MitoNEET, a key regulator of mitochondrial function and lipid homeostasis

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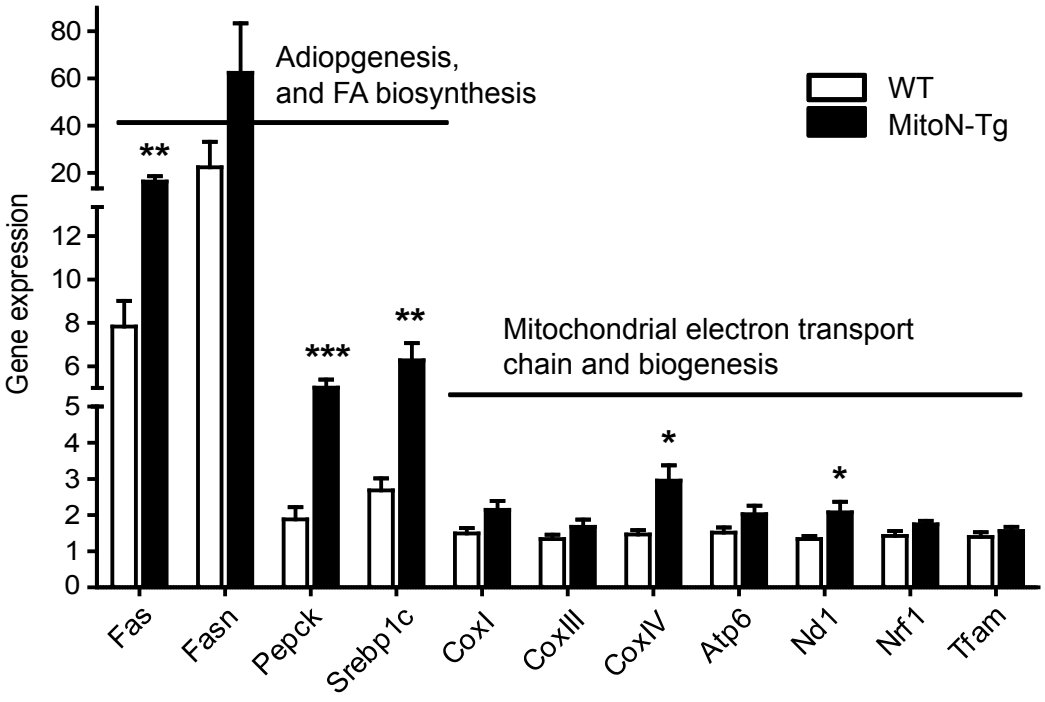
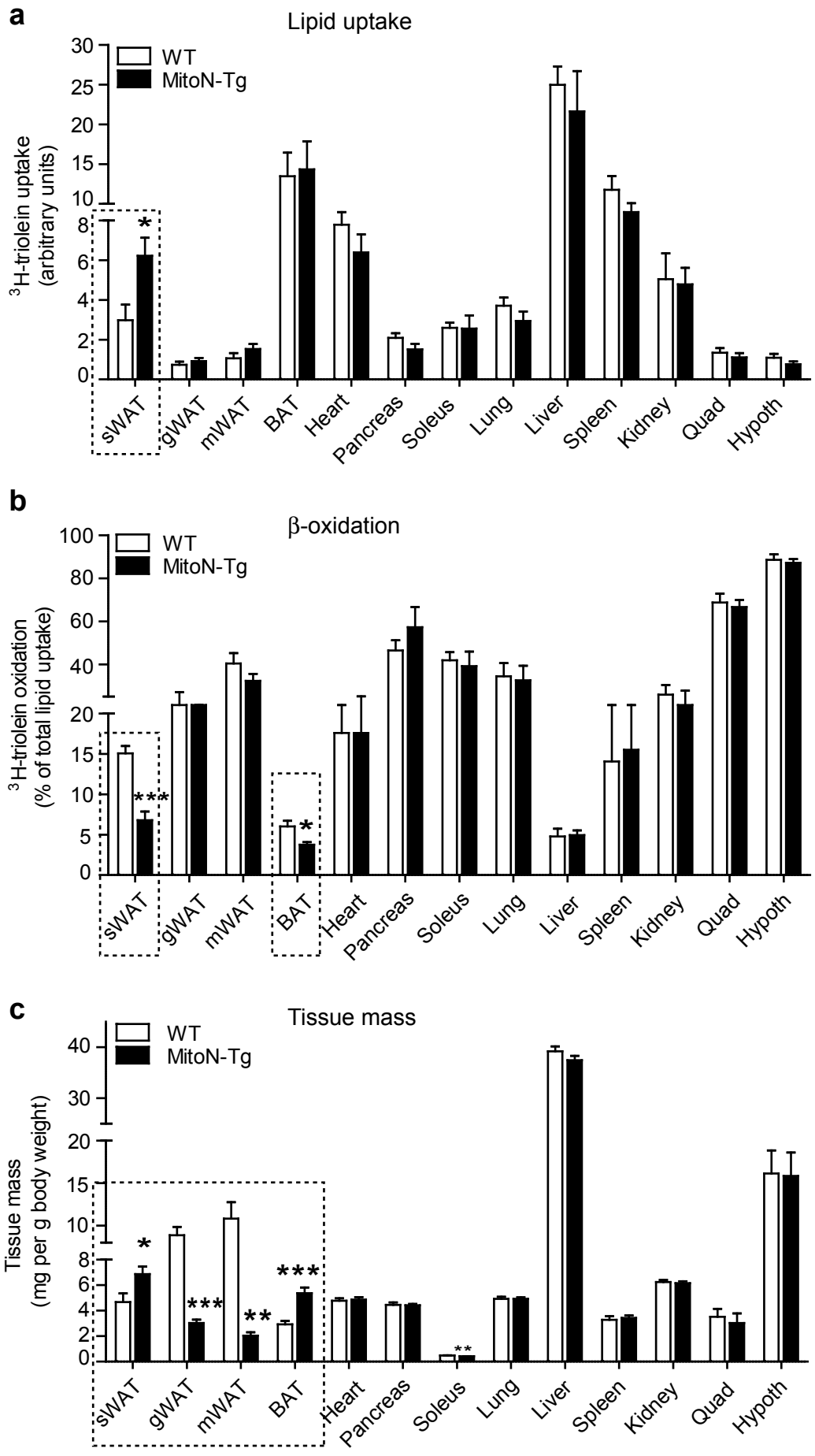
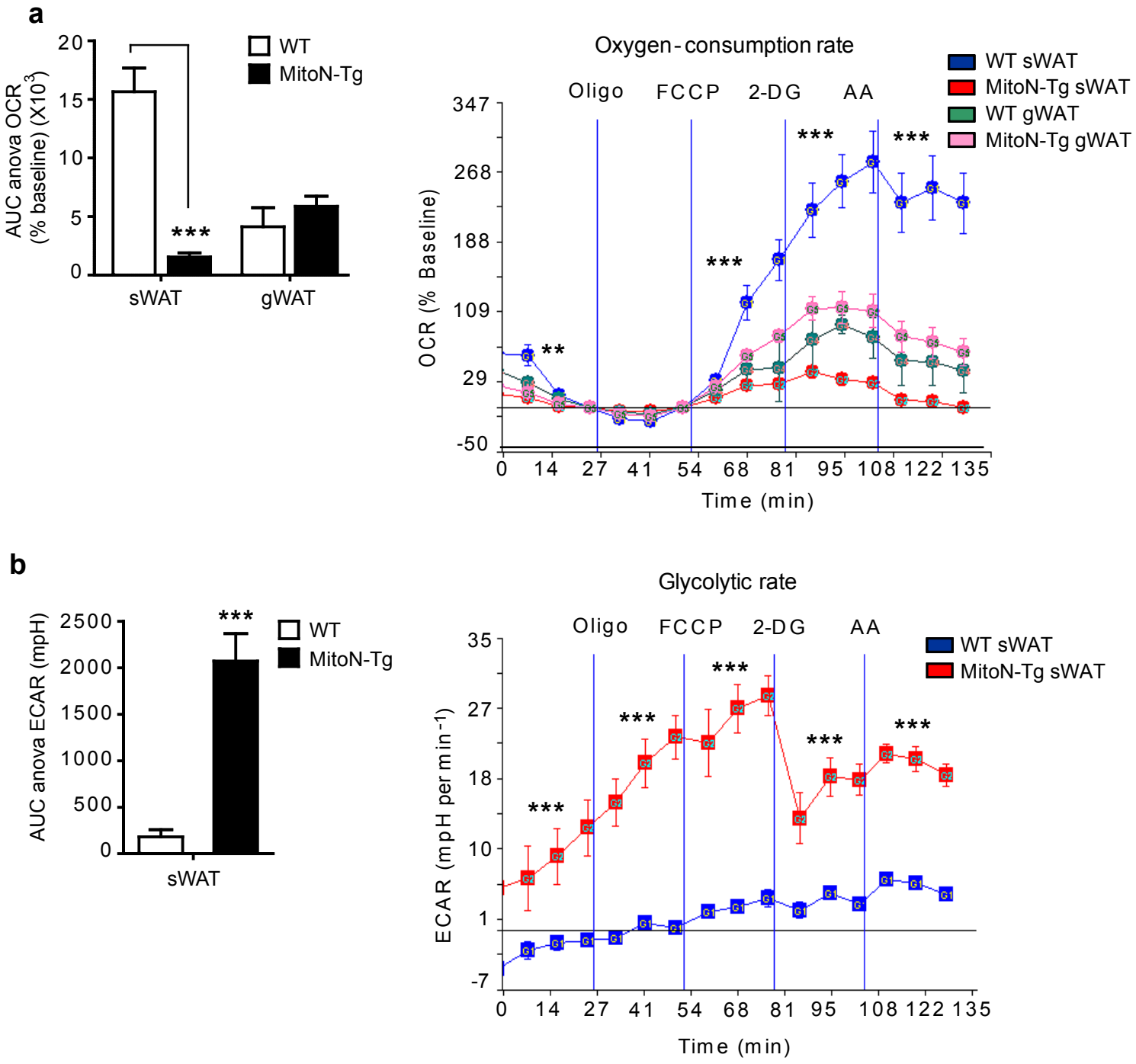
Supplementary Information



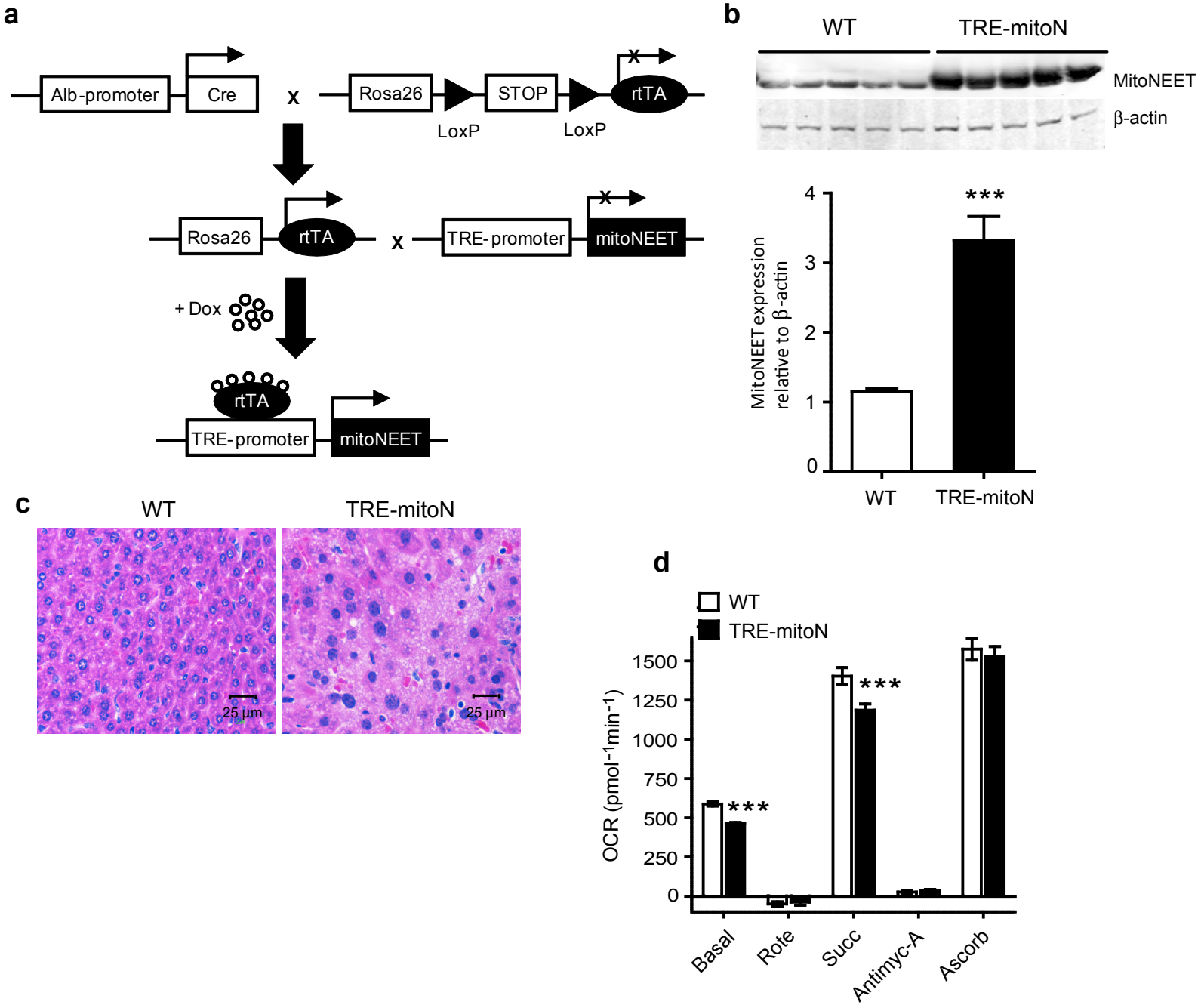
Supplemental Figure 1. Adipogenesis, FA-biosynthesis and mitochondrial pathways are upregulated in MitoN-Tg sWAT. RT-PCR confirmation of key genes obtained from microarray analyses of WT versus MitoN-Tg sWAT (*n* = 9 per group).



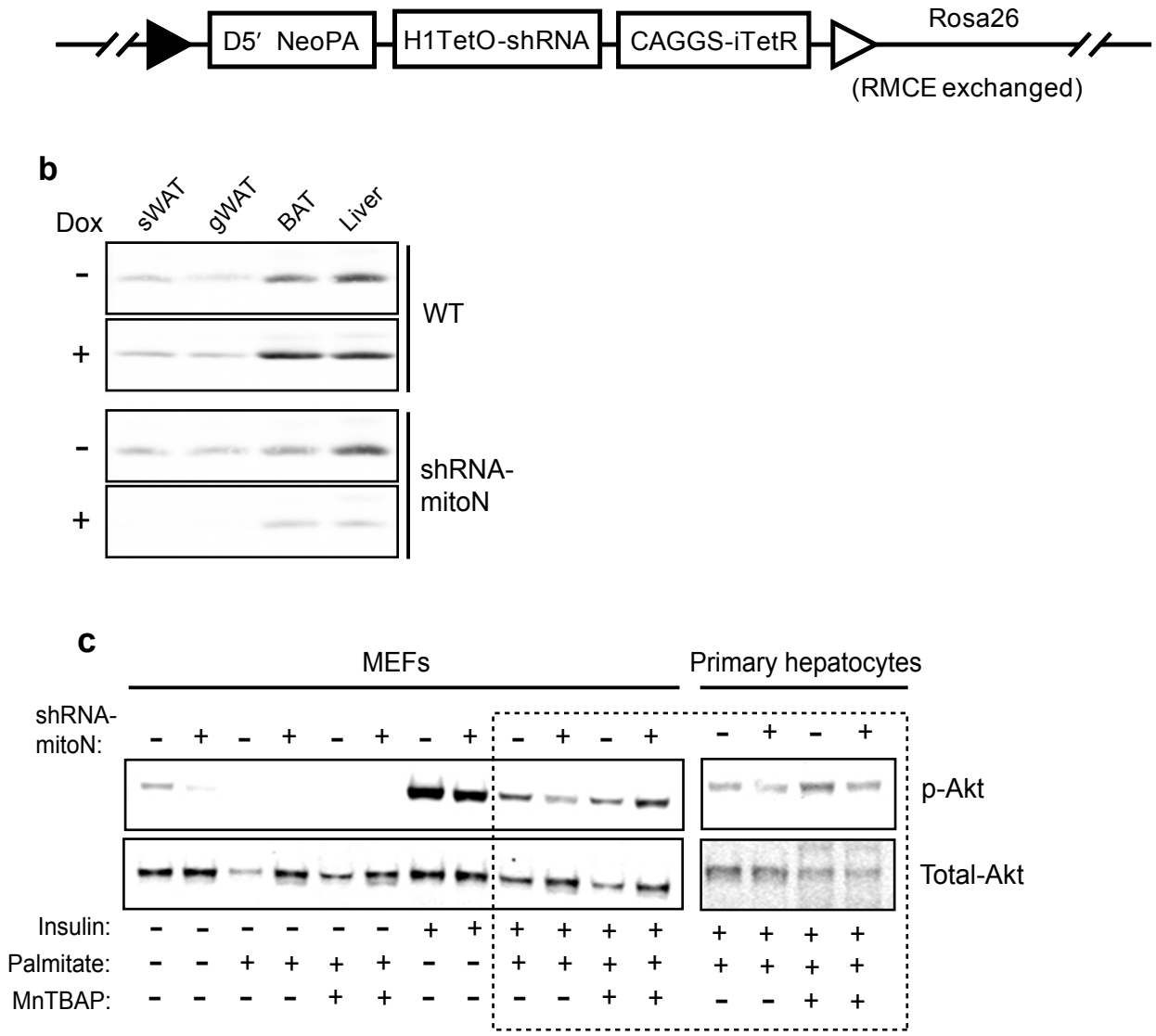
Supplemental Figure 2. MitoNEET enhances lipid-uptake and declines the rate of β-oxidation specifically in sWAT. (a) ^{3}H-triolein lipid-uptake, (b) β-oxidation and (c) tissue-mass in WT versus MitoN-Tg tissues (2 μCi/mouse in 100 ul of 5% intralipid; single tail-vein injection) (*n* = 6 per group).



Supplemental Figure 3. Adipose-specific overexpression of mitoNEET enhances glycolysis. Oxygen consumption rates (OCRs) (top-panel) in WT and MitoN-Tg whole sWAT and gWAT tissue-slices (~20 mg), in addition to extracellular acidification rates (ECARs) (bottom panel) (as a measure of glycolytic rate) in WT and MitoN-Tg sWAT tissue-slices; in response to sequential additions of oligomycin (an ATP synthase inhibitor), FCCP (a chemical uncoupler), 2-Deoxy-D-glucose (2-DG) (an inhibitor of glycolysis) and antimycin-A (complex III inhibitor) (n = 4 per group). $^{**}P < 0.01$; $^{***}P < 0.001$.



Supplemental Figure 4. Generation of an inducible liver-specific overexpression of mitoNEET. (a) Strategy of Dox-inducible overexpression of mitoNEET specifically in the liver. Liver-specific albumin (Alb)-Cre mice were crossed with the Rosa26-loxP-STOP-loxP-rtTA mice to achieve liver-specific expression of rtTA. These mice were then bred with TRE-mitoN transgenic mice. Following exposure to Dox, the resulting triple transgenic mice express mitoNEET only in the liver. (b) Representative Western blots and the average Dox-induced overexpression of mitoNEET protein in liver tissues from WT and TRE-MitoN mice following Dox-HFD-feeding (600 mg/kg Dox-HFD for 3-weeks) (*n* = 5 per group). (c) Representative H&E stain of WT and TRE-MitoN livers following Dox-HFD feeding. (d) OCRs in mitochondria isolated from WT livers and TRE-mitoN livers (*n* = 5 per group).



Supplemental Figure 5. Generation of an inducible constitutive knockdown of mitoNEET. (a) Schematic of the Dox-inducible knockdown system of mitoNEET, with the shRNA construct being targeted to the Rosa26 locus. (b) Representative Western blots demonstrating the Dox-induced knockdown of mitoNEET protein in sWAT, gWAT, BAT and liver tissues from WT and shRNA-MitoN mice following Dox-chow-feeding (600 mg/kg Dox-chow for 10 d) (+Dox) or standard chow-feeding (-Dox) (*n* = 4 per group). (c) Representative Western blots showing insulin signaling (phospho-Akt (Ser473) (p-Akt) and total-Akt) in Dox-treated (1 mg/ml) WT and shRNA-mitoN MEFs (left panel), in addition to primary hepatocytes (right panel) derived from WT mice and shRNA-mitoN mice. Expression levels were examined in response to treatments with, or without insulin, palmitate and the anti-oxidant, MnTBAP (*n* = 4 per group).

Supplemental Table 1. A summary of the differentially regulated pathways and genes identified by microarray cluster analyses from WT and MitoN-Tg sWAT. Gene abbreviations, definitions, in addition to fold-alterations and significant differences between WT and MitoN-Tg sWAT groups are indicated (n = 9 per group). *P < 0.05; **P < 0.01.

Pathway Gene	Gene definition	Fold-change	P-value
Adipogenic and lipogenic transcription factors:			
Pparγ	Peroxisome proliferator activated receptor γ	1.87	0.0067**
C/ebpα/β	CCAAT/enhancer-binding protein α/β	1.36/1.11	0.0458*/0.0382*
Srebp1c	Sterol regulatory element binding transcription factor 1	1.30	0.0500
Lxrα	Nuclear receptor subfamily 1, group H, member 3	1.30	0.0377*
Klf15	Kruppel-like factor 15	1.24	0.0429*
Adipo*q*	Adiponectin	1.50	0.0187*
TG synthesis, NEFA re-esterification and fatty acid biosynthesis:			
Lpin1	Lipin 1	1.31	0.0153*
Pepck-C	Phosphoenolpyruvate carboxykinase, soluble	1.52	0.0367*
Dgat1/2	Diacylglycerol O-acyltransferase 1	1.30/1.23	0.0580/0.0342*
Glut4	Solute carrier family 2 (facilitated glucose transporter) member 4	1.20	0.0138*
Gnpat	Glyceronephosphate O-acyltransferase	1.30	0.0343*
Fasn	Fatty acid synthase	1.20	0.0332*
Fads1/2	Fatty acid desaturase 1/2	1.57/1.20	0.0420*/0.0443*
Mcat	Malonyl CoA:ACP acyltransferase	1.26	0.0142*
Lipid-droplet associated proteins:			
Plin1	Perilipin 1	1.44	0.0374*
Fsp27	Cell death-inducing DFFA-like effector c	1.46	0.0291*
Fatty acid uptake, transport and oxidation:			
Fabp5	Fatty acid binding protein 5	1.50	0.0297*
Fatp4	Fatty acid transporter protein 4	1.20	0.0138*
Cd36	Cd36 antigen	1.15	0.0402*
Cpt1	Carnitine palmitoyl transferase I	-1.50	0.0057**
PPARα	Peroxisome proliferator activated receptor-α	-1.19	N.S
Beta-adrenergic signaling:			
Adrβ1/2/3	Adrenergic receptor β 1/2/3	1.18/1.09/1.36	N.S/N.S/0.0106*

Supplemental Table 2. A list of RT-PCR primer sequences of differentially expressed genes identified by the microarray cluster analysis from sWAT derived from WT mice versus MitoN-Tg mice.

Gene	Forward primer	Reverse primer
β-actin	5'-TACCACAGGCATTGTGATGG-3'	5'-TTTGATGTCACGCACGATTT-3'
Ppar-γ	5'-TCAGAGGGACAAGGATTCATGA-3'	5'-CACCAAAGGGCTTCCGCAGGCT-3'
C/ebp-α	5'-ACGCCGCCTTTGGCTTTC-3'	5'-TTGGCCTTCTCCTGCTGTCG-3'
C/ebp-β	5'-GCCAAGCCGAGCAAGAAGC-3'	5'-CAGGGCGAACGGGAAACC-3'
Adn	5'-GGAGATGCAGGTCTTCTTGG-3'	5'-CGAATGGGTACATTGGGAAC-3'
Pgc1-α	5'-TCCTCACACCAAACCCACAGAA-3'	5'-TTGGCTTGAGCATGTTGCGA-3'
Cd36	5'-TGAGACTGGGACCATTGGTGAT-3'	5'-CCCAAGTAAGGCCATCTCTACCAT-3'

Supplemental Table 3. *Ad libitum* chow-fed and fasted (24 h) body-weights and serum parameters in WT mice versus MitoN-Tg mice (*n* = 7 per group).

	Fed		**Fasted (24 h)**	
	WT	MitoN-Tg	WT	MitoN-Tg
Body-weight (g)	28.7 ± 0.9	28.2 ± 0.5	25.2 ± 1.1	24.9 ± 0.5
Glucose (mg/dl)	146.3 ± 5.2	138.7 ± 6.5	83.3 ± 5.0	100.1 ± 12.9
Insulin (ng/ml)	2.61 ± 0.40	1.85 ± 0.29	0.65 ± 0.05	0.78 ± 0.13
Triglyceride (mg/dl)	116.5 ± 17.3	36.9 ± 8.2**	63.1 ± 10.4	52.5 ± 6.6
FFA (mmol/l)	0.16 ± 0.02	0.12 ± 0.02	0.44 ± 0.03	0.58 ± 0.04*
Glycerol (mmol/l)	0.15 ± 0.02	0.16 ± 0.01	0.19 ± 0.01	0.26 ± 0.02**
Adiponectin (µg/ml)	6.8 ± 0.38	24.3 ± 1.18***	7.5 ± 0.29	21.7 ± 0.10***

Data obtained from 12-week old male FVB mice. ** P < 0.05; ** P < 0.01; *** P < 0.001.*

Supplemental Table 4. Chow-fed *ob/ob* mice versus MitoN-Tg *ob/ob* mice fasted (3 h) body-weights and serum parameters (*n* = 8 per group).

	ob/ob	MitoN-Tg *ob/ob*
Glucose (mg/dl)	427.4 ± 75.9	151.4 ± 17.2**
Insulin (ng/ml)	8.5 ± 2.9	5.4 ± 0.8
Triglyceride (mg/dl)	225.3 ± 58.0	94.1 ± 5.0*
Adiponectin (µg/ml)	6.7 ± 0.7	29.4 ± 2.4***

Data obtained from ~16-week old male FVB mice.
** P < 0.05; ** P < 0.01; *** P < 0.001.*

Supplementary Methods

Animals. Generation of an inducible shRNA-mitoN knockdown mouse model was developed as described by Seibler *et al*.[59] (Taconic Artemis). Briefly, the inducible shRNA transgene was constructed by introduction DNA encoding an shRNA of the sequence (5'-GGCCTTGCTACTGAAACATTTCAAGAGAATGTTTCAGTAGCAAGGCC-3') into a cassette consisting of a truncated Neomycin selectable marker, an H1-tetO promoter/operator for shRNA expression and a CAGGS-iTetR gene (for Dox-dependent repression of shRNA expression) all flanked by incompatible FRT sites for subsequent Flpe-mediated RMCE (Recombinase Mediated Cassette Exchange)[60] in ES cells. The 5' underlined 19 nucleotides are sense and the 3' underlined nucleotides are antisense to mitoNEET transcripts. The shRNA-mitoN transgene cassette was targeted to the RMCE compatible Rosa 26 locus in ES cells (strain 129S6). Six candidate shRNAs were tested in this transgenic format for optimal inducible knockdown of mitoNEET RNA in targeted ES cells. Targeted ES cells expressing the most potent shRNA molecule were utilized for generation of mice by blastocyst injection. Mice used in the present study have been backcrossed to C57/BL6 background. To generate a Dox-inducible mitoNEET overexpression mouse model, mitoNEET cDNA and a Kozak sequence of GCCGCCACC were engineered into the pTRE-tight vector (Clontech) with *Xba I* sites. The expression of mitoNEET is controlled by 7 tandem repeats of tetracycline responsive elements in front of a minimum CMV promoter in the pTRE-tight construct. A rabbit β-globin 3'UTR was included to stabilize the transcript and enhance the translation. After *Nae I*, and *ApaL I* digestion and purification, pTRE-mitoNEET DNA was injected to embryos into a pure C57/BL6 background by the transgenic core facility at UTSW. The liver specific albumin-Cre transgenic mice and the Rosa26-*loxP*-STOP-*loxP*-rtTA transgenic mice were purchased from the Jackson

Laboratories. Albumin-Cre mice were firstly bred with Rosa26-*loxP*-STOP-*loxP*-rtTA mice to obtain mice homozygous for both transgenes. To achieve inducible expression of mitoNEET, TRE-mitoNEET mice were then crossed with double homozygous albumin-Cre and Rosa26-*loxP*-STOP-*loxP*-rtTA mice. The resulting triple transgenic animals were used for experiments. Age-matched transgenic animals with albumin-Cre and Rosa26-*loxP*-STOP-*loxP*-rtTA genotype, but lacking the TRE-mitoNEET transgene were used as controls.

Transmission electron microscope (TEM). For TEM analysis, fresh tissues were fixed by perfusion with 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer. Fixed tissues were then transferred to 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, post-fixed in buffered 1% osmium tetroxide, en bloc stained in 4% uranyl acetate in 50% ethanol, dehydrated with a graded series of ethanol, then embedded in EMbed-812 resin. Thin sections were cut on a Leica Ultracut UCT ultramicrotome and post-stained with 2% uranyl acetate and lead citrate. Images were acquired on a FEI Tecnai G^2 Spirit TEM equipped with a LaB_6 source and operating at 120 kV.

Primary hepatocyte isolation and treatment. Primary hepatocytes were isolated from 12-week old male C57/BL6 WT and shRNA-mitoN mice. Following overnight plating, hepatocytes were treated with either palmitate (400 μM) or MnTBAP (1 mg ml^{-1}) for 4 h, then acutely with insulin (16 mM) for 10 min. Protein was extracted for Western blot analysis.

Mitochondrial membrane potential (ΔΨm). For ΔΨm, WT MEFs and shRNA-mitoN MEFs ($1x10^5$) were incubated with or without Dox (1 μg ml^{-1}; 48 h). Cells were treated with 50 μM TMRM for 20 min at 37 °C, with an additional well containing control FCCP (20 μM for 30 min at 37 °C). All images were obtained using a confocal microscope (Leica TCS SP5) at 63×

magnification. In parallel, for stable transfection, the pCB7-mitoNEET construct was transfected into 3T3-L1 preadipocytes using Lipofectamine 2000 (Invitrogen). The transfected cells were selected by hygromycin B (125 μg ml^{-1}), then individual cell clones were isolated, expanded and analyzed for expression levels of recombinant mitoNEET protein by Western blot. Two clonal lines, with low- and high-mitoNEET expression levels respectively, were utilized for ΔΨm analysis, along with 3T3-L1 preadipocytes pre-treated for 12 h with 1.0 μM TZD (rosiglitazone) or control vehicle. ΔΨm was measured using 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$; Sigma) staining. Briefly, cultured cells were washed with PBS, then incubated for 15 min at 37 °C in 1 ml of serum-free culture medium containing 50 nM $DiOC_6$. Following centrifugation, cells were re-suspended in PBS, then immediately analyzed by flow cytometry on a FACscan (Becton Dickinson Immunocytometry Systems).