Related to Figure 1). Adipocyte size of eWAT in young and aging mice.

Representative eWAT images from young and aging mice. Scale bar, 100μm.

Figure S2

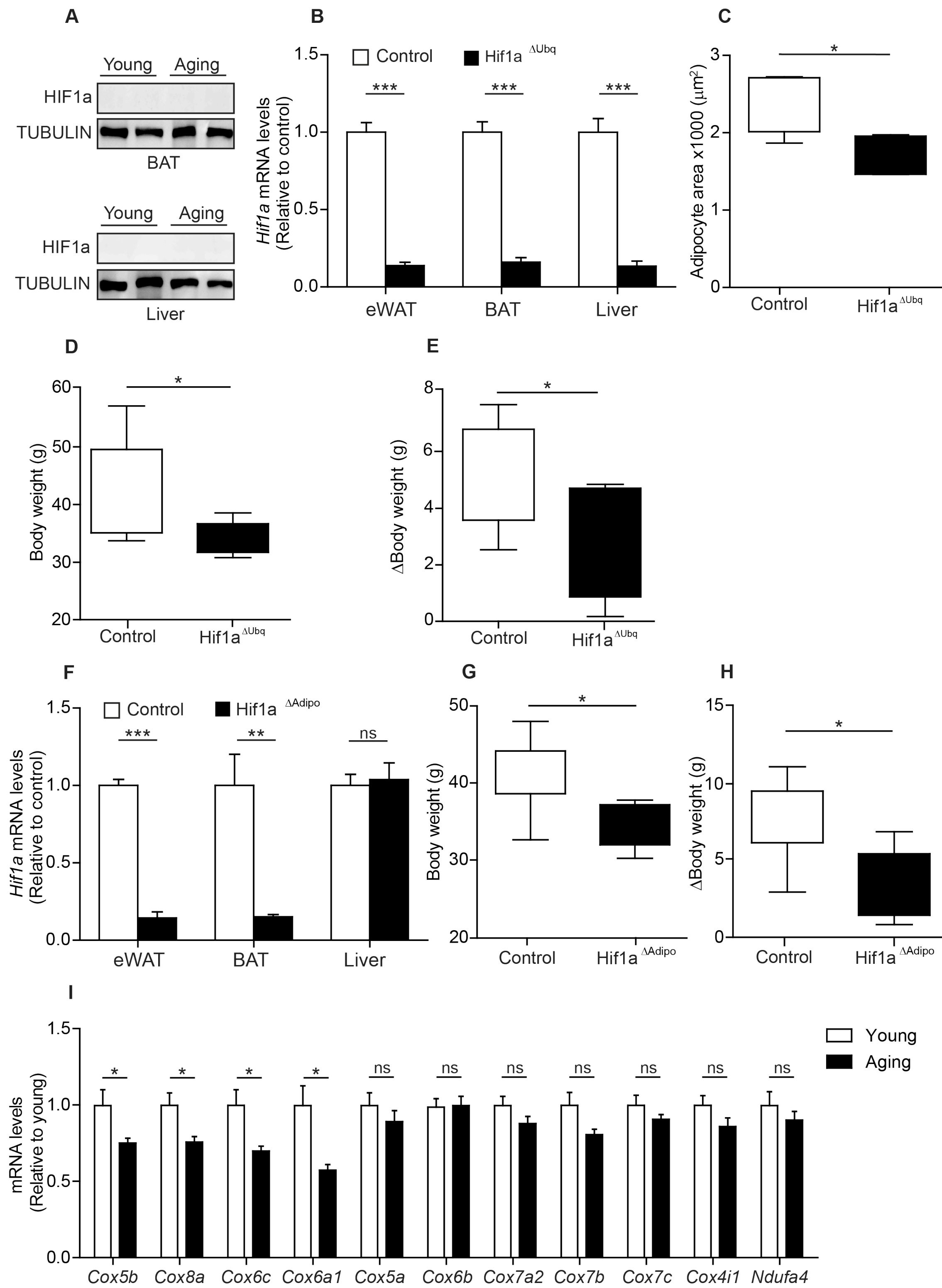

Figure S2 (Related to Figure 2). Global and adipocyte-restricted reduction of *Hif1a* gene expression and its role in age-dependent adipocyte size enlargement.

(A) Western blot analysis of HIF1A in brown adipose tissue (BAT) and liver from young and aging mice. This image corresponds to a parallel western blot shown in Figure 2A in which HIF1A protein expression was detected in eWAT. (B) Analysis of *Hif1a* mRNA levels in eWAT, brown adipose tissue (BAT) and liver from $Hif1a^{\Delta Ubq}$ (n=13; black bars) and control mice (n=16; white bars). (C) Box and whiskers plot showing average adipocyte area quantification from $Hif1a^{\Delta Ubq}$ (n=4; black bars) and control mice (n=6; white bars). (D) Box and whiskers plot showing body weight of $Hif1a^{\Delta Ubq}$ (n=7; black bars) and control mice (n=6; white bars) at 10–12 months of age. (E) Box and whiskers plot showing body weight gain in $Hif1a^{\Delta Ubq}$ (n=10; black bars) and control (n=7; white bars) mice after *Hif1a* gene inactivation. (F) Analysis of *Hif1a* mRNA levels in eWAT, BAT and liver from $Hif1a^{\Delta Adipo}$ (n=6; black bars) and control mice (n=8; white bars). (G) Box and whiskers plot showing body weight of $Hif1a^{\Delta Adipo}$ (n=6; black bars) and control mice (n=8; white bars) at 10–12 months of age. (H) Box and whiskers plot showing body weight gain in $Hif1a^{\Delta Adipo}$ (n=6; black bars) and control mice (n=8; white bars) after adipocyte-restricted *Hif1a* gene inactivation. (I) mRNA expression levels of all ten nuclear-encoded mitochondrial complex IV subunits in isolated visceral white adipocytes from eWAT of young (n=7) and aging (n=7) mice. In bar graphs, values represent mean ± SEM (error bars). In box and whiskers plots, vertical lines connect the minimum and the maximum values. Statistical significance was assessed with a two-tailed t-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, not significant).

Figure S3

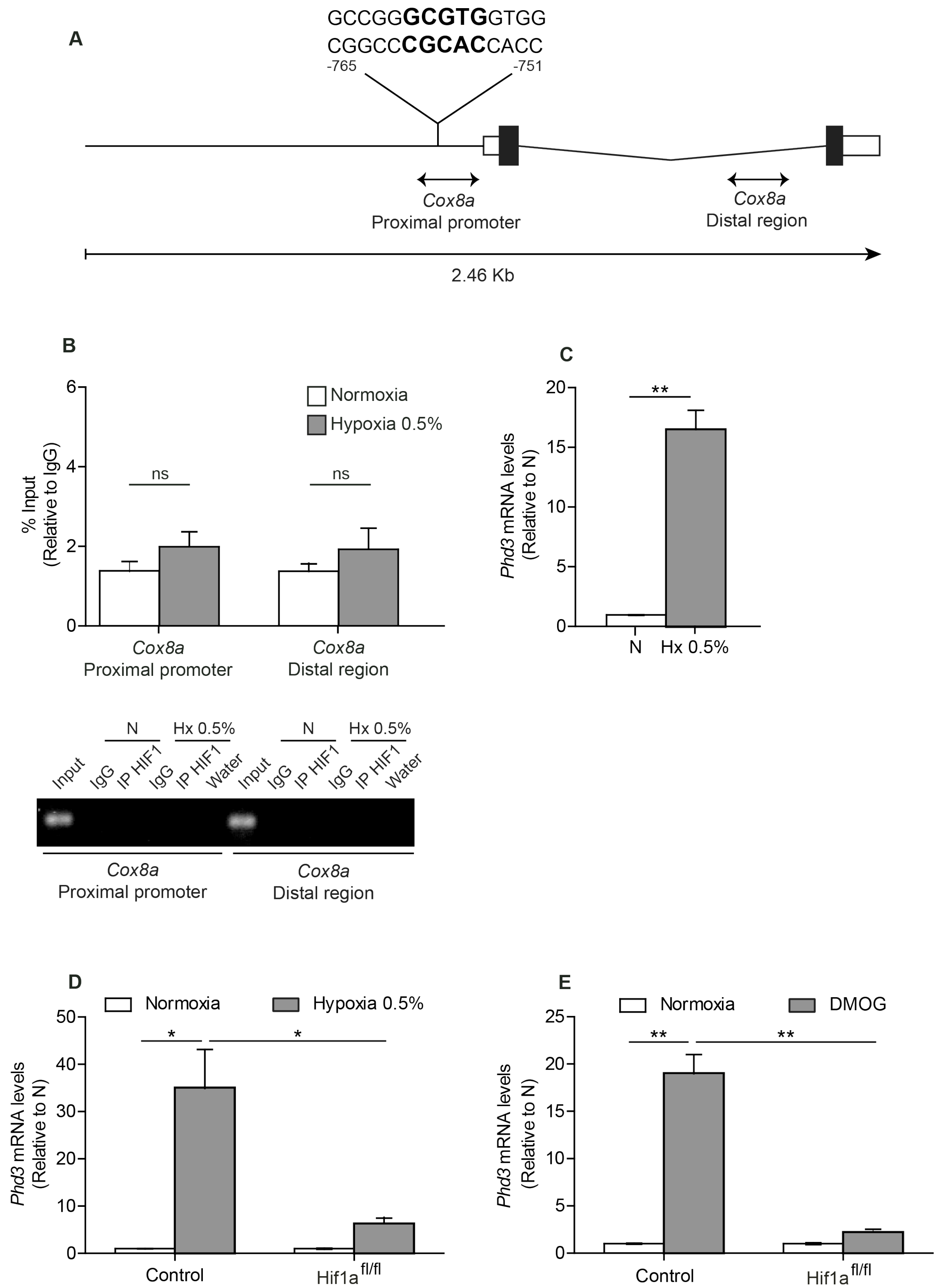

Figure S3 (Related to Figure 4). HIF1A Chip analysis of the *Cox8a* proximal promoter.

(A) Schematic representation of mouse *Cox8a* gene and its promoter region indicating the location of the proximal promoter and the distal region in which primers for ChIP analysis were designed. Nucleotide sequence corresponding to the potential HIF1A binding site is highlighted in bold. (B) ChIP assay to assess the relative HIF1A binding activity to the mouse *Cox8a* proximal promoter and distal region in 3T3-L1 cells exposed to normoxia (white bars) or hypoxia 0.5% for 18–20 hr (grey bars) (n=7 independent experiments). A representative gel showing DNA amplified in the ChIP assays is shown in the lower panel. (C) *Phd3* mRNA expression levels in 3T3-L1 cells exposed to normoxia (white bars) or hypoxia 0.5% for 72 hr (grey bars) (n= 4 independent experiments). (D) *Phd3* mRNA expression levels in control or Hif1a$^{fl/fl}$ MEF exposed to normoxia (white bars) or hypoxia 0.5% for 48 hr (grey bars) (n= 4 independent experiments). (E) *Phd3* mRNA expression levels in control or Hif1a$^{fl/fl}$ MEF under normoxia (white bars) or 48 hrs after 0.5 mM DMOG administration (grey bars) (n=4 independent experiments). In bar graphs, values represent mean ± SEM (error bars). Statistical significance was assessed with a two-tailed t-test (*, p<0.05; **, p<0.01; ns, not significant).

Figure S4

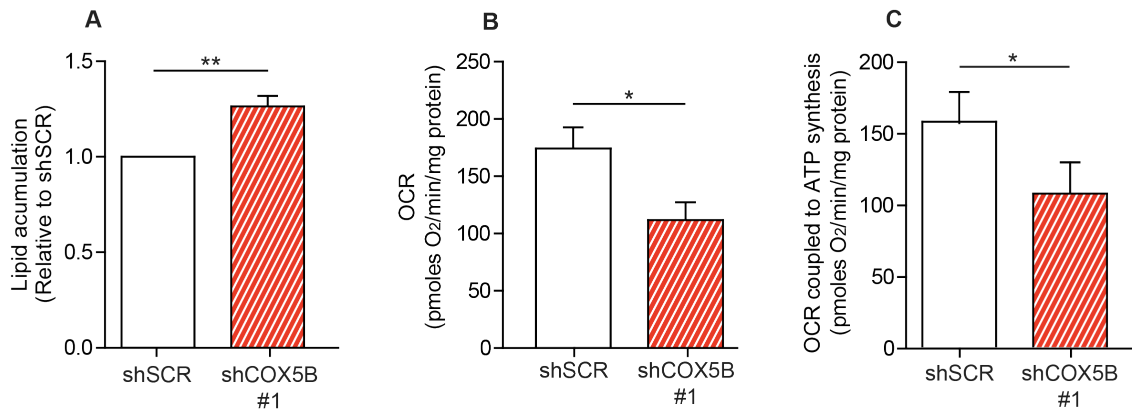

Figure S4 (Related to Figure 5) Intracellular lipid accumulation in vitro and decreases mitochondrial respiration *Cox5b* silenced 3T3-L1 cells.

(A) Nile Red fluorescence intensity in 3T3-L1-shCOX5B #1 cells (red lined bar) relative to 3T3-L1-shSCR control cells (white bar) (n=7 independent experiments). (B) Mitochondrial oxygen consumption rate (OCR) in 3T3-L1-shCOX5B #1 (red lined bar) and 3T3-L1-shSCR control cells (white bar) (n=4). (C) Mitochondrial oxygen consumption rate (OCR) coupled to ATP synthesis (or ATP turnover) in 3T3-L1-shCOX5B #1 (red lined bar) and 3T3-L1-shSCR control (white bar) (n=4). ATP turnover was calculated as the difference between basal respiration and respiration after oligomycin addition to the medium. In bar graphs, values represent mean ± SEM (error bars). Statistical significance was assessed with a two-tailed t-test (*, $p<0.05$; **, $p<0.01$).

Figure S5

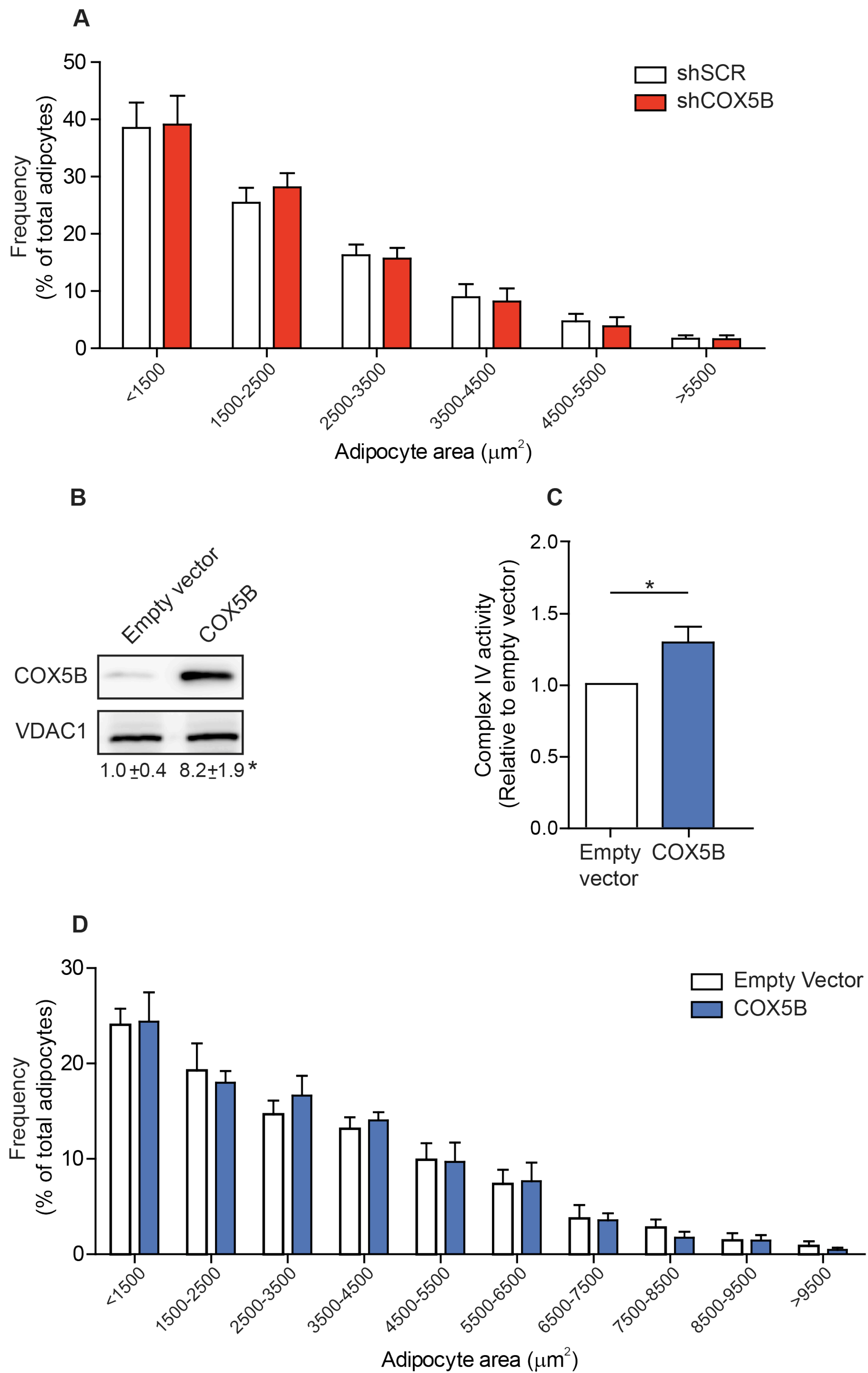

Figure S5 (Related to Figure 6). Adipocyte size frequency distribution in contralateral non-injected eWAT depot in shCOX5B and shSCR mice.

(A) Adipocyte area frequency distribution analysis in non-injected depots from shSCR (n=4; white bars) and shCOX5B (n=4; red bars) mice. (B) Western blot analysis of COX5B expression in HEK293T cells transfected with the COX5B expression vector or the corresponding empty vector. Protein expression was normalized to VDAC1; fold change relative to empty vector is shown (n=4 independent experiments). Values represent mean ± SEM. (C) Complex IV activity in HEK293T cells transfected with empty vector (white bars) or COX5B expression vector (blue bars) (n=8 independent experiments). (D) Adipocyte area frequency distribution analysis in non-injected depots from empty vector (n=4; white bars) and COX5B (n=4; blue bars) mice. In bar graphs, values represent mean ± SEM (error bars). Statistical significance was assessed with a two-tailed t-test (*, $p<0.05$).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Reagents and cell culture. The 3T3-L1 cell line (ATCC) was maintained in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% bovine calf serum (ATCC). The HEK293T cell line was maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Cultek). Immortalized mouse embryonic fibroblasts (MEF) were maintained in DMEM supplemented with 10% fetal bovine serum premium (Biowest). All media were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were routinely cultured in 21% O_2 and 5% CO_2 (normoxic conditions). For hypoxic exposure, 3T3-L1 or MEF cell culture dishes were placed into an Invivo2 400 humidified hypoxia workstation (Ruskinn Technologies, Bridgend, UK) at 0.5% O_2. Cells were regularly tested for mycoplasma and treated if necessary. For *Hif1a* gene inactivation in Hif1$^{fl/fl}$ MEF (Tello et al., 2011), cells were treated for 7 days with 4OH-Tamoxifen 1.5 μM. Lentiviral shRNA vectors for *Cox5b* were purchased from Open Biosystems. Lentiviral shRNA vectors for *Cox8a* were purchased from Sigma (Mission shRNA plasmids).

To construct the COX5B overexpression vector, we amplified mouse *Cox5b* cDNA using the following primer set (forward: 5'-GTCCCGCCCATCTTGCT-3'; reverse: 5'-GCCAGTGCAATGGCTAATCTTT-3') and cloned the purified PCR fragment into the pCR®2.1-TOPO® TA vector. *Cox5b* cDNA was obtained from the pCR®2.1-TOPO® TA vector after EcoRI (New England Biolabs) digestion and cloned into the pLVX-IRES-ZsGreen vector (Clontech) to create pLVX-COX5B-IRES-ZsGreen vector. In all figures, figure legends and in the main text, empty vector refers to pLVX-IRES-ZsGreen plasmid and COX5B expression vector refers to pLVX-COX5B-IRES-ZsGreen plasmid.

Sodium azide (Ref: S2002) and Nile Red (Ref: N3013) were purchased from Sigma-Aldrich.

Animal models. All mice used in this study were males of C57/BL6J background. For global *Hif1a* gene inactivation, *Hif1a*^{floxed}-UBC-Cre-ER^{T2} mice were generated from B6.129-*Hif1a*^{tm3Rsjo}/J mice (The Jackson Laboratory, stock no. 007561) that harbor two loxP sites flanking exon 2 of the murine *Hif1a* locus (Ryan et al., 2000). These mice were crossed with B6.Tg (UBC-Cre/ER^{T2}) 1Ejb/J mice (The Jackson Laboratory, stock no. 008085), which ubiquitously express a tamoxifen-inducible CRE recombinase (Cre-ER^{T2}). *Hif1a*^{floxed}-UBC-Cre-ER^{T2} mice and their corresponding control mice were generated through the appropriate crosses.

For adipocyte restricted *Hif1a* gene inactivation, *Hif1a*^{floxed}-adiponectin-Cre-ER^{T2} mice were generated by crossing B6.129-*Hif1a*^{tm3Rsjo}/J mice with Adipoq-CreER^{T2} mice, which express Cre-ER^{T2} in adipocytes (Sassmann et al., 2010). *Hif1a*^{floxed}-adiponectin-Cre-ER^{T2} mice and their corresponding control mice were generated through the appropriate crosses.

Mice were kept under specific pathogen-free conditions at the animal facility at the Autonomous University of Madrid (UAM). For adipocyte-specific *Hif1a* gene inactivation, 20–25 week-old *Hif1a*$^{\text{floxed}}$-adiponectin-Cre-ER$^{\text{T2}}$ and the corresponding *Hif1a*$^{\text{floxed}}$ control mice were given *ad libitum* access for 25–30 days to Teklad CRD TAM^{400}/CreER tamoxifen pellets (The Harlan Laboratory) that contain 400 mg tamoxifen citrate/kg. For global *Hif1a* gene inactivation, 10–15 week-old *Hif1a*$^{\text{floxed}}$-UBC-Cre-ER$^{\text{T2}}$ and the corresponding *Hif1a*$^{\text{floxed}}$ control mice were given *ad libitum* access for 10–15 days to Teklad CRD TAM^{400}/CreER tamoxifen pellets (The Harlan Laboratory). After this period, animals were returned to a standard mouse chow diet (*EURodent Diet 22%*, http://www.labdiet.com/cs/groups/lolweb/@labdiet/documents/web_content/mdrf/mdi4/~edisp/ducm04_028432.pdf). Those male mice that were not fed with tamoxifen diet for gene inactivation were used for age-dependent WAT analysis and lentiviral injections. In all figures, figure legends and in the main text, *Hif1a*$^{\text{floxed}}$-adiponectin-Cre-ER$^{\text{T2}}$ (or Hif1a$^{\Delta\text{Adipo}}$) and *Hif1a*$^{\text{floxed}}$-UBC-Cre-ER$^{\text{T2}}$ (or Hif1a$^{\Delta\text{Ubq}}$) refer to male mice that have been exposed to tamoxifen diet. Control refers to tamoxifen diet exposed *Hif1a*$^{\text{floxed}}$ male mice. Those tamoxifen-treated Hif1a$^{\Delta\text{Ubq}}$ and Hif1a$^{\Delta\text{Adipo}}$ mice that showed *Hif1a* mRNA reduction less than 80% in isolated white adipocytes in Hif1a$^{\Delta\text{Adipo}}$ or WAT in Hif1a$^{\Delta\text{Ubq}}$ mice were excluded.

Chromatin immunoprecipitation (ChIP) assays. For ChIP assays, MEF were cultured on 15 cm plates and exposed to hypoxia (0.5% oxygen) or left under normoxic conditions for 18–20 hr. Subsequently, cells were fixed with a final concentration of 1% (v/v) formaldehyde for 15 min at room temperature, which was stopped by the addition of 125 mM glycine. Cells were washed three times with cold PBS and harvested by scraping in 5 ml of PBS. Cell pellets were resuspended in lysis buffer 1 (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 and 0.25% Triton X-100 - supplemented with protease inhibitors) and incubated on ice for 10 min. Pellets were then resuspended in lysis buffer 2 (10 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA and 0.5 mM EGTA - supplemented with protease inhibitors) and placed in a rotating wheel for 10 min at room temperature. Nuclei pellets from these lysates were resuspended in ChIP SDS sonication buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.1, 5 mM EDTA and 0.5% SDS - supplemented with protease inhibitors) and placed in a rotating wheel for 20 min at 4°C. Nuclei lysates were sonicated to shear the DNA under conditions established to ensure that the DNA fragments were between 300 and 1000 bp. After the removal of the insoluble material by centrifugation, samples were diluted in ChIP Triton dilution buffer (100 mM Tris-HCl pH 8.6, 100 mM NaCl, 5mM EDTA and 5% Triton X-100 - supplemented with protease inhibitors) and pre-cleared with 100 µl of protein G sepharose for 30–120 min at 4°C. From each sample, 100 µl was removed and stored (serving as the input), while the remaining sample was immunoprecipitated overnight at 4°C with a rabbit polyclonal anti-HIF1alpha antibody ChIP grade (AbCam, Ref: ab2185) or a rabbit normal IgG control antibody (Santa Cruz, Ref; sc-2027 X). Immunocomplexes were recovered by addition of 100 µl of Protein G sepharose to the samples that were then sequentially washed twice for 5 min in ChIP Triton dilution buffer, mixed micelle wash buffer (0.2% SDS, 1% Triton X-100, 5% sucrose, 5 mM EDTA, 20 mM Tris-HCl pH 8.1 and 150 mM NaCl), buffer 500 (1% Triton X-100, 1 mM EDTA, 0.1% deoxycholate, 50 mM HEPES pH 7.5 and 500 mM NaCl) and LiCl/detergent wash buffer (250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA and 10 mM Tris-HCl pH 8.0). The complexes were then

washed twice with TE buffer (10 mM Tris-HCl and 1 mM EDTA) and eluted with an elution buffer (1% SDS and 100 mM $NaHCO_3$). DNA-protein cross-linking was reversed in the input and eluted samples by the addition of 200 mM NaCl and incubating overnight at 65 °C. Proteins were removed by the addition of proteinase K (100 µg/sample), 10 mM EDTA, 100 mM Tris-HCl and RNase A (10 µg/sample) for 1 hr at 45 °C. Phenol:chloroform:isoamyl alcohol (500 µl) was added to samples and the upper phase was precipitated in absolute ethanol for 1 hr at -20°C. Finally, DNA was resuspended in 50 µl of water. Immunoprecipitated DNA was quantified by PCR using the following primers: *Cox5b* proximal promoter: forward 5´-GTGCGGCGTCTACTTTTAGC -3´; reverse 5´-TCCGTTGGTGGGAGTCTAAC-3´; *Cox5b* distal region: forward 5´- CCCAAGATTTGCTGGCATCT -3´; reverse 5´-GAGTGGTGGCATTGTCCTTTAAT -3´. *Cox8a* proximal promoter: forward 5´-AGAGGAGAAAGGAACAGAAAGAATAAAG -3´; reverse 5´- CATGCCTTGTTGCCTCTCTTC -3´. *Cox8a* distal region: forward 5´- TCACCAGCATGAAGATTTGACAT -3´; reverse 5´-CCTACCAGCACTAGGGCTAAAGAC -3´.

Western blot and antibodies. 3T3-L1 cells were lysed in Laemmli buffer. Pulverized mouse tissues and isolated adipocytes were homogenized in RIPA buffer supplemented with 1% Triton, 0.2% SDS and an EDTA-free protease inhibitor (Roche), and protein extracts were quantified using BCA technology. Western blotting was performed using 8–15% SDS-polyacrylamide gels and membranes were probed with rabbit polyclonal antisera raised against voltage-dependent anion channel 1 (VDAC1; Ref: ab15895, Abcam), NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4 (NDUFA4; Ref: BS3883, Bioworld) and hypoxia-inducible factor 1 alpha (HIF1A ; Ref: 10006421, Cayman), or mouse monoclonal antibodies raised against cytochrome c oxidase subunit Vb (COX5B; Ref: ab110263, Abcam), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9 (NDUFA9; Ref: ab14713, Abcam), ubiquinol-cytochrome c reductase core protein 1 (UQCRC1; Ref: ab110252, Abcam), succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (SDHA; Ref: ab1377040, Abcam), mitochondrial encoded cytochrome c oxidase I (mt-Co1; Ref: ab14705, Abcam) and alpha tubulin (Ref: T6199, Sigma). Immunoreactivity was detected by enhanced chemiluminescence (Immun-Star WesternC Kit, Biorad, and SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific) and visualized with a digital luminescent image analyzer (Image Quant LAS4000 Mini; GE Healthcare). Quantification of western blots was performed using the gel analyzer tool from ImageJ (NIH).

Mitochondrial ETC complex enzymatic activity. Homogenates of 3T3-L1, HEK293T cells or isolated adipocytes were prepared by three freeze/thaw cycles in PBS followed by centrifugation at 500 × *g* for 10 min. The supernatants were then centrifuged at 16,000 × *g* for 30 min. Pellets were suspended in PBS followed by spectrophotometric analysis of isolated respiratory chain complex activities at 30°C using a JASCO UV-630 spectrophotometer. <u>Complex I</u> activity was determined at 340 nm after the addition of 0.13 mM NADH, 0.4 mM decylubiquinone and 2 µg/ml antimycin A and controlling for rotenone sensitivity. <u>Complex II</u> activity was measured at 600 nm after the addition of 10 mM succinate, 30 µM dichlorphenolindophenol (DCPIP), 0.4 mM decylubiquinone, 2 µg/ml antimycin A and 3 µM rotenone.

Complexes I + III activity was assessed at 550 nm in the presence of 0.1 mM cytochrome c, 0.1 mM NADH and 0.5 mM KCN and controlling for antimycin A sensitivity. Complexes II + III activity was determined at 550 nm in presence of 0.1 mM cytochrome c, 3 mM succinate and 0.5 mM KCN and controlling for antimycin A sensitivity. Complex IV activity was determined at 550 nm in the presence of 1 mg/ml reduced cytochrome c as described (Balsa et al., 2012) and controlling for azide sensitivity. All activities were expressed relative to μg of total protein. All chemicals were obtained from Sigma Aldrich.

Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE). Five million 3T3-L1 cells were washed twice in ice-cold PBS, disrupted by mechanical force and centrifuged for 5 min at 2500 rpm at 4ºC. Isolated adipocytes were prepared by three freeze/thaw cycles in PBS followed by centrifugation at 500 × *g* for 10 min. The supernatants were then centrifuged at 16,000 × *g* for 30 min. 3T3-L1 cell pellets and pellets of isolated adipocytes were first incubated for 10 min at 4ºC in PBS with 1 volume of Digitonin 8 mg/ml. Subsequently, 1 ml of PBS was added and the samples were centrifuged for 5 min at 10,000 rpm at 4ºC. After washing, the mitochondrial pellet was resuspended in 1.5 M aminocaproic acid, 50 mM Bis-Tris/HCl pH 7. Protein concentration was determined by BCA assay. Mitochondria were resuspended at a concentration of 10 μg/μl in 1.5 M aminocaproic acid, 50 mM Bis-Tris/HCl pH 7 and solubilized with 3g/g n-dodecyl β-D-maltoside (DDM). After centrifugation at 13,000 rpm for 30 min at 4ºC, the supernatant was collected and sample buffer (5% Commassie Brilliant Blue G-250 5% in 1.5 M aminocaproic acid solution) was added. Samples (50–100 μg) were loaded and run on a 3–20% gradient BN-PAGE gel as described (Schagger, 1995). Proteins were electroblotted onto PVDF membranes and immunoassayed with mouse monoclonal antisera raised against mt-CO1 (Invitrogen) and rabbit polyclonal antisera raised against UQCRC2 (Protein Tech).

RNA extraction, RT-PCR analysis and primers. Mice were anesthetized by intraperitoneal administration of ketamine (Ketolar® 50 mg/ml) and xylazine (Rompun® 20 mg/ml), and the tissues of interest were removed and snap-frozen in liquid nitrogen. Subsequently, tissues or isolated adipocytes were homogenized in Trizol (Invitrogen), following two freeze/thaw cycles, and total RNA was isolated using the RNeasy RNA Extraction Kit (Qiagen). The cDNA template was prepared by reverse transcription of RNA (1 μg) using Improm-II reverse transcriptase (Promega) and gene expression was measured using a Power SYBR Green PCR Master Mix kit (Applied Biosystems). The amplification data were analyzed using StepOne Software version 2.0 (Applied Biosystems). The same protocol was used for isolated adipocytes, 3T3-L1, and HEK 293T cell lines.

The following primer sets were used: Mouse *Hprt*: forward 5′-GTTAAGCAGTACAGCCCCAAA -3′; reverse 5′-AGGGCATATCCACAACAAACTT -3′. Mouse *b-Actin*: forward 5′-CGATGCCCTGAGGCTCTTT -3′; reverse 5′- TGGATGCCACAGGATTCCA -3′ Mouse *Cox5b*: forward 5′- GGAGATCATGATAGCAGCACAGA- 3′; reverse 5′-AGCTGCCTTTGGAGGTAGCA -3′. Mouse *Cox8a*: forward 5′-GGGCTCAGGTCCACTCGAA -3′; reverse 5′-AAGACAACACACGAAGCAGGAA -3′; Mouse *Cox6a1:* forward 5′-CGAGAGACCCCCGTTCGT -3′; reverse 5′-GTTGTGGAAGAGGGTATGGTTACC -3′. Mouse *Cox6c:* forward 5′-GGAGTTGCCGCTGCCTATAA -3′; reverse 5′-AATTCTGCATACGCCTTCTTTCTT -3′. Mouse *Cox5a:* forward 5′-CGCATCTTGGAGGTTGTTAAG -3′; reverse 5′-TGGATGACATAGGGATAGATTTCCT -3′. Mouse *Cox6b:* forward 5′-CCAGACTAAGAACTGTTGGCAGAAC -3′; reverse 5′-CCCTTGGCCGTCATTGC -3′. Mouse *Cox7c:* forward 5′-CCGTCGCAGCCACTATGAG -3′; reverse 5′-TCCAAAGTACACGGTCATCATAGC -3′. Mouse *Cox7b:* forward 5′-GTTGCCCTTAGCCAAAAACG -3′; reverse 5′-CCACTTGCTGAATGCTTCGA -3′. Mouse *Cox7a2:* forward 5′-TCACGAAGGCATTTTGAAAACA -3′; reverse 5′-CCCCGCCTTTCAGATGAAC -3′. Mouse *Cox4il:* forward 5′-CCGACTGGAGCAGCCTTTC -3′; reverse 5′-TCGTTAAACTGGATGCGGTACA -3′. Mouse *Ndufa4:* forward 5′-CGCTTGGCACTGTTTAATCCA -3′; reverse 5′-TCCATGGCTCTGGGTTGTTC -3′. Mouse *Hif1a*: forward 5′-CACCGATTCGCCATGGA -3′; reverse 5′- TCGACGTTCAGAACTCATCTTTTT -3′. Mouse *Phd3*: forward 5′- TGGACAACCCCAATGGTGAT -3′; reverse 5′-GCAGGACCCCTCCATGTAACT -3′. Human *HPRT*: forward 5′-ATTGTAATACCAGTCAACAG -3′; reverse 5′-GCATTGTTTTGCCAGTGTCAA -3′.

Oxygen consumption in eWAT. Respiration of WAT mitochondria was measured at 37°C by high-resolution respirometry with the Oxygraph-2k (Oroboros Instruments) as previously described (Sebastian et al., 2012). Mice were anesthetized with isofluorane, and eWAT was removed and placed on a 15 ml tube containing 3–6 ml of ice-cold homogenization buffer (0.25 M sucrose, 50 mM KCl, 5 mM EDTA, 1 mM sodium pyrophosphate, 5 mM $MgCl_2$, pH 7.4 and protease inhibitors, Roche). eWAT depots were homogenized in ice-cold homogenization buffer (using a Teflon pestle) and homogenates were centrifuged at 740 × *g* for 5 min at 4ºC. The supernatants were centrifuged again at 9,000 × *g* for 15 min at 4ºC. Mitochondrial cell pellets were resuspended in 100 µl of homogenization buffer and protein concentration was determined by BCA assay. Fifty micrograms of mitochondria were loaded into the Oxygraph chamber containing 2 ml of air-saturated respiration medium (10 mM Ca-EGTA buffer (2.77

mM CaK_2EGTA + 7.23 mM K_2EGTA), 20 mM imidazole, 20 mM taurine, 50 mM K-Mes, 3 mM K_2HPO_4, 6.5 mM $MgCl_2$, 5.7 mM ATP, 15 mM phosphocreatine, and 0.5 mM DTT (pH 7.1)). All respiration measurements were made in duplicate with the following protocol: resting respiration (state 2, absence of adenylates) was assessed by the addition of 10 mM glutamate and 2 mM malate as a complex I substrate, and then state 3 respiration was assessed by the addition of 2.5 mM ADP. The addition of 10 mM succinate induced state 3 respiration with parallel electron input to complexes I and II, and state 4 respiration was determined after addition of 2 µM oligomycin. The addition of 0.5 µM rotenone resulted in inhibition of complex I, thereby allowing examination of O_2 flux with the complex II substrate alone, whereas 2.5 µM antimycin A was added to inhibit complex III to observe non-mitochondrial respiration. The concentrations of substrates and inhibitors used were based on prior experiments conducted for optimization of the titration protocols. Mitochondrial oxygen consumption rate (OCR) coupled to ATP synthesis (or ATP turnover) was calculated as the difference between State 3 and State 4 in the presence of glutamate, malate, ADP and succinate.

Fatty acid oxidation. 3T3-L1 cells were incubated in fully supplemented DMEM medium with 100 µM unlabeled palmitate and 50 µM carnitine. Cells were incubated for 6 hr in medium containing 2 µCi/ml [9,10-^{3}H]-palmitate (De Bock et al., 2013). Thereafter, the supernatant was transferred into glass vials sealed with rubber stoppers. ^3H_2O was captured in hanging wells containing a Whatman paper soaked with H_2O over a period of 48 hr at 37°C to reach saturation (Aragones et al., 2008). Radioactivity was determined by liquid scintillation counting.

Nile Red staining. 3T3-L1 cells were plated at 80% confluence in DMEM medium supplemented with bovine calf serum. After one day, cells reached confluence and one day later the culture medium was replaced by fresh DMEM medium supplemented with FBS or with FBS and 5 mM sodium azide. On the third day, cells were trypsinized and centrifuged for 5 min at 1200 rpm. The cell pellet was suspended and fixed with 2% paraformaldehyde at 4ºC for 15 min. Cells were centrifuged again, resuspended in PBS with a final Nile Red concentration of 1 mg/ml and kept at 4ºC in the dark for 30 min. After two washes with cold PBS, cells were analyzed in a Cytomics FC 500 MPL flow cytometer (Becton Dickinson). Nile Red fluorescence was measured in the FL2 emission channel and 10,000 events were analyzed for each sample.

Human samples. Visceral adipose tissue (VAT) samples and subcutaneous adipose tissue (SAT) samples were obtained from participants recruited at the Endocrinology Service of the Hospital of Girona, "Dr Josep Trueta". Anthropometric and clinical parameters of the human cohort were (values represent mean ± standard deviation): VAT samples: Sex (male/female): 7/17; age (years): 44 ± 10.3; BMI (kg/m2): 31.2 ± 6.1; fasting glucose (mg/dL): 91.1 ± 11.2; HDL cholesterol (mg/dL): 55.6 ± 14.5; fasting triglycerides (mg/dL): 83 (67-191)* (*Median; interquartile range). Age quartiles for correlations with Cox5b

expression (quartile 1: < 36 years; quartile 2: 36–43 years; quartile 3: 43–51 years; quartile 4: >51 years; SAT samples: Sex (male/female): 3/27; age (years): 41.2 ± 11.6; BMI (kg/m2): 42.4 ± 9.8; fasting glucose (mg/dL): 95 (81.7-102.2)*; HDL cholesterol (mg/dL): 54.8± 18.5; fasting triglycerides (mg/dL): 99 (75.5-137.5)* (*Median; interquartile range). All subjects were of Caucasian origin and reported that their body weight had been stable for at least three months before the study. They had no systemic disease other than obesity and were free of any infections in the previous month before the study. Liver diseases and thyroid dysfunction were specifically excluded by biochemical work-up. Adipose tissue samples were obtained during surgical procedures and immediately transported to the laboratory (5-10 min). Tissue handling was carried out under strictly aseptic conditions. The samples were washed in PBS, cut into small pieces (100 mg), and immediately flash-frozen in liquid nitrogen and then stored at -80ºC.

RNA purification was performed using the RNeasy Lipid Tissue Mini Kit (QIAgen, Izasa SA, Barcelona, Spain) and the integrity of the RNA was checked with the Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Gene expression was determined by real time PCR with a LightCycler® 480 Real-Time PCR System (Roche Diagnostics SL, Barcelona, Spain), using TaqMan® technology suitable for relative genetic expression quantification. The commercially available and pre-validated TaqMan® primer/probe sets used were: endogenous cyclophilin A (*PPIA*, Hs99999904_m1, housekeeping gene) and target genes cytochrome c oxidase subunit Vb (*COX5B*, Hs00976765_g1) and voltage-dependent anion channel 1 (*VDAC1*, Hs01631624_gH). These assays were purchased from Life Technologies S.A. (Madrid, Spain). All values were normalized to cyclophilin A expression.

Cellular oxygen consumption. Oxygen consumption rate (OCR) was measured using the XF24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA). 3T3-L1-shSCR or 3T3-shCOX5B cells (6×10^4) were plated per well (3–4 wells per treatment for each independent experiment) one day before the experiment. Cells were preincubated with unbuffered DMEM supplemented with 25 mM glucose, 1 mM pyruvate, and 2 mM glutamine for 1 hr at 37°C in an incubator without CO_2 regulation. OCR measurements were programmed with successive injections of unbuffered DMEM, 5 µg/ml oligomycin, and 1 µM rotenone plus 1 mM antimycin A. Protein concentration was quantified by the BCA assay to normalize the OCR. Calculations were performed following the manufacturer´s instructions. After measuring basal respiration, oligomycin was added to inhibit respiration (by blocking H^+-ATPase), therefore, the amount of oxygen used to produce ATP by OXPHOS is estimated from the difference with basal oxygen consumption (i.e. coupling efficiency or ATP turnover).

Viral infection. For lentiviral infection in vitro, HEK293T cells were transfected with 8 µg of each lentiviral vector and 5 µg of pmd2.G and pCMV8.9 lentivirus packaging plasmids using Lipofectamine 2000 (Invitrogen), according to the manufacturer´s instructions. Cell culture supernatants were harvested 24 hr after transfection, filtered through a 0.45 µm filter and added to 3T3-L1 cells. This step was repeated for the next 2 days.

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