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Supplemental Information

MOXI Is a Mitochondrial Micropeptide

That Enhances Fatty Acid β-Oxidation

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SUPPLEMENTAL INFORMATION

Figures S1-S4.

Tables S1-S3. Related to Figure 2.

Supplemental Experimental Procedures.

Supplemental References.

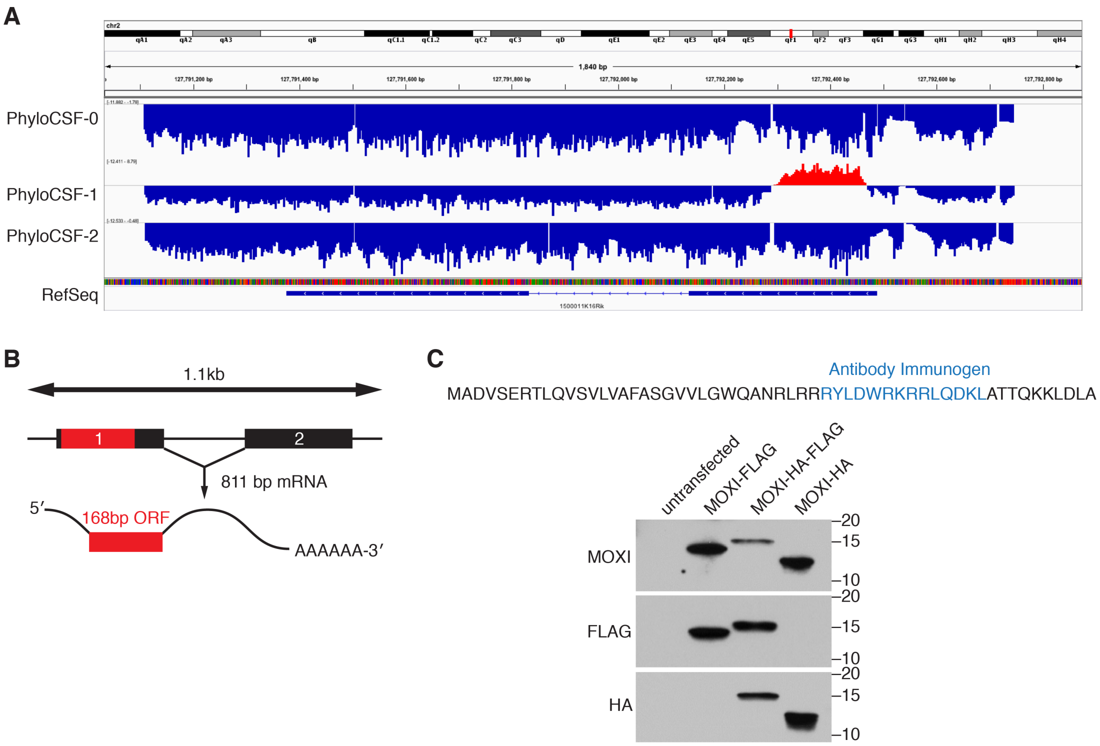

Figure S1. Overview of the *Moxi* Locus and Antibody Validation. Related to Figure 1.

(**A**) PhyloCSF score plot for the *Moxi* locus as seen in the UCSC genome browser using the PhyloCSF track hub. The region highlighted in red indicates a positive PhyloCSF score and corresponds with the MOXI ORF.

(**B**) In mice, *Moxi* is transcribed from a 1.1 kilobase (kb) locus on chromosome 2 to produce a transcript of approximately 800 base pairs (bp). The predicted open reading frame (highlighted in red) is contained entirely within the first exon. The transcript is annotated as *1500011K16Rik* and *LINC00116* in the mouse and human genomes, respectively.

(**C**) A custom antibody was raised against the MOXI peptide and validated by Western blot analysis of HEK293 cells transfected with the indicated fusion proteins. Further validation was performed in skeletal muscle and heart tissues from wild-type and MOXI KO mice (Figures 3A and 3B).

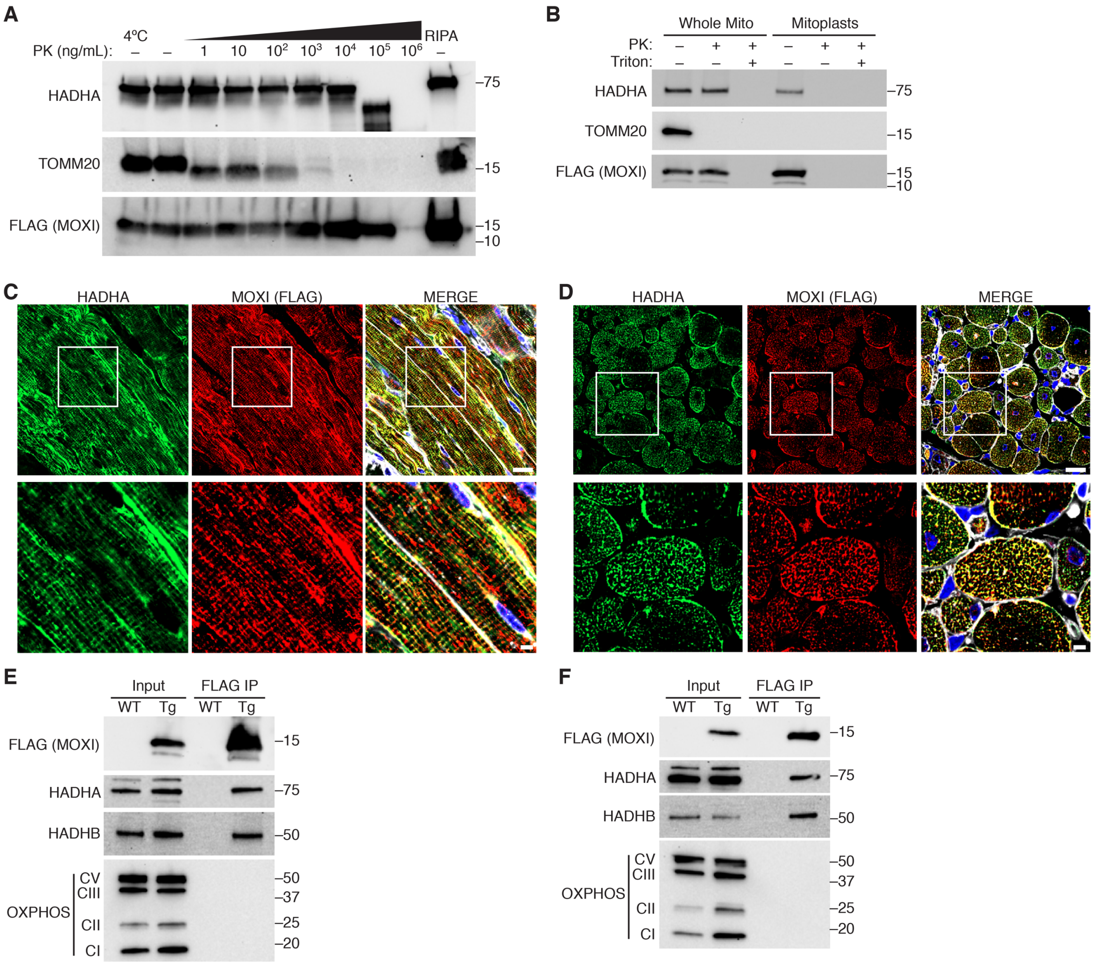

Figure S2. Validation of Proper IMM Localization of MOXI-FLAG in MCK Transgenic Mice and its Interaction with the MTP. Related to Figure 2.

(**A**) Mitochondria were isolated from quadriceps muscle from adult MCK-MOXI-FLAG transgenic mice and subjected to proteinase K (PK) digestion at the indicated concentrations at 37°C unless otherwise indicated. Far right, mitochondria lysed in RIPA buffer serves as a control for the total mitochondrial fraction. Western blots were performed for markers of the OMM (TOMM20), IMM (HADHA) or MOXI-FLAG (FLAG).

(**B**) Adult mouse quadriceps mitochondria from MCK-MOXI-FLAG transgenic mice were subjected to PK proteolysis to digest exposed proteins in the presence or absence of detergent (Triton). Osmotic shock was used to isolate mitoplasts which were also subjected to PK digestion. Western blot analysis was performed using antibodies for MOXI-FLAG (FLAG) or for the sub-mitochondrial markers HADHA and TOMM20.

(**C, D**) Longitudinal (**C**) and transverse (**D**) sections of quadriceps muscle from MCK-MOXI-FLAG TG mice were immunostained with antibodies for HADHA (green) and FLAG (red) and imaged by confocal microscopy. Co-localization of MOXI-FLAG with the IMM protein HADHA is evident by yellow signal in the merged image. The merge image also includes DAPI (blue), which labels the nuclei of the muscle fibers, and wheat germ agglutinin (WGA, white), which marks the membranes of the fibers. Low magnification images are displayed on top (scale bar, 20μm) and high magnification images are below (scale bar, 5μm).

(**E, F**) Western blots analysis of FLAG IPs from gastrocnemius/plantaris (**E**) and heart (**F**) homogenates of WT and MCK-MOXI-FLAG TG mice using antibodies for HADHA, HADHB, FLAG, and an OXPHOS cocktail that detects Complex I, II, III and V of the OXPHOS complex. The interaction of MOXI-FLAG with the MTP in the IMM appears to be specific as subunits of the highly abundant OXPHOS complex are excluded from the bound fractions.

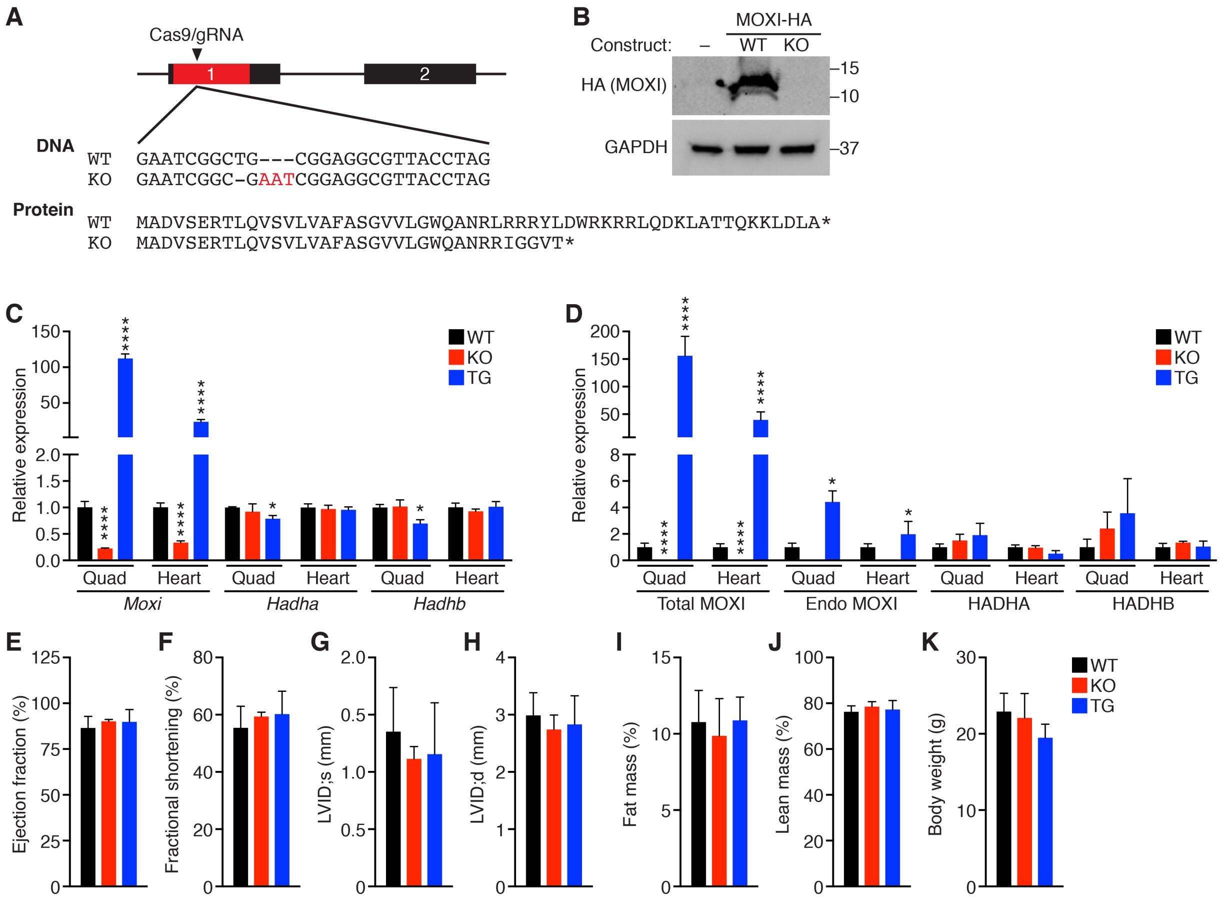

Figure S3. Generation and Baseline Phenotyping of MOXI knockout and transgenic mice. Related to Figure 3.

(**A**) A CRISPR gRNA was generated to target the coding sequence of MOXI. An allele containing a 1-bp deletion and 3-bp insertion that creates a frame-shift and premature stop codon was chosen for further experiments.

(**B**) The mutant MOXI sequence created by the CRISPR strategy was cloned and analyzed for stability by Western blot. When transfected in HEK293 cells, the wild-type MOXI-HA fusion protein produces a stable protein while the knockout clone cannot be detected.

(**C**) RNA expression levels of the indicated genes were analyzed in quadriceps and heart tissue of WT, KO and TG mice by qRT-PCR. Data are expressed as mean ± SD for n = 4 mice with significance indicated as $*p < 0.05$, $****p < 0.001$.

(**D**) Quantification of protein expression levels by Western blot analysis of quadriceps and heart tissue of WT, KO and TG mice. Data are expressed as mean ± SD for n = 8 mice with significance indicated as $*p < 0.05$, $****p < 0.001$. Total MOXI expression (FLAG-tagged and untagged MOXI) and the endogenous (Endo) MOXI protein were quantified separately.

(**E-H**) Echocardiography was performed on adult male mice and cardiac function (**E**, **F**) and dimensions (**G**, **H**) were calculated from M-mode images. Data are expressed as mean ± SD for n = 12 WT and KO mice and n = 7 TG mice. LVID, left ventricular internal diameter; s, systole; d, diastole.

(**I-K**) Body composition was measured in adult male mice. Fat mass (**I**) and lean mass (**J**) were analyzed by live magnetic resonance imaging (MRI) and were expressed as a percentage of total body weight (**K**) for n = 10 mice.

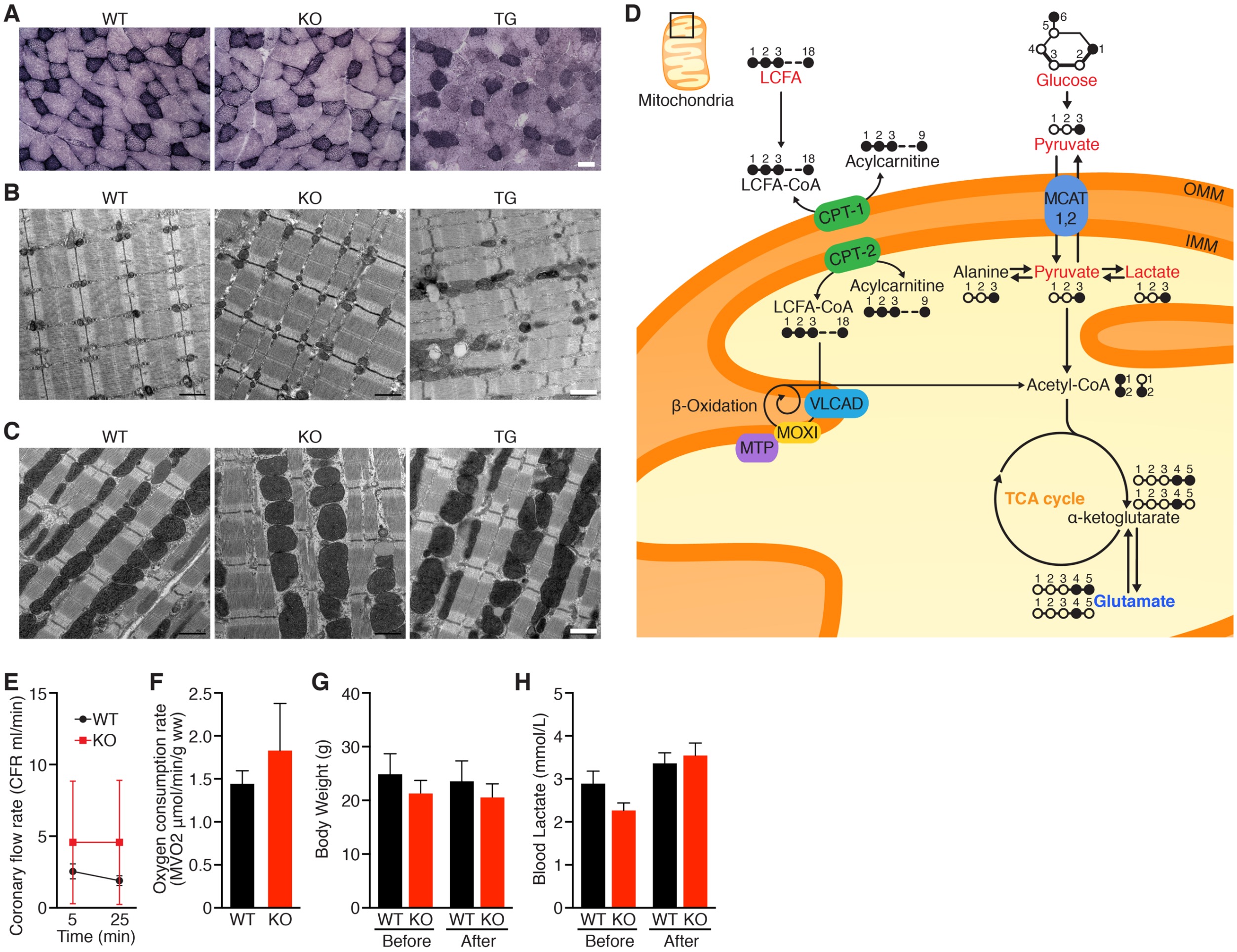

Figure S4. Mitochondrial Abnormalities in KO and TG Mice. Related to Figures 3 and 4.

(**A**) Brightfield images of transverse cryosections of quadriceps muscle from WT, KO and TG mice after succinate dehydrogenase (SDH) staining. Representative images from n = 4 mice are shown. Scale bar, 100µm.

(**B, C**) Transmission electron micrographs of quadriceps muscle (**B**) and heart (**C**) from WT, KO and TG mice at low magnification. Scale bar, 1µm. Representative images from n = 3 mice are shown.

(**D**) Illustration of carbohydrate vs long chain fatty acid substrate selection metabolism used for ^{13}C-NMR isotopomer and flux analyses (related to Figure 4A-F).

(**E**, **F**) Coronary flow rates (**E**) and oxygen consumption rates (**F**) were assessed during perfusion of WT and KO hearts for ^{13}C-NMR isotopomer studies. Data are presented as mean ± SEM for n = 4 mice per genotype.

(**G, H**) Body weight (**G**) and blood lactate levels (**H**) measured in WT and KO mice before and after forced treadmill running (related to Figure 4G and 4H). Data are represented as mean ± SD for n = 14 mice.

Table S1. Unique peptides identified by mass spectrometry of the trifunctional enzyme subunit alpha (HADHA). Percent Coverage: 40.6%. Related to Figure 2.

Position	**Probability**	**Charge / Sequence**	**Number of instances**
39 - 46	0.9858	THINYGVK	1
47 - 53	0.9994	GDVAVIR	3
54 - 60	0.9855	INSPNSK	1
119 - 125	0.9984	ISQEGQR	3
126 - 132	0.1546	MFEKLEK	3
191 - 205	0.9996	MVGVPAAFDMMLTGR	35
215 - 230	0.9996	MGLVDQLVEPLGPGIK	9
215 - 235	0.9996	MGLVDQLVEPLGPGIKSPEER	3
268 - 279	0.9996	LTTYAMTVPFVR	15
280 - 289	0.0589	QQVYKTVEEK	1
285 - 292	0.1252	TVEEKVKK	1
310 - 326	0.9996	AGLEQGSDAGYLAESQK	8
327 - 334	0.9996	FGELALTK	11
327 - 337	0.9996	FGELALTKESK	2
338 - 350	0.9977	ALMGLYNGQVLCK	3
387 - 399	0.9996	TLLKDTTVTGLGR	3
391 - 399	0.9996	DTTVTGLGR	1
400 - 406	0.9995	GQQQVFK	2
416 - 422	0.9972	ALTSFER	2
461 - 489	0.9996	EVESVTPEHCIFASNTSALPINQIAAVSK	2
506 - 516	0.9994	MQLLEIITTDK	6
517 - 531	0.9996	TSKDTTASAVAVGLR	2
520 - 531	0.9996	DTTASAVAVGLR	5
520 - 534	0.9996	DTTASAVAVGLRQGK	3
532 - 540	0.3681	QGKVIIVVK	1
541 - 549	0.9996	DGPGFYTTR	2
550 - 560	0.9996	CLAPMMSEVMR	23
561 - 569	0.9996	ILQEGVDPK	14
632 - 644	0.4449	SGKGFYIYQEGSK	2
635 - 644	0.9996	GFYIYQEGSK	2
647 - 660	0.9995	SLNSEMDNILANLR	8
661 - 676	0.9996	LPAKPEVSSDEDVQYR	5
720 - 728	0.9996	FVDLYGAQK	1

Table S2. Unique peptides identified by mass spectrometry of the trifunctional enzyme subunit beta (HADHB). Percent Coverage: 37.9%. Related to Figure 2.

Position	**Probability**	**Sequence**	**Number of instances**
43 - 53	0.311	SKKTLAKPNMK	2
46 - 53	0.9519	TLAKPNMK	2
63 - 73	0.9996	IPFLLSGTSYK	4
83 - 91	0.9996	AALSGLLHR	4
119 - 129	0.9996	EAALGAGFSDK	2
183 - 189	0.974	MMLDLNK	2
183 - 191	0.3303	MMLDLNKAK	3
231 - 239	0.9996	LAAAFAVSR	7
240 - 248	0.9996	MEQDEYALR	22
256 - 269	0.9754	AQDEGHLSDIVPFK	2
279 - 292	0.9996	DNGIRPSSLEQMAK	9
327 - 335	0.9996	ALAMGYKPK	5
340 - 349	0.9996	DFIYVSQDPK	2
340 - 362	0.9996	DFIYVSQDPKDQLLLGPTYATPK	3
350 - 362	0.9996	DQLLLGPTYATPK	18
393 - 406	0.9995	AMDSDWFAQNYMGR	8
408 - 417	0.74	TKVGSPPLEK	2
449 - 475	0.9813	DGGQYALVAACAAGGQGHAMIVEAYPK	2

Table S3. Unique peptides identified by mass spectrometry of MOXI-FLAG. Percent Coverage: 58.8%. Related to Figure 2.

Position	**Probability**	**Charge / Sequence**	**Number of instances**
8 - 29	0.9993	TLQVSVLVAFASGVVLGWQANR	1
8 - 31	0.9993	TLQVSVLVAFASGVVLGWQANRLR	2
52 - 58	0.9961	KLDLASR	6
62 - 80	0.9993	DHDGDYKDHDIDYKDDDDK	41
69 - 80	0.0554	DHDIDYKDDDDK	1

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals. All animal work described in this manuscript has been approved and conducted under the oversight of the UT Southwestern Institutional Animal Care and Use Committee. Transgenic and knockout mouse lines were generated on a pure C57BL/6N background. All functional experiments were performed using 12-16 week old male mice.

Identification of conserved small open reading frames. RNA transcripts annotated as noncoding were analyzed using PhyloCSF on the UCSC genome browser (Kent et al., 2002; Lin et al., 2011; Raney et al., 2014). PhyloCSF was used to calculate conservation scores for potential open reading frames (ORFs) in the three reading frames on both strands of DNA (Lin et al., 2011). Regions that scored positively by PhyloCSF were further analyzed manually. The MOXI ORF was found contained within the noncoding transcript *1500011K16Rik* in mice and *LINC00116* in humans.

In vitro transcription and translation. The full-length *Moxi* and *Pln* RNAs were subcloned into pcDNA3.1(+) (Invitrogen) containing the T7 RNA polymerase promoter. Frameshift (FS) mutations were introduced immediately after the endogenous ATG start codon of each transcript to disrupt the open reading frames of the micropeptides using site directed mutagenesis (QuikChange Lightening Site Directed Mutagenesis kit, Agilent Technologies). Coupled in vitro transcription and translation assays were performed using digested linear plasmids and the TNT T7 Coupled Wheat Germ Extract System (Promega), as per the manufacturer's protocol. Reactions were setup with radiolabeled [^{35}S]methionine (PerkinElmer). Products were resolved on 18% SDS–polyacrylamide gels, dried, and exposed to film for 48 hours at room temperature.

Quantitative mRNA measurement. Total RNA was extracted from adult mouse tissues using Trizol and reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad) with random primers. Quantitative Polymerase Chain Reaction (qPCR) samples were assembled using KAPA SYBR Fast qPCR Master Mix (SIGMA). Assays were performed using a 7900HT Fast Real-Time PCR machine (Applied Biosystems). Expression amount was normalized to 18S mRNA and was represented as fold change. The following oligonucleotides were ordered from Integrated DNA Technologies to measure transcript abundance:

18s Forward: 5′- ACC GCA GCT AGG AAT AAT GGA **-3′**

18s Reverse: 5′- GCC TCA GTT CCG AAA ACC A **-3′**

MOXI Forward: 5′- GTG TCC GTG CTA GTG GCT TT **-3′**

MOXI Reverse: 5′- CCA GGT CCA GCT TTT TCT GA **-3′**

Hadha Forward: 5′- TGC ATT TGC CGC AGC TTT AC **-3′**

Hadha Reverse: 5′- GTT GGC CCA GAT TTC GTT CA **-3′**

Hadhb Forward: 5′- ACT ACA TCA AAA TGG GCT CTC AG **-3′**

Hadhb Reverse: 5′- AGC AGA AAT GGA ATG CGG ACC **-3′**

Tissue western blot analysis. Tissues were collected and snap frozen in liquid nitrogen. Frozen samples were pulverized and then homogenized in RIPA buffer (SIGMA) with added cOmplete, EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitors (Roche) on ice using a glass WHEATON Dounce tissue grinder ('tight'). Protein concentration was determined using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific). Samples were separated on Mini-PROTEAN TGX Precast Gels (Bio-Rad) or bis/acrylamide gels made by standard gel preparation. Gels were transferred to PVDF membrane (Millipore, Immobilon-P), blocked in 5% milk/TBST and then incubated in primary antibodies: MOXI (New England Peptide), 1:500; HADHA (Abcam) 1:1,000; HADHB (BETHYL Laboratories), 1:500; and GAPDH (Millipore), 1:10,000. Western blots were washed in TBST, incubated with fluorescent or HRP-conjugated secondary antibodies (Bio-Rad), and then developed using a ChemiDoc MP Imaging System (Bio-Rad) or autoradiograph film.

Cell culture, transfections and immunostaining. C2C12 myoblasts and HEK293 cells (ATCC) were grown and maintained in DMEM containing 10% FBS and penicillin-streptomycin. C2C12 cells were transfected with Lipofectamine 2000 (Invitrogen) and HEK293 cells were transfected with FuGENE 6 Transfection Reagent (Promega) according to the manufacturer's protocol. For immunostaining, C2C12 cells were plated on MatTek glass bottom dishes and transfected for 24 hours. Cells were washed with PBS and then fixed with 4% paraformaldehyde. Fixed cells were permeabilized with 0.1% Triton-X100, blocked with 1% BSA/PBS, and then incubated in primary antibody (rabbit FLAG or mouse FLAG M2, SIGMA) diluted in blocking buffer at 4°C overnight. The following day, cells were washed and incubated with Alexa Fluor (Invitrogen) fluorescent secondary antibodies and DAPI (SIGMA). Samples were imaged using a Zeiss LSM-800 confocal with a 40X oil objective.

Mitochondrial subfractionation and proteinase K digestion assays. Quadriceps muscles were isolated from mice and homogenized with a Teflon Potter dounce in isolation buffer (75 mM sucrose, 225 mM mannitol, 5 mM HEPES and 1 mM EGTA, pH 7.4). Homogenates were centrifuged at 2,000xg and then the supernatants were transferred to fresh tubes and re-centrifuged at 14,000xg. The pellets were resuspended in isolation buffer and spun again at 14,000xg. The pellet was resuspended in assay buffer (125 mM KCl, 20 mM HEPES, 2 mM $MgCl_2$, 2 mM KH_2PO_4 and 0.04 mM EGTA, pH 7.2). For proteinase K gradient experiments, mitochondrial samples were incubated with a range of PK concentrations from 1 ng/mL to 1 mg/mL at 37°C and then analyzed by gel electrophoresis and Western blotting. For subfractionation experiments, mitochondria were swollen in hypotonic 10 mM KH_2PO_4 buffer (pH 7.4) and then one-third the volume of hypertonic buffer (1.8 M sucrose, 10 mM $MgCl_2$, pH 7.4) was added to shrink the mitochondria. Samples were sonicated and then centrifuged at 12,000xg. The pellet was resuspended in hypertonic buffer and centrifuged at 12,000xg. The pellet derived from this spin was the mitoplast fraction. This was then either kept as the total mitoplast fraction or re-swollen in hypotonic buffer and shrunk in hypertonic buffer to disrupt the inner mitochondrial membrane. Where indicated, samples were treated with Triton X-100 at a final concentration of 1%.

Generation of mouse lines. Animal work described in this manuscript has been approved and conducted under the oversight of the UT Southwestern Institutional Animal Care and Use Committee. All mouse lines were generated on a pure C57BL/6N background. Muscle creatine kinase (MCK) transgenic (TG) mice were made as previously described by placing the murine MOXI cDNA fused with a C-terminal FLAG tag under the control of a 4.8-kb fragment of the MCK promoter (Sternberg et al., 1988) and deriving transgenic mice on a C57BL/6N background. All analyses were done within the same line using wild-type littermates as controls. For genotyping, tail biopsies were collected and treated with an alkaline lysis buffer (25 mM NaOH, 0.2 mM EDTA) at 95°C, followed by neutralization with 40 mM Tris-HCl. Transgenic mice were genotyped based on the presence or absence of the hGH sequence and myogenin primers were used as a positive control. The following primers were used for genotyping:

hGH Forward: 5'- GTC TGA CTA GGT GTC CTT CT -**3'**

hGH Reverse: 5'- CGT CCT CCT GCT GGT ATA G -**3'**

Myogenin Forward: 5'- TTA CGT CCA TCG TGG ACA GC -**3'**

Myogenin Reverse: 5'- TGG GCT GGG TGT TAG CCT TA -**3'**

KO mice were generated using the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) gene-editing system by pronuclear and cytoplasmic injection of mouse embryos with Moxi guide RNA (gRNA) and Cas9 mRNA as previously described (Long et al., 2014). Briefly, a gRNA targeting the first exon of *Moxi* was designed using http://crispr.mit.edu/ and cloned into Addgene plasmid #42230 (Cong et al., 2013), a gift from Feng Zhang, using the following primers:

Moxi guide Forward: 5′- CAC CGG GCA AGC GAA TCG GCT GCG G **-3′**

Moxi guide Reverse: 5′- AAA CCC GCA GCC GAT TCG CTT GCC **-3′**

Cleavage efficiency was tested in cell culture using 10T1/2 mouse fibroblasts. The gRNA was transcribed in vitro and spin-column purified, while the Cas9 mRNA was obtained commercially (TriLink Biotechnologies). Mouse embryos were injected with an equal ratio (w/w) of gRNA and Cas9 mRNA into the pronucleus and cytoplasm and transferred to a surrogate dam for gestation. Mosaic C57BL/6N F_0 founders were identified by T7E1 assay on tail biopsies, and positive founders were bred to wild-type C57BL/6N mice to isolate knockout alleles. Mutants in the F1 generation were identified by T7E1 assay, and the alleles were TOPO TA cloned (Thermo Fisher Scientific) and sequenced. A founder with a 1-base pair deletion and 3-base pair insertion that disrupts the MOXI ORF was chosen for further analysis. Tail DNA was isolated as described above and mice were genotyped using the following primers:

MOXI KO genotyping Forward: 5′- AAC GCG GAG CCC ATC AGA AA -**3′**

MOXI KO genotyping Reverse: 5′- CCC TCA GCA AAC TGA GCT GT -**3′**

PCR products were run on a 1.5% agarose gel, gel purified, and submitted for sequencing using the MOXI KO genotyping Forward primer.

The guide RNA that we selected to use to generate our MOXI KO mice did not have any predicted off-targets with fewer than 3 base pair mismatches. For thoroughness, we verified that there were no off-target editing events in our KO mice by sequencing the top 25 predicted genome-wide off-target sites. Notably, of these top 25 sites, only 6 are located in exons of coding genes and all of these have greater than 4 base pair mismatches to the guide indicating unlikely targeting. The primer sequences used to amplify and sequence these regions are as follows:

Locus	**Gene**	**Forward**	**Reverse**
chr7:-81596050		GAGGAGTCTGGTTTGGGTGA	CCCACAAACTCGGCATAGAT
chrX:-34348198		AAAAGGGAAGCAGAGGAAGC	CTGTGCTCTGACGTGGGTTA
chr7:-125280421	Smg1	ATCAACATCTCGGCCCTCTA	CATGAACCACCACTGTGGAA
chr6:-39424255		CACAGTTGGGTGACAGTTGG	CAAATGCCTCTTCTGCAACA
chr14:+56111847	Lrrc16b	TCTCTCTGGAGAGCGGAGTC	GGATGCTCAGCTCCTGGTAA
chr6:+87119752		CCAGCCACAGAAGCAATTTT	AACAACCTCCTGGCACAAAC
chr7:+145057591		CCCAGCTGTGTGACAGAGAA	TAAGGGTAGCAGGAGGCAGA
chr6:+7529542		ATTGTGGGAATGCAAGAAGG	CGTGGTGAAATGCTGTTTTG
chr5:-93026160		AAGTGGAAACGCAGCAGATT	ACCGGACATAGGCTGTTTTG
chr4:+128369270		GGAGCTGGTGTTATGGTGGT	TGCCAGCTGTCTTTCAGATG
chr9:+58244183		CGGTCGCTGAGATAAGGAAA	GGCATGAGGTGATTTGAGGT
chr12:-86217015	Ltbp2	TTCTCCGAGTCTGGACAGGT	CGCCATACAGCCCTTACCTA
chr1:+190967046		CCCCAGACAGCTAGCTCAAG	GGACTGCGTTATCCACAGGT
chr3:-127540076	Ap1ar	GTTCTGTCAAGGCCCAAAAG	GATGGGGAACTGCTGCTG
chr1:-120927914	Gli2	GGAAGGGGAAGAGGAGTGAG	CACCAAGCTCCACCATCTCT
chr14:+122020530	Dock9	CTCACTGTGCAACAGCACCT	CGGTTGTCATGTCTTGGATG
chr2:+164721570	Zfp335	TTTGTAAAAGCGGGAACCTG	GGCTCTGGTAGGATGGTGAA
chr11:-104624333		CCTTCCTCCAGCTTCTCTCC	TAAAGAGCAAGCACCCTCCA
chr18:-28221255		AAGCCTGAAAGGGTCAAACA	AGTAAATACCGAGGCCAAAGC
chr2:-166962614		TGCACACACAATCACAGGAA	TGGATCTCATTCACGACTGC
chr10:-74305478		CCAGTTGCTCTGCCAAATAA	GTTTGGGAGGTTTGGTCTGA
chr10:+31055340	Tpd52l1	ACCTTGAAGCACATGCCTTT	GCCCAACCCTACAGTTCAAA
chr7:+59316103		TTGCCCTGGGAAAATAATTAAA	CATTTCCTAAGCCCGGTATG
chr3:-55458365		TGATATGGTGGTAGGCAGCA	AAGTCACCTGTGGCCATCTT
chr12:+45076178		CTCCCTGAAACCTGCAAAAG	TGTTTGGAGGAATGGTCACA

There were no off-target CRISPR editing events identified in any of the regions analyzed.

Tissue immunoprecipitations (IPs) and proteomics. For the identification of MOXI-interacting proteins, quadriceps muscles were isolated from MCK-MOXI-FLAG TG mice and wild-type littermate controls. Tissues were homogenized with a Teflon Potter dounce in IP Buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol, pH 7.4) containing cOmplete, EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitors (Roche). Homogenates were spun at 2,000xg at 4^{o}C and supernatants were transferred to a clean tube. Samples were lightly sonicated on ice and then incubated with Anti-FLAG M2 magnetic beads (SIGMA) that were pre-blocked with 1% BSA. Samples were rotated overnight at 4^{o}C and then washed 10 times with IP Wash Buffer (25 mM Tris, 700 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol, pH 7.4). Bound proteins were eluted using 3XFLAG peptide (SIGMA) in IP Buffer at 4^{o}C as per manufacturer's instructions. Tissue homogenates, FLAG eluted proteins and the unbound protein fractions were run on a 4-20% Mini-PROTEAN TGX gel (Bio-Rad) and silver stained using a SilverQuest Silver Staining Kit (Invitrogen). Silver stained bands that were unique to the TG sample were cut out and submitted to the University of Texas Southwestern Medical Center Proteomics Core facility for protein identification using LC-MS/MS as previously described (Garg et al., 2014). Proteins that were identified by Mass Spec were validated by Western blotting using the following antibodies: FLAG M2-Peroxidase (HRP), 1:1,000 (SIGMA); HADHA, 1:1,000 (Abcam); HADHB, 1:500 (BETHYL Laboratories); Total OXPHOS rodent WB antibody cocktail, 1:1,000 (Abcam). Tissue IPs were repeated using gastrocnemius/plantaris muscles and hearts from WT and TG mice and results were validated by Western blot.

For the identification of endogenous MOXI protein by mass spec analysis, quadriceps muscles were isolated from adult wild-type mice and homogenates were made as described above. Samples were immunoprecipitated with MOXI primary antibody (New England Peptide) and the bound proteins were captured using magnetic Dynabeads (Invitrogen) and eluted by boiling in Laemmli Buffer (Bio-Rad). Eluted proteins were run on a 4-20% Mini-PROTEAN TGX gel (Bio-Rad) and coomassie stained using EZBlue Gel Staining Reagent (SIGMA). The region of the gel corresponding to the molecular weight of MOXI (below 10kDa) was isolated and submitted to the University of Texas Southwestern Medical Center Proteomics Core facility for protein identification using elastase in-gel digestion and LC-MS/MS analysis as previously described (Garg et al., 2014).

Immunoprecipitations in transfected cells. Co-IPs were performed as previously described (Anderson et al., 2015; Nelson et al., 2016). Briefly, HEK293 cells were transfected with expression plasmids encoding Myc-HADHA, HADHB-FLAG or empty FLAG vector control, and MOXI-HA or HA-DWORF using FuGENE 6 Transfection Reagent (Promega). Cells were washed with PBS and then scraped and collected in IP Buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol, pH 7.4) containing cOmplete, EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitors (Roche). The same protocol detailed above for tissue IPs was followed for lysate preparation, FLAG IP and FLAG elution in transfected cells. Western blots were performed using the following antibodies: FLAG M2-Peroxidase (HRP), 1:1,000 (SIGMA); HADHA, 1:1,000 (Abcam); Myc, 1:1,000 (Invitrogen); HA clone (5B1D10), 1:1,000 (Invitrogen) and GAPDH (Millipore), 1:10,000.

Transthoracic echocardiography. Cardiac function and heart dimensions were determined in 12-16 week old male mice by two-dimensional echocardiography using a Visual Sonics Vevo 2100 Ultrasound (Visual Sonics, Canada) on unanesthetized mice. Motion (M)-mode tracings were used to measure anterior and posterior wall thicknesses at end diastole and end systole. Left ventricular (LV) internal diameter (LVID) was measured as the largest anteroposterior diameter in either diastole (LVIDd) or systole (LVIDs). A single observer blinded to mouse genotypes performed echocardiography and data analysis. Fractional shortening (FS) was calculated according to the following formula: FS(%) = [(LVIDd-LVIDs)/LVIDd] X 100. Ejection fraction (EF%) was calculated by: EF(%) = EDV-ESV/EDV (ESV, end systolic volume; EDV, end diastolic volume).

Body composition measurements. Live 12-16 week old male mice were analyzed for total body fat, lean tissue and body water content using an EchoMRI quantitative magnetic resonance system (Echo Medical Systems).

Histology and immunofluorescence. For enzyme histochemistry, skeletal muscle tissues were isolated and embedded in a mixture of OCT (Fisher) and gum tragacanth (Sigma-Aldrich) and flash-frozen in a 2-methybutane reservoir submerged in liquid nitrogen, followed by cryostat sectioning at 10 μM. Sections were submitted to the University of Texas Southwestern Medical Center Molecular Pathology Core for enzyme histochemistry (succinate dehyrdeogenase, SDH). For immunofluorescent staining, frozen sections were air-dried, fixed with 1% PFA and permeabilized with PBST (0.3% Tween-20/PBS). Sections were blocked with 5% donkey serum (Sigma-Aldrich) in PBST and then incubated in primary antibody in blocking buffer at 4°C in a humidified chamber using the following antibodies: HADHA (Abcam), FLAG M2 (SIGMA), Desmin (Dako), Laminin (SIGMA). The following day, samples were washed and incubated with the appropriate Alexa Fluor (Invitrogen) fluorescent secondary antibodies, Wheat Germ Agglutinin (Invitrogen) and DAPI (SIGMA). Samples were washed again and coverslips were mounted using VECTASHIELD Antifade Mounting Media (VECTOR Laboratories). Confocal images were taken with a Zeiss LSM-800 using a 40X oil objective. For routine histology, samples were isolated and fixed in 4% (vol/vol) paraformaldehyde in PBS for 48 hours at room temperature with gentle shaking. Samples were dehydrated, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) using standard procedures.

Transmission electron microscopy. 12-week old male mice were perfusion fixed by transcardial perfusion using 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Tissues were collected and samples were processed by the University of Texas Southwestern Medical Center Electron Microscopy Core facility.

Briefly, fixed tissues were post-fixed, stained, dehydrated, and embedded in EMbed-812 resin. Tissue sections were cut and post-stained, and images were acquired on a FEI Tecnai G2 Spirit TEM.

DNA extraction from tissue for mtDNA quantification. Tissues from 12-16 week old male mice were homogenized in Trizol and then separated using chloroform. The aqueous phase containing RNA was removed and back extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate, 1M Tris) was added to the remaining interphase and organic phase. Samples were mixed, incubated at room temperature, and then centrifuged at 3,000xg at 4°C. The upper phase containing the DNA was transferred to a new tube and precipitated with isopropanol with polyacryl carrier. Samples were mixed and then centrifuged at 12,000xg at 4°C. Pellets were washed with 75% ethanol four times and then air dried and resuspended in 8 mM NaOH. After DNA was resuspended, 1 M Hepes was added to a final concentration of 10 mM and 100 mM EDTA was added to a final concentration of 1 mM. qPCR was performed using KAPA SYBR Fast qPCR Master Mix (SIGMA) as described above. The following primer sets were used for qPCR:

NADH dehydrogenase subunit 1 (MT-ND1) Forward: **5′-** CCC ATT CGC GTT ATT CTT **-3′**

NADH dehydrogenase subunit 1 Reverse: 5′- AAG TTG ATC GTA ACG GAA GC **-3′**

Lipoprotein Lipase (LPL) Forward: **5′-** GGA TGG ACG GTA AGA GTG ATT C **-3′**

Lipoprotein Lipase Reverse: 5′- ATC CAA GGG TAG CAG ACA GGT **-3′**

Treadmill exercise. 12-16 week old male mice were run on Exer-3/6 treadmill apparatus (Columbus Instruments) with mild electrical stimulus. Two days before the experiment, mice were acclimatized to a single lane treadmill by performing a 10 m/min run for 10 min. For the experimental test, the treadmill was set to ramp from 0 to 10 m/min over a period of 5 minutes and then stay at 10 m/min for an additional 5 minutes. The treadmill speed then incrementally increased (1m/min every 5 min) to a maximum speed of 20 m/min until exhaustion. Exhaustion was defined by failure to run for greater than 10 sec.

Mitochondrial isolations for functional analysis. Quadriceps muscle and hearts were isolated from 12-16 week old male mice and homogenized in ice cold isolation buffer (10 mM MOPS, 210 mM mannitol, 70 mM sucrose and 1mM EDTA, pH 7.4) with a Teflon Potter dounce. Homogenates were centrifuged at 800xg and the supernatants were collected and centrifuged again at 8,000xg. The pellet was resuspended in isolation buffer and then centrifuged a final time at 8,000xg. The pellet was resuspended in STE buffer (250 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) for fatty acid oxidation rate assays or isolation buffer for mitochondrial respiratory function analysis.

Long chain fatty acid oxidation assays. Oxidation of [1-^{14}C]palmitic acid by mitochondria isolated from quadriceps muscle or hearts of WT and MOXI KO mice was analyzed using a detailed published protocol (Huynh et al., 2014). Briefly, mitochondria were isolated as described above and resuspended in STE buffer (250 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Mitochondria were incubated in oxidation reaction buffer (100 mM sucrose, 10 mM Tris-HCl, 5 mM KH_2PO_4, 0.2 mM EDTA, 80 mM KCl, 1 mM $MgCl_2$, 2 mM L-carnitine, 0.1 mM malate, 0.05 mM coenzyme A, 2 mM ATP, 1 mM DTT and 0.7% BSA/0.1 mM palmitate/0.4 µCi ^{14}C-palmitate) for one hour and then transferred to tubes containing 1 M perchloric acid. Fully oxidized CO_2 was captured on Whatman filter paper discs treated with 1 M NaOH placed in the lids of the tubes. After incubation for one hour with gentle agitation, the Whatman filter paper discs were transferred to scintillation vials for counting and the reaction mixture was then centrifuged at 14,000xg. The supernatant containing the acid-soluble metabolites (ASMs) was transferred to a scintillation vial for counting and analysis. Where indicated, samples were pre-incubated with 40 µM etomoxir for 5 minutes on ice before addition of the oxidation reaction buffer.

Analysis of mitochondrial respiratory function. Isolated mitochondria were diluted to 0.25 mg/mL (heart) or 0.5 mg/mL (quadriceps) in respiratory buffer composed of 10 mM MOPS, 210 mM mannitol, 70 mM sucrose and 5 mM K_2HPO_4 at pH 7.4 containing the following respiratory substrates: 1 mM malate and palmitoylcarnitine (25 µM for heart, 50 µM for quad) or 100 µM pyruvate. For measurements with quadriceps mitochondria, 1.25 mM $MgCl_2$ was also included in the respiratory buffer. State 3 respiration was initiated after 2 minutes with the addition of ADP at a final concentration of 0.25 mM. Rates of mitochondrial respiration were evaluated at room temperature using a Neofox oxygen chamber with a 175 µL volume (Instech Laboratories) (Crewe et al., 2013).

^{13}C-NMR isotopomer analysis. Hearts were quickly excised after cervical dislocation of mice. Hearts were cannulated via aorta, connected to a perfusion column apparatus and maintained at 37 °C with a controlled temperature bath. Hearts were perfused for 30 min at 100 cm H_2O pressure with a modified Krebs-Henseleit (KH) buffer containing 8 mM [1,6-^{13}C]glucose, 1.2 mM [3-^{13}C]lactate, 0.12 mM [3-^{13}C]pyruvate, 0.4 mM [U-^{13}C]long chain fatty acids

(LCFA), 0.75% bovine serum albumin (BSA) and 2.5 mM $CaCl_2$ equilibrated with 95:5 O_2:CO_2. Cardiac function was monitored during the perfusion with a fluid-filled catheter in the left ventricle. Coronary flow samples were collected at 5 and 25 minutes to measure oxygen consumption using a blood gas analyzer (Instrumentation Laboratory, Lexington, MA). After 30 min of perfusion, hearts were snap-frozen in liquid nitrogen. The frozen tissues were pulverized in liquid nitrogen and extracted with perchloric acid (4%), neutralized, and reconstituted in D_2O containing 1 mM EDTA and 0.5 mM 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) standard. Proton-decoupled ^{13}C-NMR spectra of heart extracts were acquired at 600 MHz spectrometer (Bruker Corporation, USA) equipped with 5-mm cryoprobe. ^{13}C-NMR multiplets from glutamate were deconvoluted using ACD/SpecManager (ACD Labs, Canada) and multiplet ratios to determine the relative oxidation of [1,6-$^{13}C_2$]glucose, [U-^{13}C]LCFA, and unlabeled endogenous substrates (e.g., triglycerides and glycogen). Multiplet ratios were submitted as input in tcaCALC v.2.07 for isotopomer analysis (Baskin et al., 2014). Data presented as the mean ± SEM (n = 4 per group) and analyzed with Welch's t-test for statistical significance.

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