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Supplemental Information

Mitoregulin Controls β-Oxidation in Human and Mouse Adipocytes

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Figure S1

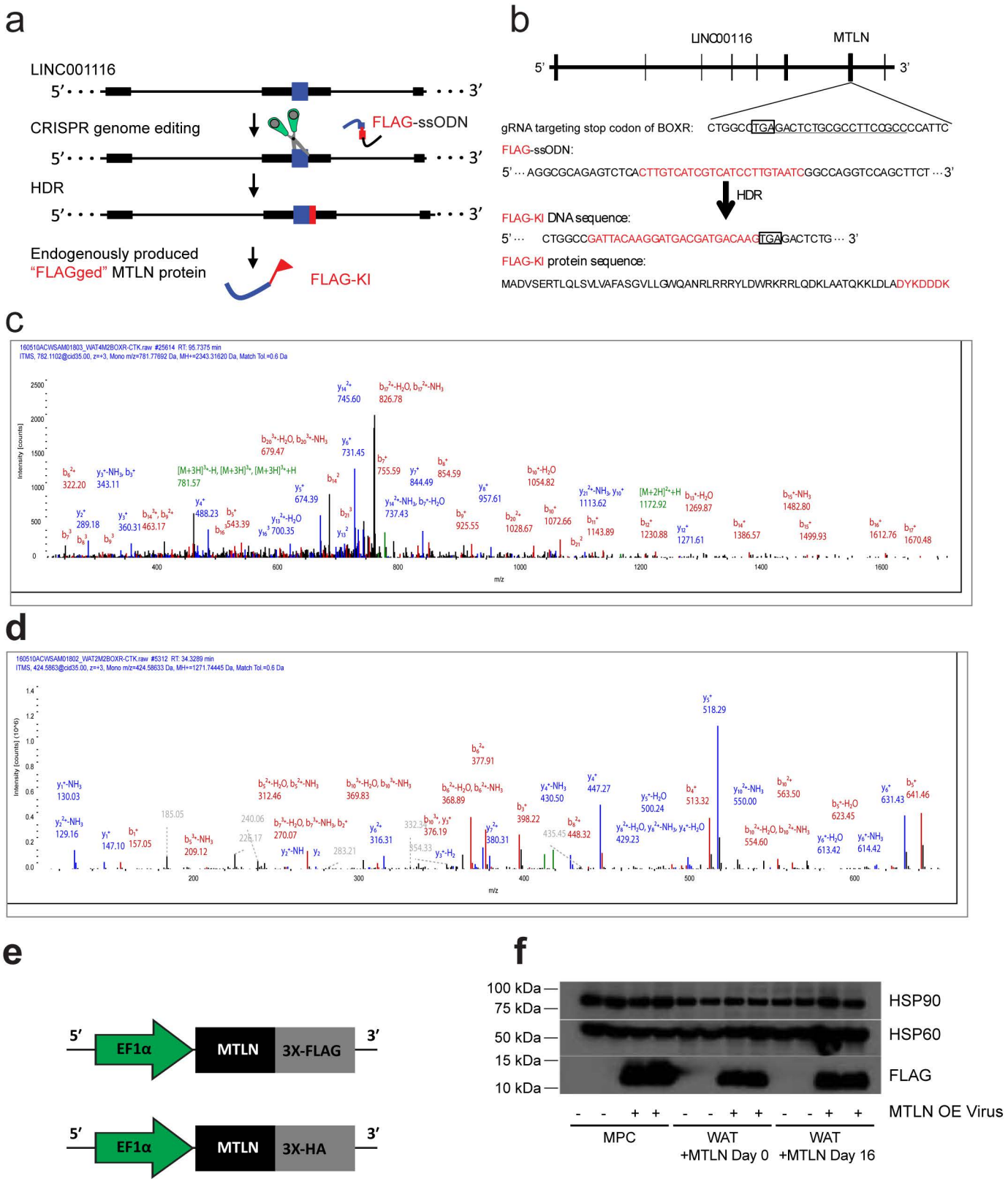

a
LINC001116
5′ · · · 3′
CRISPR genome editing
FLAG-ssODN
5′ · · · 3′
HDR
5′ · · · 3′
Endogenously produced
"FLAGged" MTLN protein
FLAG-KI
b
LINC00116
MTLN
5′
3′
gRNA targeting stop codon of BOXR: CTGGCCTGAGACTCTGCGCCTTCCGCCCCATTC
FLAG-ssODN:
5′ ··· AGGCGCAGAGTCTCACTTGTCATCGTCATCCTTGTAATCGGCCAGGTCCAGCTTCT ··· 3′
HDR
FLAG-KI DNA sequence:
5′ ··· CTGGCCGATTACAAGGATGACGATGACAAGTGAGACTCTG ··· 3′
FLAG-KI protein sequence:
MADVSERTLQLSVLVAFASGVLLGWQANRLRRRYLDWRKRRLQDKLAATQKKLDLADYKDDDK
c
160510ACWSAM01803_WAT4M2BOXR-CTK.raw #25614 RT: 95.7375 min
ITMS, 782.1102@cid35.00, z=+3, Mono m/z=781.77692 Da, MH+=2343.31620 Da, Match Tol.=0.6 Da
Intensity [counts]
m/z
d
160510ACWSAM01802_WAT2M2BOXR-CTK.raw #5312 RT: 34.3289 min
ITMS, 424.5863@cid35.00, z=+3, Mono m/z=424.58633 Da, MH+=1271.74445 Da, Match Tol.=0.6 Da
Intensity [counts] (10^6)
m/z
e
5′
EF1α
MTLN
3X-FLAG
3′
5′
EF1α
MTLN
3X-HA
3′
f
100 kDa
75 kDa
50 kDa
15 kDa
10 kDa
HSP90
HSP60
FLAG
- - + + - - + + - - + +
MTLN OE Virus
MPC
WAT
+MTLN Day 0
WAT
+MTLN Day 16

Figure S2

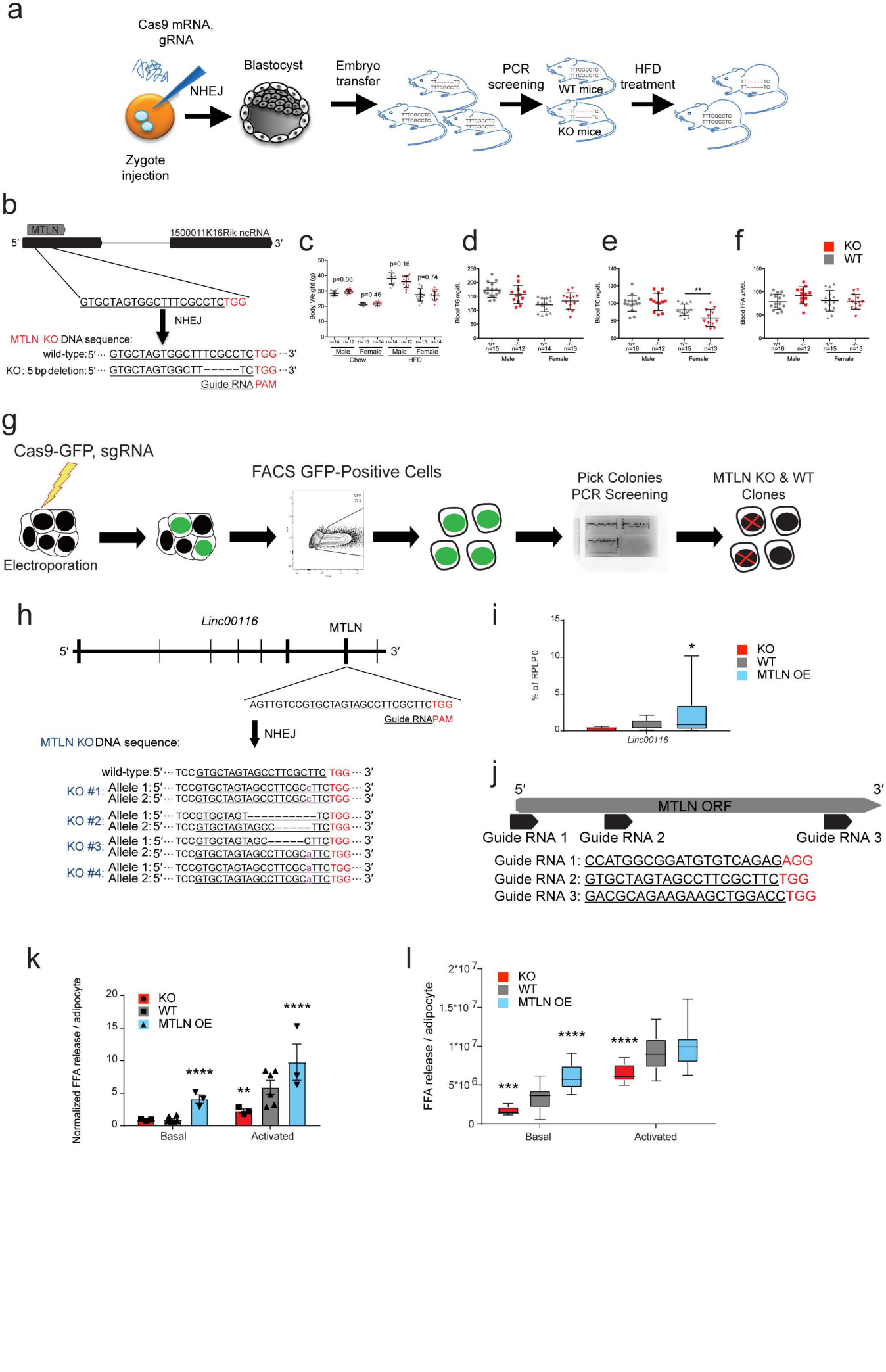

a
Cas9 mRNA, gRNA
NHEJ
Blastocyst
Zygote injection
Embryo transfer
PCR screening
WT mice
KO mice
HFD treatment
b
MTLN
1500011K16Rik ncRNA
5′
3′
GTGCTAGTGGCTTTCGCCTCTGG
NHEJ
MTLN KO DNA sequence:
wild-type: 5′··· GTGCTAGTGGCTTTCGCCTCTGG ··· 3′
KO: 5 bp deletion: 5′··· GTGCTAGTGGCTT−−−−−TCTGG ··· 3′
Guide RNA PAM
c
Body Weight (g)
p=0.06
p=0.46
p=0.16
p=0.74
Male Female Male Female
Chow HFD
d
Blood TG mg/dL
Male Female
e
Blood TC mg/dL
Male Female
f
KO
WT
Blood FFA μm/dL
Male Female
g
Cas9-GFP, sgRNA
Electroporation
FACS GFP-Positive Cells
Pick Colonies PCR Screening
MTLN KO & WT Clones
h
Linc00116
MTLN
5′
3′
AGTTGTCCGTGCTAGTAGCCTTCGCTTCTGG
Guide RNAPAM
NHEJ
MTLN KO DNA sequence:
wild-type: 5′··· TCCGTGCTAGTAGCCTTCGCTTC TGG ··· 3′
KO #1: Allele 1: 5′··· TCCGTGCTAGTAGCCTTCGCcTTCTGG ··· 3′
Allele 2: 5′··· TCCGTGCTAGTAGCCTTCGCcTTCTGG ··· 3′
KO #2: Allele 1: 5′··· TCCGTGCTAGT−−−−−−−−−TCTGG ··· 3′
Allele 2: 5′··· TCCGTGCTAGTAGCC−−−−−TTCTGG ··· 3′
KO #3: Allele 1: 5′··· TCCGTGCTAGTAGC−−−−−CTTCTGG ··· 3′
Allele 2: 5′··· TCCGTGCTAGTAGCCTTCGCaTTCTGG ··· 3′
KO #4: Allele 1: 5′··· TCCGTGCTAGTAGCCTTCGCaTTCTGG ··· 3′
Allele 2: 5′··· TCCGTGCTAGTAGCCTTCGCaTTCTGG ··· 3′
i
% of RPLP0
Linc00116
KO
WT
MTLN OE
j
5′
3′
MTLN ORF
Guide RNA 1
Guide RNA 2
Guide RNA 3
Guide RNA 1: CCATGGCGGATGTGTCAGAGAGG
Guide RNA 2: GTGCTAGTAGCCTTCGCTTCTGG
Guide RNA 3: GACGCAGAAGAAGCTGGACCTGG
k
Normalized FFA release / adipocyte
KO
WT
MTLN OE
Basal
Activated
l
FFA release / adipocyte
KO
WT
MTLN OE
Basal
Activated

Figure S3

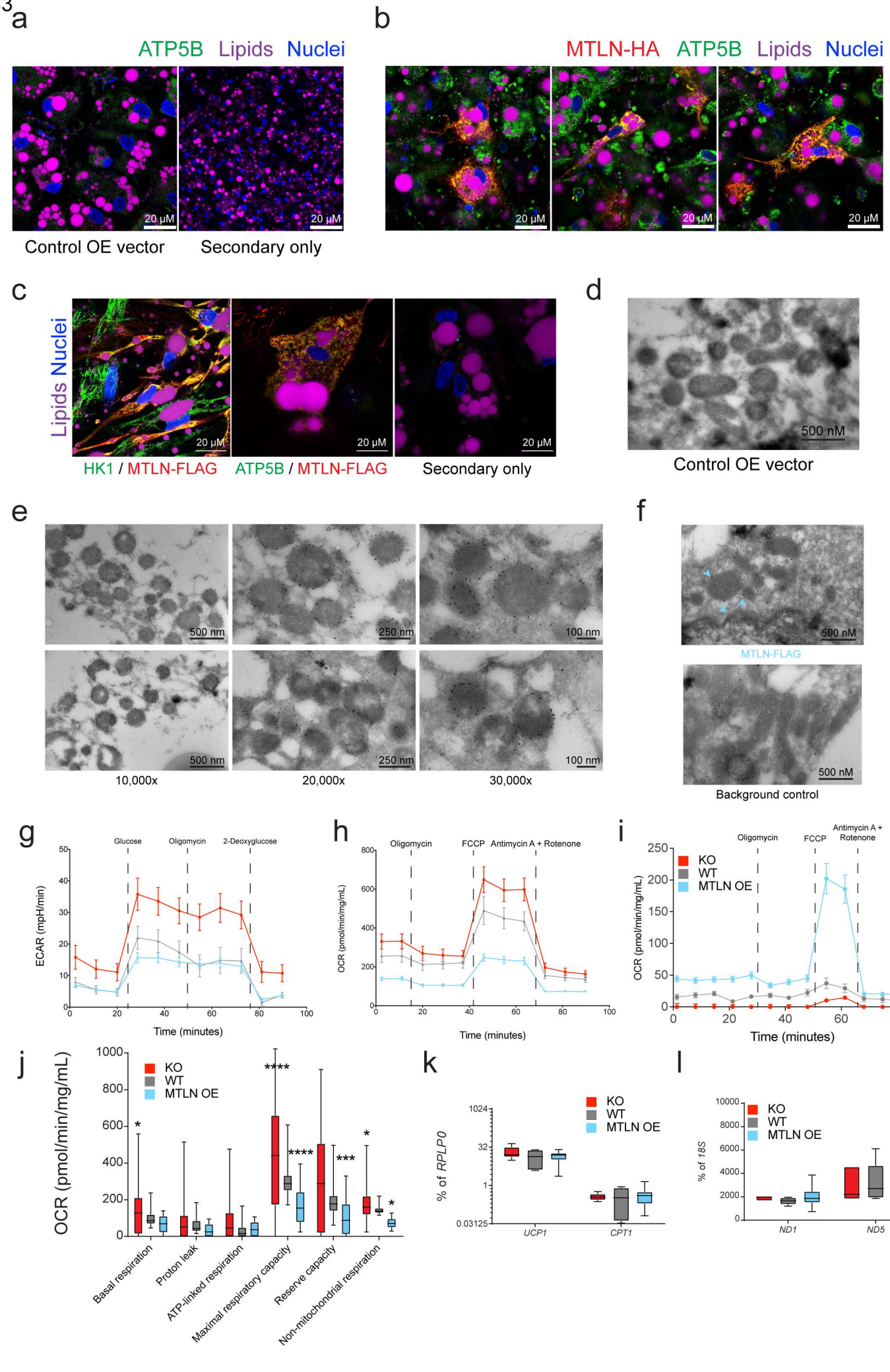
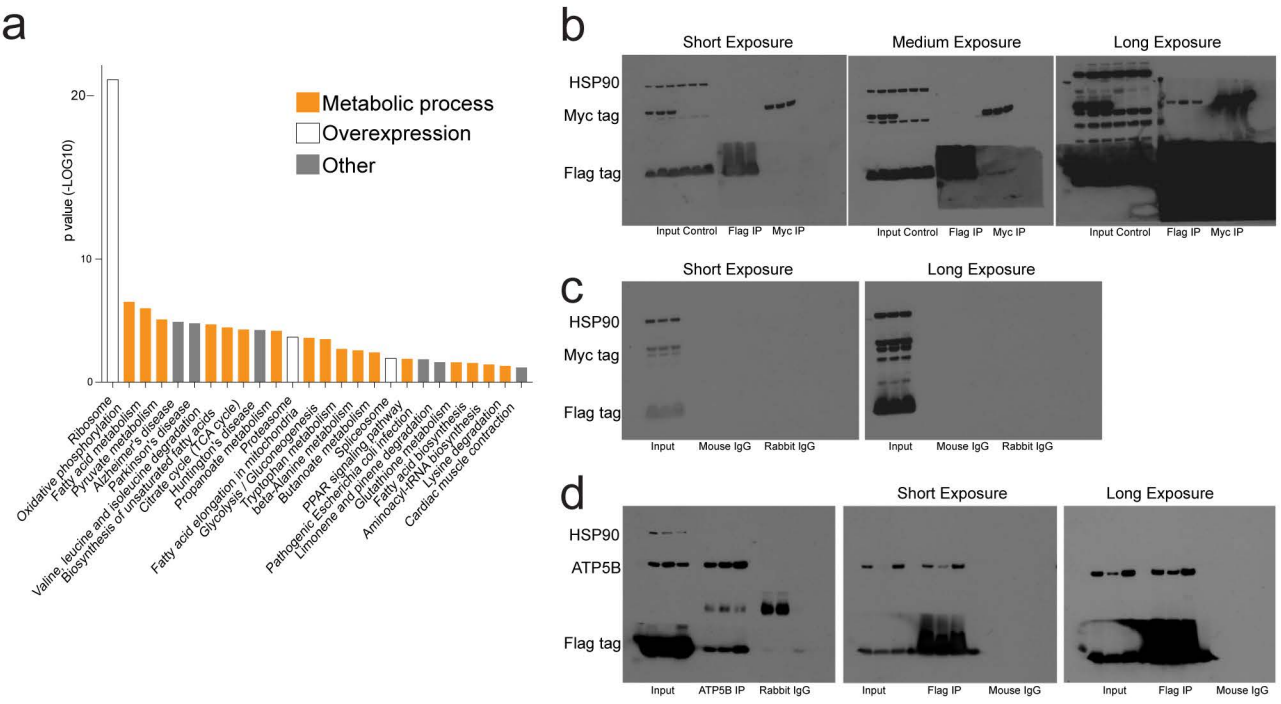

a
ATP5B Lipids Nuclei
20 μM
Control OE vector
Secondary only
b
MTLN-HA ATP5B Lipids Nuclei
c
Lipids Nuclei
HK1 / MTLN-FLAG
ATP5B / MTLN-FLAG
Secondary only
d
500 nM
Control OE vector
e
500 nm
250 nm
100 nm
10,000x
20,000x
30,000x
f
MTLN-FLAG
Background control
g
Glucose
Oligomycin
2-Deoxyglucose
ECAR (mpH/min)
Time (minutes)
h
Oligomycin
FCCP
Antimycin A + Rotenone
OCR (pmol/min/mg/mL)
Time (minutes)
i
KO
WT
MTLN OE
Time (minutes)
j
OCR (pmol/min/mg/mL)
Basal respiration
Proton leak
ATP-linked respiration
Maximal respiratory capacity
Reserve capacity
Non-mitochondrial respiration
k
% of RPLP0
UCP1
CPT1
l
% of 18S
ND1
ND5

Figure S4


a
Metabolic process
Overexpression
Other
p value (-LOG10)
20
10
0
Ribosome
Oxidative phosphorylation
Fatty acid metabolism
Pyruvate metabolism
Alzheimer's disease
Parkinson's disease
Valine, leucine and isoleucine degradation
Biosynthesis of unsaturated fatty acids
Citrate cycle (TCA cycle)
Huntington's disease
Propanoate metabolism
Proteasome
Fatty acid elongation in mitochondria
Gluconeogenesis
Glycolysis / Gluconeogenesis
Tryptophan metabolism
beta-Alanine metabolism
Butanoate metabolism
Spliceosome
PPAR signaling pathway
Pathogenic Escherichia coli infection
Limonene and pinene degradation
Glutathione metabolism
Fatty acid degradation
Fatty acid biosynthesis
Aminoacyl-tRNA biosynthesis
Lysine degradation
Cardiac muscle contraction
b
Short Exposure
Medium Exposure
Long Exposure
HSP90
Myc tag
Flag tag
Input Control
Flag IP
Myc IP
c
Short Exposure
Long Exposure
HSP90
Myc tag
Flag tag
Input
Mouse IgG
Rabbit IgG
d
Short Exposure
Long Exposure
HSP90
ATP5B
Flag tag
Input
ATP5B IP
Rabbit IgG
Flag IP
Mouse IgG

Supplemental Figure 1. MTLN-Flag methodology, related to Figure 1. a) Schematic of the experimental procedure for knocking in a FLAG-tag at the c-terminus of MTLN in hPSCs. b) Partial sequence of the MTLN smORF, FLAG knock-in ssODN, and guide RNA used to generate knock-ins. The resultant DNA and peptide sequence after genome editing is also shown. c) Mass spectrometry spectrum identifying the 2.34 kDa peptide of MTLN. d) Mass spectrometry spectrum identifying the 1.27 kDa peptide of MTLN. e) Schematic of the MTLN-FLAG and MTLN-HA expression constructs inserted into a lentiviral packaging vector. f) Immunoblot demonstrating overexpression of lentiviral MTLN-FLAG in MPCs, MPCs differentiated into hPSC-adipocytes (WAT), or hPSC-adipocytes transduced with MTLN-FLAG after differentiation (WAT+MTLN Day 16).

Supplemental Figure 2. Supplemental data describing mouse and *in vitro* models of MTLN function, related to Figure 2. a) Experimental design for the creation of $MTLN^{-/-}$ mice and HFD treatment. b) Schematic of the murine *1500011K16Rik* ncRNA gene and MTLN smORF. The guide RNA is underlined, the protospacer-adjacent motif (PAM) is in red. Knockout mutation sequence indicated below. c) Body weight of mice on chow or HFD (10.5 weeks). d-f) Serum concentrations of TG (d), total cholesterol (e), and FFA (f) on chow diet. g) Experimental design for knocking out MTLN in hPSCs. h) Schematic of the human *LINC00116* gene and MTLN smORF. The guide RNA is underlined, the PAM is in red. Knockout line indel sequences indicated below. i) qRT-PCR analysis of *MTLN* expression in differentiated hPSC-adipocytes. n=18 for WT, 30 for KO, 48 for OE. j) Pool of guide RNA sequences used in SGBS knockout experiments. k) Concentration of FFAs released into the medium of differentiated murine pre-adipocytes under basal or forskolin stimulated conditions. n=6 for WT, 3 for KO and OE. l) Concentration of FFAs released into the medium of hPSC-adipocytes under basal or forskolin stimulated conditions. n=30/21 for WT, 42/42 for KO, 66/90 for OE basal/stimulated. Data are means ± SD. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, one- or two-way ANOVA. All n's are independent biological replicates performed in technical duplicate.

Supplemental Figure 3. Extended data related to subcellular localization and energetic effects of MTLN, related to Figure 3. a) Confocal microscopy depicting ATP5B (green), neutral lipids (pink), and nuclei (blue) in hPSC-adipocytes. The left panel is an exposure-matched control OE vector as a negative control indicating HA antibody background staining. The right panel is an exposure-matched secondary antibody-only image as a negative control indicating background fluorescence. Scale bar, 20 μm. b) Confocal microscopy showing MTLN-HA (red), ATP5B (green), neutral lipids (purple), and nuclei (blue) in differentiated hPSC-adipocytes in triplicate replicating experiments. Scale bar, 20 μm. c) Confocal microscopy showing MTLN-FLAG (red), HK1/ATP5B (green), neutral lipids (purple), and nuclei (blue) in hPSC-adipocytes. The right panel is an exposure-matched secondary antibody-only image as a negative control indicating background fluorescence. Scale bar, 20 μm. d) Anti-HA immunogold-labeled hPSC-adipocytes with a control OE vector serves as a negative control for transmission electron microscopy studies. e) Immunogold labeling of MTLN-HA in differentiated hPSC-adipocytes. Replicate experiments at three magnification levels are shown. f) Immunogold labeling of MTLN-FLAG in differentiated hPSC-adipocytes. Arrows point to gold particles localized at the mitochondrial membrane. g) ECAR of hPSC-adipocytes during serial stimulation with glucose, oligomycin, and 2-deoxyglucose. n=30. h) OCR of hPSC-adipocytes during serial stimulation with oligomycin, CCCP, and antimycin A/rotenone. n=30. i) OCR of hPSC-adipocytes during starvation followed by serial stimulation with palmitate, oligomycin, CCCP, and antimycin A/rotenone. n=35. j) Comparison of basal and ATP-linked respiration, as well as maximal and spare respiratory capacity of hPSC-adipocytes. n=30. k) *UCP1* and *CPT1* mRNA expression analysis of MTLN KO, WT, and MTLN OE hPSC-adipocytes. n=9. l) *ND1* and *ND5* mtDNA quantity analysis of MTLN KO, WT and MTLN OE hPSC-adipocytes. n=6 for WT and KO, 9 for OE. Data are means ± SD. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, one- or two-way ANOVA. All n's are independent biological replicates performed in technical duplicate.

Supplemental Figure 4. Extended data related to biological pathway analysis and MTLN interactions, related to Figure 4. a) Gene ontology analysis describing the biological pathways enriched in the co-immunoprecipitated proteins bound to MTLN-Flag. Orange: mitochondrial processes. White: protein translation and overexpression. Grey: Other. Modified Fisher Exact P-Value, EASE Score. Data in Supplemental Table 4. b-d) uncropped immunoblots used in Figure 4.

Supplemental Table 4. Kegg Pathway Analysis of Immunoprecipitated Proteins, Related to Supplementary Figure 4a.

Term	PValue	FDR
Ribosome	2.81E-25	3.29E-22
Oxidative phosphorylation	2.94E-07	3.43E-04
Fatty acid metabolism	1.01E-06	0.001177483
Pyruvate metabolism	8.15E-06	0.009534287
Alzheimer's disease	1.26E-05	0.014744305
Parkinson's disease	1.61E-05	0.01885183
Valine, leucine and isoleucine degradation	2.03E-05	0.023750611
Biosynthesis of unsaturated fatty acids	3.56E-05	0.041635892
Citrate cycle (TCA cycle)	5.27E-05	0.061626906
Huntington's disease	5.76E-05	0.067281384
Propanoate metabolism	6.76E-05	0.078968375
Proteasome	2.19E-04	0.255326078
Fatty acid elongation in mitochondria	2.56E-04	0.298580235
Glycolysis / Gluconeogenesis	3.24E-04	0.378657897
Tryptophan metabolism	0.001928104	2.231394172
beta-Alanine metabolism	0.002633853	3.03670699
Butanoate metabolism	0.003878293	4.441943803
Spliceosome	0.011819171	12.97912799
PPAR signaling pathway	0.012556727	13.73555596
Pathogenic Escherichia coli infection	0.014189132	15.38839481
Limonene and pinene degradation	0.023492963	24.26852227
Glutathione metabolism	0.025034765	25.65491006
Fatty acid biosynthesis	0.027528922	27.84859109
Aminoacyl-tRNA biosynthesis	0.037197044	35.80420646
Lysine degradation	0.048336348	43.97110827
Cardiac muscle contraction	0.063839579	53.76136456