Supplemental Information
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Exercise and PGC1α-Independent Synchronization of Type I Muscle Metabolism and Vasculature by ERRγ

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Succinate Dehydrogenase (SDH) Staining. SDH staining was performed on 6μM cryo-sections of gastrocnemius. Briefly, WT and transgenic sections were incubated at 37°C for 10 min in substrate buffer [0.2M Phosphate buffer containing sodium succinate (250mg/10ml) and NBT (10mg/10ml)]. Following incubation, sections were washed three times with water following by two washes each with increasing and decreasing concentrations of acetone (30%, 60%, 90%). Finally, the sections were washed three times with water and mounted in an aqueous mounting media.

Immunohistochemistry. Gastrocnemius muscles isolated from WT and transgenic mice were equilibrated in 30% sucrose (in PBS) for 2-3 hr and frozen in OCT. Cryo-sections (10μM) were fixed (4% paraformaldehyde-PBS), permeabilized (0.3% Triton X-PBS) and blocked (normal goat serum-PBS) before antibody treatment. Further, the sections were incubated overnight at 4°C with anti-PECAM 1 antibody (1:25 in PBS, SEROTEC), washed three times with PBS, incubated with anti-rat secondary antibody (1:250, ALEXA FLOR 344), washed three times with PBS and mounted in VECTASHIELD. For negative controls,

primary antibody was replaced with normal goat serum-PBS for overnight incubation.

Alkaline phosphatase staining. For AP staining, 10μM muscle sections were fixed in ice-cold acetone (5 min, -20°C), incubated in Tris-buffered Naphthol AS-MX phosphate/ N, N Dimethylformamide solution (30 min, 37°C), rinsed with distilled water (3x2min) and mounted with aqueous media.

Cell culture, in vitro angiogenesis and Vegfa ELISA. C2C12 myoblasts were grown in 20% FBS-DMEM and differentiated in 2% horse serum-DMEM [with penicillin/streptomycin]. Conditioned media from two day differentiated WT and ERRγ over-expressing C2C12 myotubules were used in the in vitro angiogenesis assay. Murine endothelial SVEC4-10 cells were cultured and maintained in DMEM containing 10% fetal bovine serum and penicillin/streptomycin. On the day of the experiment 4 X 10^5 cells/500μl/well were plated in matrigel-coated 12-well plates. The cells were immediately treated for 7 hr with C2C12 cell conditioned media (250μl), followed by evaluation of tube formation. Vegfa concentration in the conditioned media was measured using commercial Elisa kit according manufacturer's instructions [Research & Diagnostics].

Treadmill endurance test. WT and transgenic mice were acclimated to treadmill running (8 meters/min for 15 min) every other day for 1 week before the test. For the endurance testing, the mice were run on a treadmill at 5° inclination as the speed was gradually increased to 14 meters/min. After reaching 14 m/min, mice were run to exhaustion at constant speed. Endurance was measured as the function of time and distance ran.

Figure S1. Oxidative Biomarker Expression in Wild-Type and Transgenic Mice

Protein expression levels of myoglobin and cytochrome c (cycs) in wild-type, TG 425 and TG 421 quadriceps (N=3).

Figure S2. ERRγ in Cultured Muscle Cells

(A) ERRγ knockdown in primary myoblast. Primary myoblast were prepared from soleus and red gastrocnemius and infected with either control or ERRγ siRNA. Expression of ERRγ and oxidative biomarkers (cycs, ucp3, Acscl1, Cox6a2, Ppara) was measured in control (open bars) and ERRγ (closed bars) knockdown primary muscle cells. Data is presented as mean±SD. (*) Indicates statistically significant difference between control and ERRγ knockdown cells ($p<0.05$, unpaired Student's t-test). (B-C) Mitochondrial bioenergetics in wild-type and ERRγ over-expressing C2C12 cells. (B) Basal oxygen consumption rate (OCR) representing mitochondrial respiration. (C) Basal extracellular acidification rate (ECAR) representing glycolysis. Data are presented as mean±SD. (*) Indicates statistically significant difference between the two groups. ($p<0.05$, unpaired Student's t-test).

Figure S3. ERRγ Activates Vegfa Promoter

Vegfa gene 5' of the transcriptional start site containing promoter region was PCR cloned from mouse genomic DNA and sub-cloned up-stream of luciferase gene in pGL3 vector. All three isoforms of ERR (ERRα, ERRβ and ERRγ) transcriptionally activated Vegfa promoter. Data presented as mean±SD.

Figure S4. Physiological Changes in Wild-Type and ERRγ Transgenic Mice

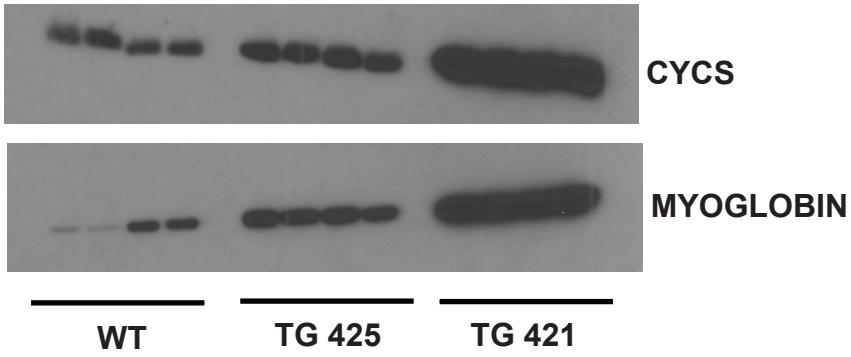
(A) The ambulatory activity, measured using CLMAC units, is comparable between the wild-type and the transgenic mice. Data are presented as mean±SEM. (B) Average weight gain in wild-type and transgenic mice on high fat diet (N=6). Data are presented as mean±SD. (*) Indicates statistically significant difference between the two groups. ($p<0.05$, unpaired Student's t-test).

Figure S5.

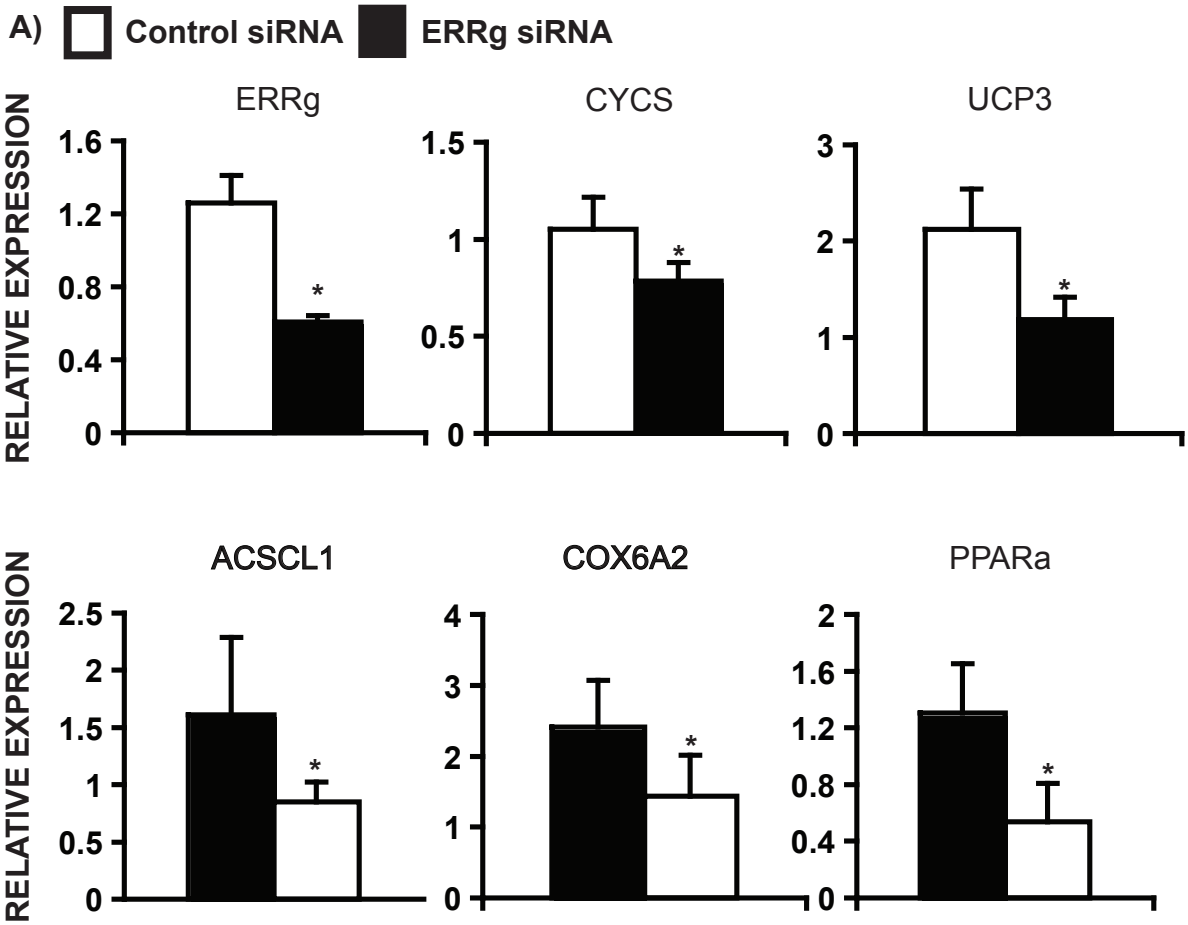
(A) PGC1α acetylation in the skeletal muscle. Nuclear extracts were prepared from freshly isolated quadriceps from wild-type and ERRGO mice using a commercially available kit according to the manufacturer's instructions (Thermo Scientific* NE-PER* Nuclear and Cytoplasmic Extraction Kit, Cat no. PI-78833). PGC1α was immunoprecipitated using anti-PGC1α antibody (Santacruz, Cat no. sc-13067) from the nuclear extracts and acetylation levels detected using anti-acetyl lysine antibody (Cell Signaling, Cat no. 9441S). *Upper panel.* Representative blots of acetylated and total PGC1α immunoprecipitated from wild-type and ERRGO nuclear extracts. *Lower panel.* Densitometric analysis (using Image J) presented as the ratio of acetylated to total PGC1α in the wild-type and ERRGO muscles. There is no statistically significant difference between the two groups. (B) Phospho-ACC levels in wild-type and ERRγ transgenic muscle. ACC phosphorylation was measured in murine quadriceps using an antibody that specifically detects phospho-ACC (Cell Signaling, Cat no. 3661). The blot represents phospho-ACC levels in wild-type and ERRγ transgenic quadriceps from N=3 samples in each group. (C) ATP levels in wild-

type and ERRγ over-expressing C2C12 cells. Absolute ATP level in C2C12 cells was measured using the ATP Bioluminescent Assay Kit according to manufacturer's instruction (Sigma, Cat No. FLAA-1KT). Briefly, $5x10^4$ cells were lysed with 100µl ATP releasing reagent for 10 minutes and combined with 100µl water. The standards (20, 10, 2, 1, 0.2, and 0.1 µM) were made by mixing 100µl ATP releasing reagent with 100µl ATP solutions. Next, 100µl ATP assay solution was added to a 96-well black plate with solid bottom and mixed with 100µl samples or standards. Luminescence was measured using the EnVision plate reader (PerkinElmer) and absolute ATP levels were calculated. ATP levels in wild-type and ERRγ over-expressing C2C12 cells are presented as mean±SD (nmol per 2 x 10^4 cells). * Represents statistically significant difference between groups ($p<0.05$, unpaired Student's t-test).

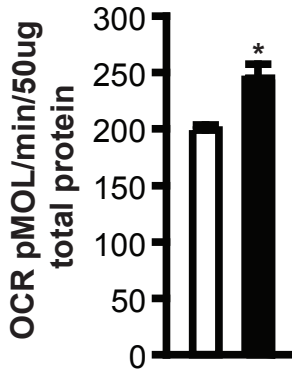
(S1)



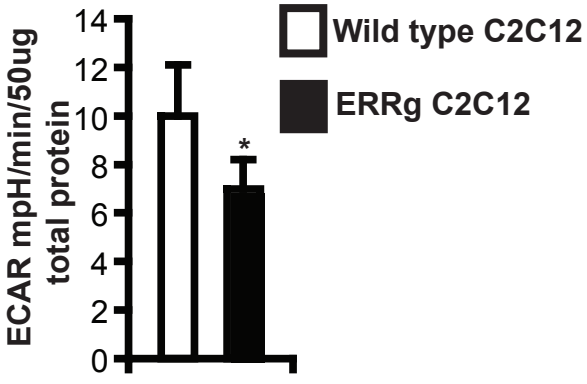
(S2 A)



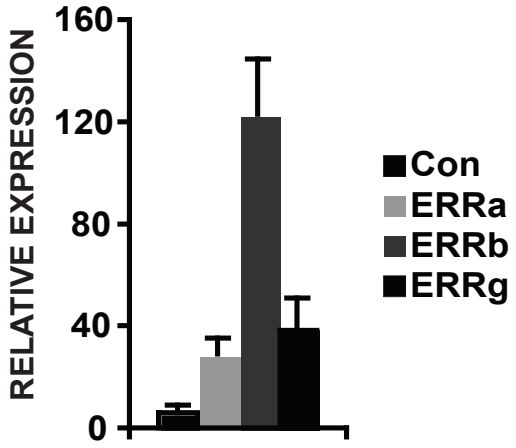
(S2 B)



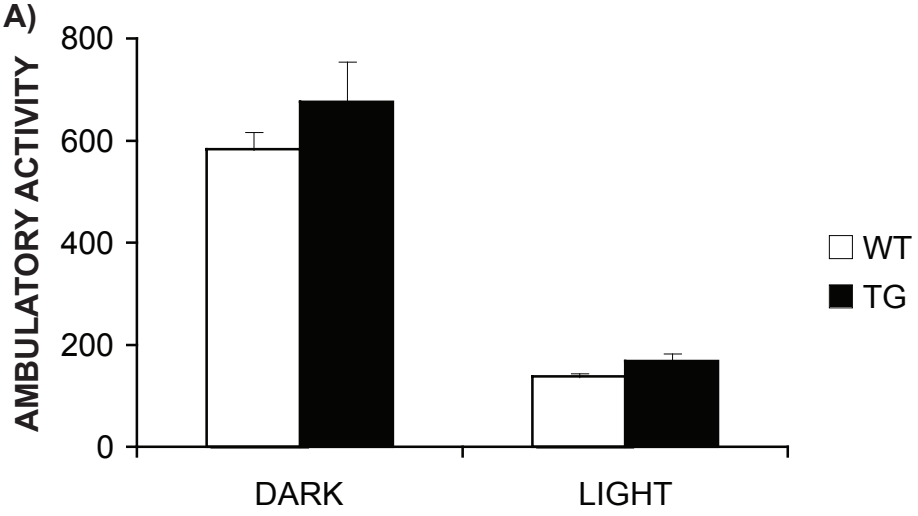
(S2 C)



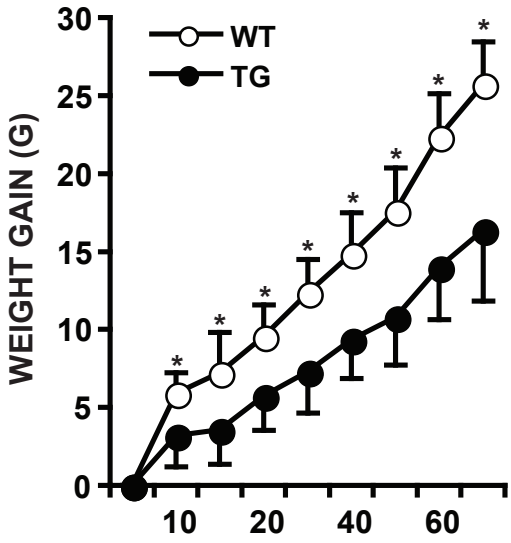
(S3)


RELATIVE EXPRESSION
160
120
80
40
0
Con
ERRa
ERRb
ERRg

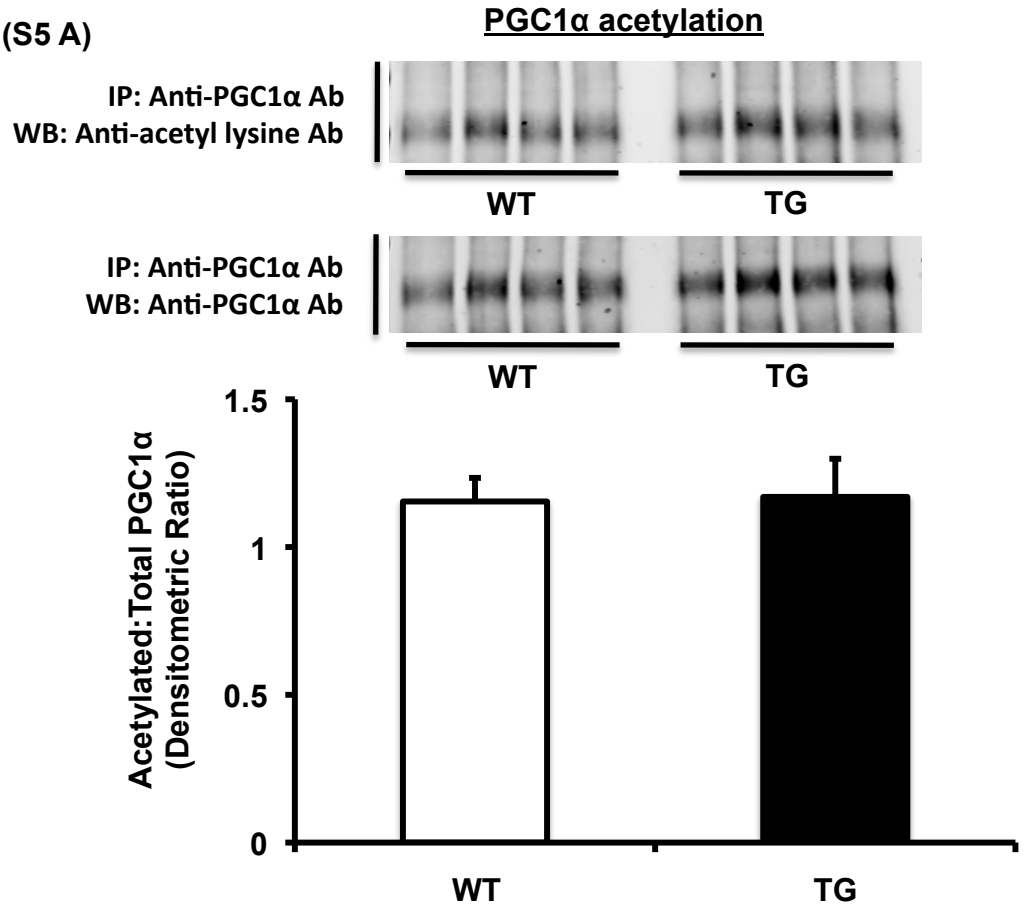
(S4 A)


AMBULATORY ACTIVITY
800
600
400
200
0
DARK
LIGHT
WT
TG

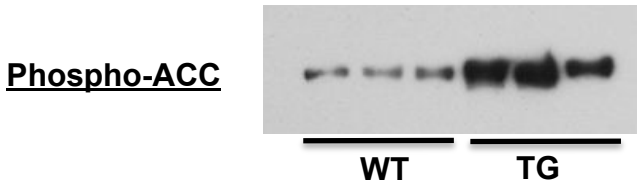
(S4 B)


WEIGHT GAIN (G)
30
25
20
15
10
5
0
10
20
40
60
WT
TG
*

(S5 A)


PGC1α acetylation
IP: Anti-PGC1α Ab
WB: Anti-acetyl lysine Ab
WT
TG
IP: Anti-PGC1α Ab
WB: Anti-PGC1α Ab
WT
TG
Acetylated:Total PGC1α
(Densitometric Ratio)
1.5
1
0.5
0
WT
TG

(S5 B)


Phospho-ACC
WT
TG

(S5 C)

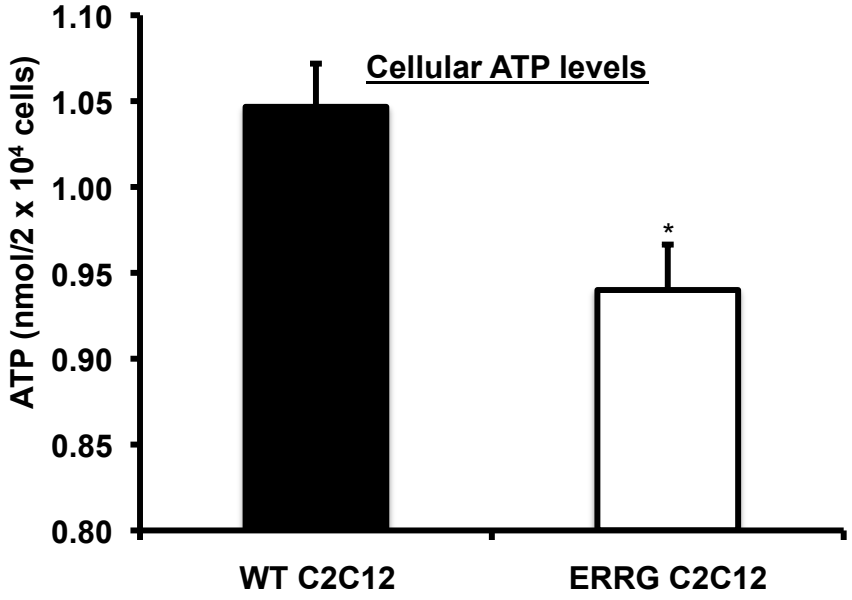

Cellular ATP levels
ATP (nmol/2 x 10⁴ cells)
1.10
1.05
1.00
0.95
0.90
0.85
0.80
*
WT C2C12
ERRG C2C12

Table S1. Global gene expression was compared between wild type and ERRγ transgenic quadriceps. The positively regulated genes were subjected to gene ontology classification. The genes linked to mitochondrial respiration and/or fatty acid oxidation are described below (N=3, each pooled from 3 mice, $p<0.05$, Bonferroni's multiple comparison test).

Locus	Fold	Description
1300010F03Rik	2.135	RIKEN cDNA 1300010F03 gene
1700020C11Rik	2.604	RIKEN cDNA 1700020C11 gene
1700034H14Rik	1.742	RIKEN cDNA 1700034H14 gene
Acaa1a	2.169	acetyl-Coenzyme A acyltransferase 1A
Acaa2	3.473	acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)
Acadl	2.093	acyl-Coenzyme A dehydrogenase, long-chain
Acadm	1.931	acyl-Coenzyme A dehydrogenase, medium chain
Acads	1.713	acyl-Coenzyme A dehydrogenase, short chain
Acadvl	2.2	acyl-Coenzyme A dehydrogenase, very long chain
Acat1	1.943	acetyl-Coenzyme A acetyltransferase 1
Acot1	2.031	acyl-CoA thioesterase 1
Acot11	2.356	acyl-CoA thioesterase 11
Acot2	3.018	acyl-CoA thioesterase 2
Acot7	1.952	acyl-CoA thioesterase 7
Acsl1	2.564	acyl-CoA synthetase long-chain family member 1
Adh1	1.843	alcohol dehydrogenase 1 (class I)
Ak3l1	7.733	adenylate kinase 3 alpha-like 1
Akap1	2.569	A kinase (PRKA) anchor protein 1
Aldh2	2.484	aldehyde dehydrogenase 2, mitochondrial
Atad3a	1.78	ATPase family, AAA domain containing 3A
Atp5h	1.685	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d
Bcat2	1.793	branched chain aminotransferase 2, mitochondrial
Bdh1	2.899	3-hydroxybutyrate dehydrogenase, type 1
Cabc1	5.199	chaperone, ABC1 activity of bc1 complex like (S. pombe)
Cat	1.919	catalase
Cds2	1.854	CDP-diacylglycerol synthase (phosphatidate cytidylyltransferase) 2
Chkb	2.021	choline kinase beta

Cox15	1.727	COX15 homolog, cytochrome c oxidase assembly protein (yeast)
Cox6a2	1.643	cytochrome c oxidase, subunit VI a, polypeptide 2
Cpt1b	1.698	carnitine palmitoyltransferase 1b, muscle
Cpt2	1.815	carnitine palmitoyltransferase 2
Ctsb	2.539	cathepsin B
D10Jhu81e	1.709	DNA segment, Chr 10, Johns Hopkins University 81 expressed
Dci	2.037	dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenyme A isomerase)
Decr1	2.745	2,4-dienoyl CoA reductase 1, mitochondrial
Dhrs4	2.103	dehydrogenase/reductase (SDR family) member 4
Dlat	2.027	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)
Ech1	2.297	enoyl coenzyme A hydratase 1, peroxisomal
Etfb	1.695	electron transferring flavoprotein, beta polypeptide
Etfdh	1.962	electron transferring flavoprotein, dehydrogenase
Fabp3	3.658	fatty acid binding protein 3, muscle and heart
Fdft1	2.287	farnesyl diphosphate farnesyl transferase 1
Gcdh	2.246	glutaryl-Coenzyme A dehydrogenase
Gfm1	2.099	G elongation factor, mitochondrial 1
Ggtla1	1.767	gamma-glutamyltransferase-like activity 1
Glrx5	2.036	glutaredoxin 5 homolog (S. cerevisiae)
Glud1	1.812	glutamate dehydrogenase 1
Got2	2.262	glutamate oxaloacetate transaminase 2, mitochondrial
Hadh	1.969	hydroxyacyl-Coenzyme A dehydrogenase
Hadha	2.415	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit
Hadhb	1.775	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit
Hba-a1	4.779	hemoglobin alpha, adult chain 1
Herc2	1.786	hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like domain (RLD) 2
Hibadh	1.685	3-hydroxyisobutyrate dehydrogenase
Hsdl2	2.283	hydroxysteroid dehydrogenase like 2
Hspa9	1.673	heat shock protein 9
Idh3b	1.749	isocitrate dehydrogenase 3 (NAD+) beta
Ivd	1.843	isovaleryl coenzyme A dehydrogenase
Ldhd	2.18	lactate dehydrogenase D

Lpl	1.873	lipoprotein lipase
Me3	1.657	malic enzyme 3, NADP(+)-dependent, mitochondrial
Mfn1	1.855	mitofusin 1
Mlycd	1.761	malonyl-CoA decarboxylase
Mrm1	1.868	mitochondrial rRNA methyltransferase 1 homolog (S. cerevisiae)
Mrpl14	2.465	mitochondrial ribosomal protein L14
Mrpl19	1.875	mitochondrial ribosomal protein L19
Mrpl3	1.744	mitochondrial ribosomal protein L3
Mrpl9	1.734	mitochondrial ribosomal protein L9
Msrb2	2.412	methionine sulfoxide reductase B2
Mterfd3	2.284	MTERF domain containing 3
Mtx2	1.738	metaxin 2
Mut	1.666	methylmalonyl-Coenzyme A mutase
Ndufab1	1.645	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1
Ndufb2	1.734	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2
Ndufs8	1.895	NADH dehydrogenase (ubiquinone) Fe-S protein 8
Ndufv1	1.648	NADH dehydrogenase (ubiquinone) flavoprotein 1
Nnt	4.809	nicotinamide nucleotide transhydrogenase
Nrip1	1.821	nuclear receptor interacting protein 1
Nudt8	2.662	nudix (nucleoside diphosphate linked moiety X)-type motif 8
Osbpl1a	2.225	oxysterol binding protein-like 1A
Pdk4	2.692	pyruvate dehydrogenase kinase, isoenzyme 4
Phca	2.679	phytoceramidase, alkaline
Pisd	1.679	phosphatidylserine decarboxylase
Pitpnc1	2.322	phosphatidylinositol transfer protein, cytoplasmic 1
Pla2g4b	4.406	phospholipase A2, group IVB (cytosolic)
Plcb4	2.431	phospholipase C, beta 4
Plcd1	2.009	phospholipase C, delta 1
Ppara	2.444	peroxisome proliferator activated receptor alpha
Ppif	1.989	peptidylprolyl isomerase F (cyclophilin F)
Ppm1k	1.759	protein phosphatase 1K (PP2C domain containing)
Prdx5	1.655	peroxiredoxin 5
Prdx6	1.643	peroxiredoxin 6
Qk	1.875	quaking
Rtn4ip1	2.032	reticulon 4 interacting protein 1
Sdhb	1.699	succinate dehydrogenase complex, subunit B, iron sulfur (Ip)
Sfxn5	1.992	sideroflexin 5

Slc25a20	2.989	solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20
Slc25a22	2.49	solute carrier family 25 (mitochondrial carrier, glutamate), member 22
Slc25a4	3.435	solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 4
Slc27a1	1.769	solute carrier family 27 (fatty acid transporter), member 1
Slc40a1	3.279	solute carrier family 40 (iron-regulated transporter), member 1
Sod2	1.766	superoxide dismutase 2, mitochondrial
Sorl1	2.367	sortilin-related receptor, LDLR class A repeats-containing
Tfam	1.754	transcription factor A, mitochondrial
Timm44	1.683	translocase of inner mitochondrial membrane 44
Tomm22	2.072	translocase of outer mitochondrial membrane 22 homolog (yeast)
Txn2	2.021	thioredoxin 2
Ucp3	2.838	uncoupling protein 3 (mitochondrial, proton carrier)
Ung	3.577	uracil DNA glycosylase
Uqcrq	2.108	ubiquinol-cytochrome c reductase, complex III subunit VII

Table S2. List of contractile genes induced by ERRγ in quadriceps of the transgenic mice (N=3, each pooled from 3 mice, $p<0.05$, Bonferroni's multiple comparison test).

Locus	Fold	Description
Abra	2.915	actin-binding Rho activating protein
Actn2	4.281	actinin alpha 2
Ankrd2	9.885	ankyrin repeat domain 2 (stretch responsive muscle)
Csrp3	8.534	cysteine and glycine-rich protein 3
Kcnj8	1.824	potassium inwardly-rectifying channel, subfamily J, member 8
Myh2	5.84	myosin, heavy polypeptide 2, skeletal muscle, adult
Myoz2	3.67	myozenin 2
Nrap	1.66	nebulin-related anchoring protein
Spna2	1.804	spectrin alpha 2
Tnnc1	3.256	troponin C, cardiac/slow skeletal
Tnni1	4.827	troponin I, skeletal, slow 1
Tnnt1	15	troponin T1, skeletal, slow
Tpm3	3.49	tropomyosin 3, gamma

Table S3. Following list of angiogenic genes were up-regulated in the quadriceps of ERRγ transgenic mice as compared to wild type mice (N=3, each pooled from 3 mice, $p<0.05$, Bonferroni's multiple comparison test).

Locus	Fold	Description
Cdh5	1.755	cadherin 5
Crhr2	2.072	corticotropin releasing hormone receptor 2
Cxcl12	2.05	chemokine (C-X-C motif) ligand 12
Efnb2	2.16	ephrin B2
Egfl7	1.958	EGF-like domain 7
Epas1	1.867	endothelial PAS domain protein 1
Fgf1	4.123	fibroblast growth factor 1
Flt1	1.85	FMS-like tyrosine kinase 1
Gja1	1.704	gap junction membrane channel protein alpha 1
Kdr	1.718	kinase insert domain protein receptor
Notch4	2.254	Notch gene homolog 4 (Drosophila)
Nrp1	1.816	neuropilin 1
Pdgfrb	1.895	platelet derived growth factor receptor, beta polypeptide
Plcd1	2.009	phospholipase C, delta 1
Qk	1.875	quaking
Rhob	1.702	ras homolog gene family, member B
Sox17	1.98	SRY-box containing gene 17
Vegfa	2.505	vascular endothelial growth factor A
Vegfb	2.341	vascular endothelial growth factor B
Vezf1	1.958	vascular endothelial zinc finger 1

Table S4. Following list of transcriptional regulators are targets of ERRγ in the quadriceps of transgenic mice (N=3, each pooled from 3 mice, $p<0.05$, Bonferroni's multiple comparison test).

LOCUS	DESCRIPTION	FOLD
Esrrb	estrogen related receptor, beta	3
Ppara	peroxisome proliferator activated receptor alpha	2.444
Ppard	peroxisome proliferator activator receptor delta	2.065
Ppargc1b	peroxisome proliferative activated receptor, gamma, coactivator 1 beta	1.988