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Supplemental Information

Estrogen-Related Receptors Mediate the Adaptive Response of Brown Adipose Tissue to Adrenergic Stimulation

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SUPPLEMENTAL FIGURES

Figure S1

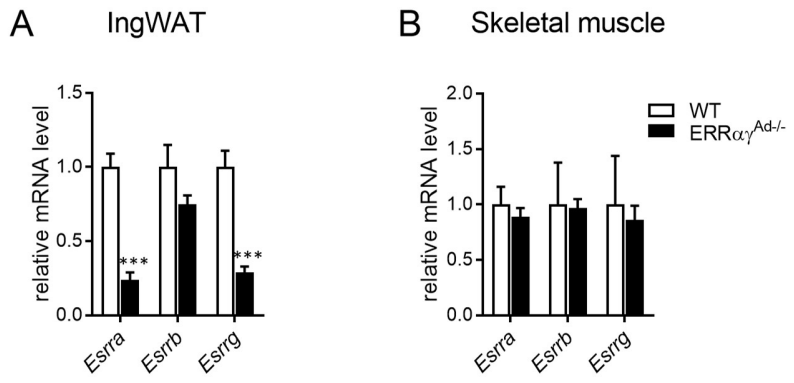

Figure S1. Related to Figure 1. ERR mRNA expression in inguinal WAT and skeletal muscle. (A) Relative *Esrra*, *Esrrb* and *Esrrg* mRNA levels in inguinal WAT (IngWAT) of WT and ERRαγ$^{Ad-/-}$ mice, normalized to *Ppia*. Data are mean ± SEM (n = 7-14). ***p < 0.001.
(B) Relative *Esrra*, *Esrrb* and *Esrrg* mRNA levels in skeletal muscle (plantaris) of WT and ERRαγ$^{Ad-/-}$ mice, normalized to *Ppia*. Data are mean ± SEM (n = 6).

Figure S2

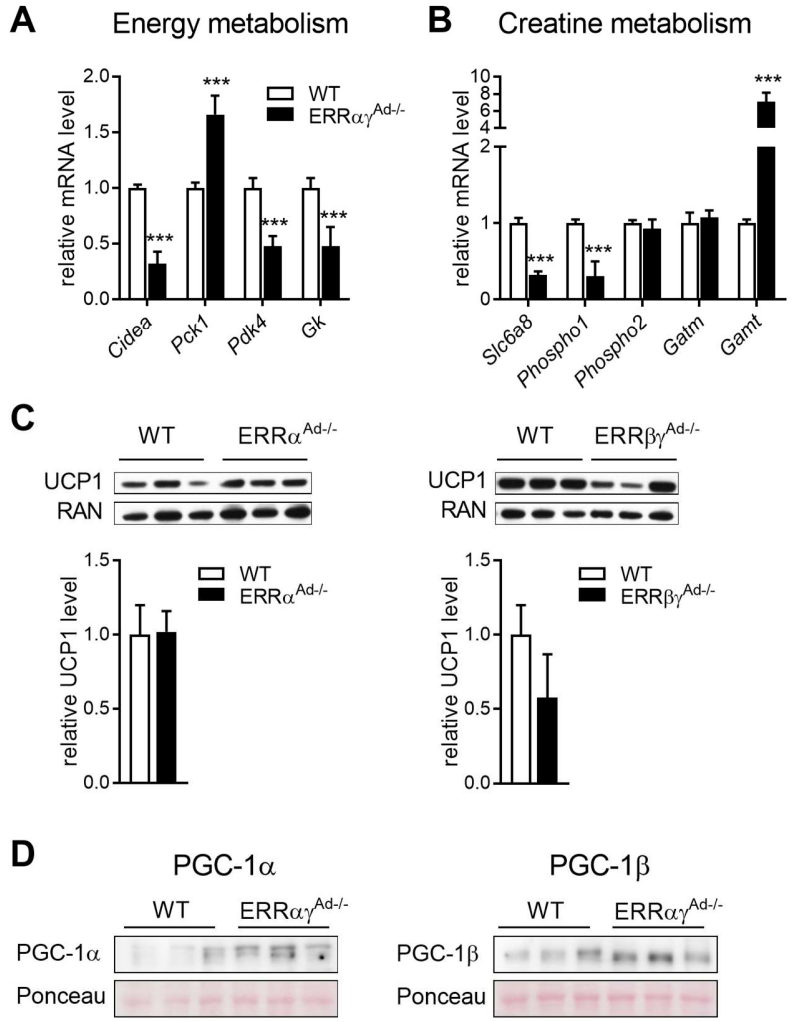

Figure S2. Related to Figure 2. Gene and protein expression levels in BAT of WT and ERR$^{Ad-/-}$ mice. (A,B) Relative mRNA levels of energy handling (A) and creatine metabolism (B) genes in BAT of WT and ERRαγ$^{Ad-/-}$ mice, normalized to *Ppia*. Data are mean ± SEM (n = 9-18). ***p < 0.001.
(C,D) Representative western blots of UCP1 and RAN (loading control) and their quantification, in BAT from ERRα$^{Ad-/-}$ (C) or ERRβγ$^{Ad-/-}$ (D), relative to WT of each group. Data are mean ± SD (n = 3).
(E) Representative western blots of PGC-1α and PGC-1β in BAT of WT and ERRαγ$^{Ad-/-}$ mice (n=3). Ponceau stain is shown as loading control.

Figure S3

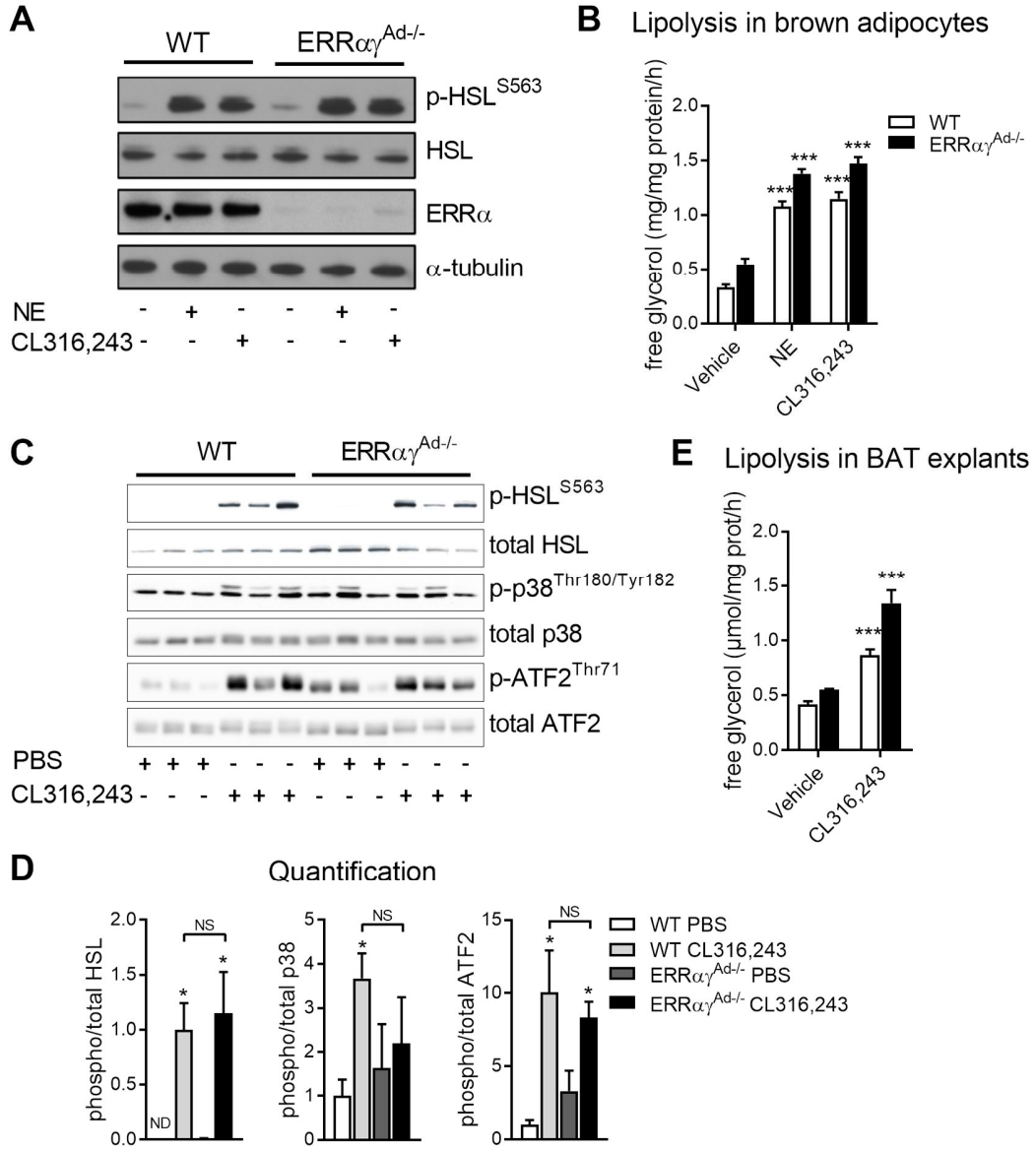

Figure S3. Related to Figure 3. Adrenergic signaling in brown adipocytes and BAT of WT and ERRαγ$^{Ad-/-}$ mice. (A) Representative western blots of phosphorylated hormone-sensitive lipase (HSL), total HSL, ERRα, and α-tubulin (loading control) in primary brown adipocytes of WT and ERRαγ$^{Ad-/-}$ mice, treated with vehicle, 100 nM norepinephrine (NE) or 50 nM CL316,243 for 10 min.
(B) Lipolysis in primary brown adipocytes of WT and ERRαγ$^{Ad-/-}$ mice, treated as in panel A for 2 hrs. Data are mean ± SEM (n = 4). ***$p < 0.001$ vs. vehicle in the same genotype.
(C,D) Western blots of phosphorylated and total HSL, p38 MAPK and ATF2 in BAT of WT and ERRαγ$^{Ad-/-}$ mice treated with PBS or CL316,243 for 45 min. Quantification data are mean ± SEM (n = 3). For p-p38, quantification is of the upper band. *$p < 0.05$ vs. vehicle in the same genotype. ND, not detected, NS, not significant.
(E) Lipolysis in BAT explants of WT and ERRαγ$^{Ad-/-}$ mice, treated ex vivo with vehicle or 1 µM CL316,243 for 2 hrs. Data are mean ± SEM (n = 3). ***$p < 0.001$ vs. vehicle in the same genotype.

Figure S4

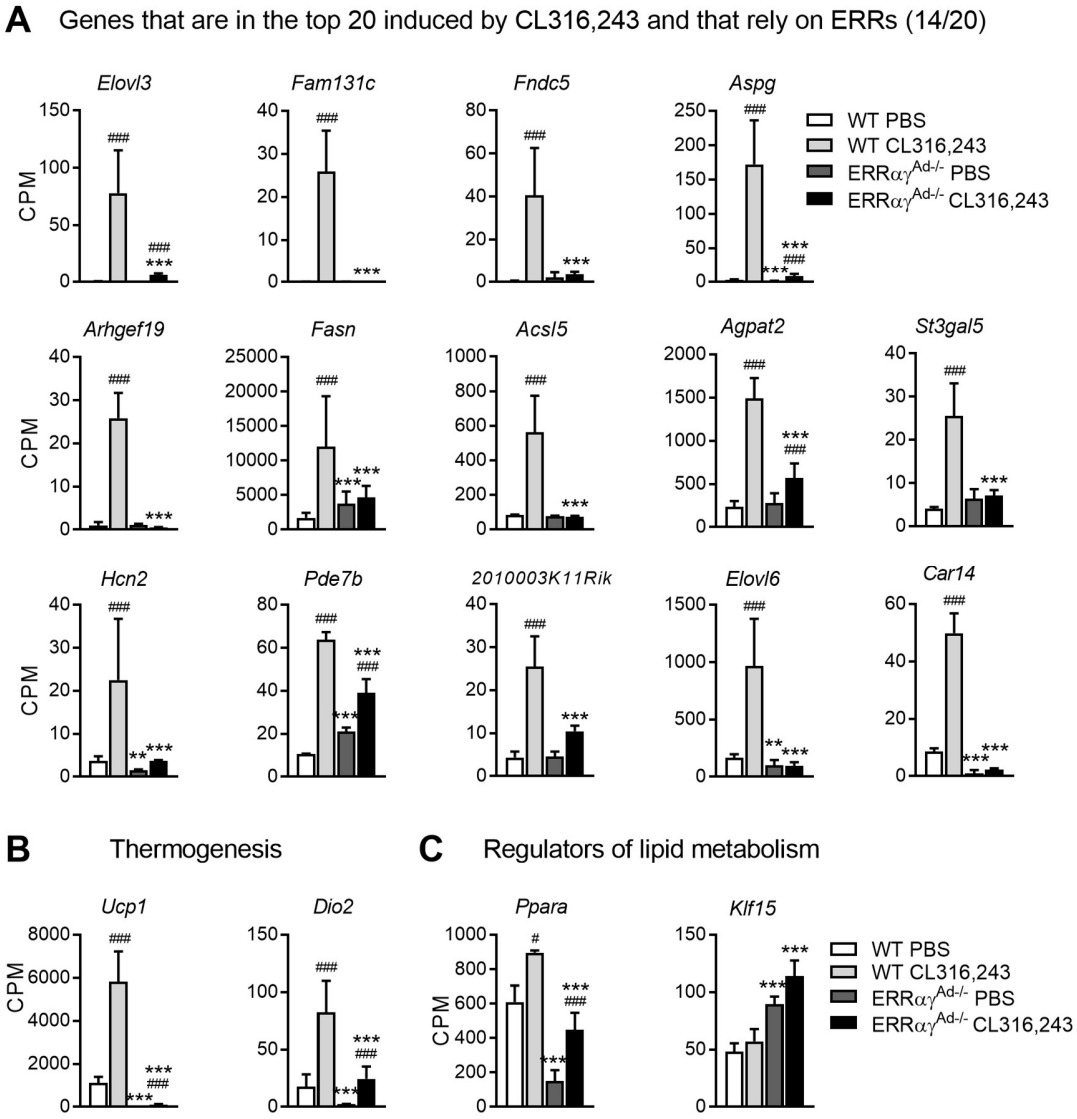

Figure S4. Related to Figure 4. Gene expression levels in BAT of WT and ERRαγ$^{Ad-/-}$ mice treated with CL316,243. (A) Expression levels of genes that are in the top 20 induced by the ten days of CL316,243 in WT BAT and dependent on ERRs for their induction. Fourteen of the top 20 CL316,243-induced genes relied on ERRs and are shown here. Several of these genes have been reported as highly induced also by cold in BAT (Marcher et al., 2015). Data are mean ± SD of CPM (counts per million reads), measured by RNA-sequencing (n = 3).
(B,C) Expression levels of thermogenesis genes *Ucp1*, *Dio2* (B), and regulators of lipid metabolism *Ppara* and *Klf15* (C) in BAT of WT and ERRαγ$^{Ad-/-}$ mice, treated with PBS or CL316,243 for 10 days. Data are mean ± SD of CPM (counts per million reads), measured by RNA-sequencing (n = 3).
(A-C) **p < 0.01, ***p < 0.001 vs. WT in the same treatment; $^{\#}$p < 0.05, $^{\#\#\#}$p < 0.001 vs. PBS in the same genotype.

Figure S5

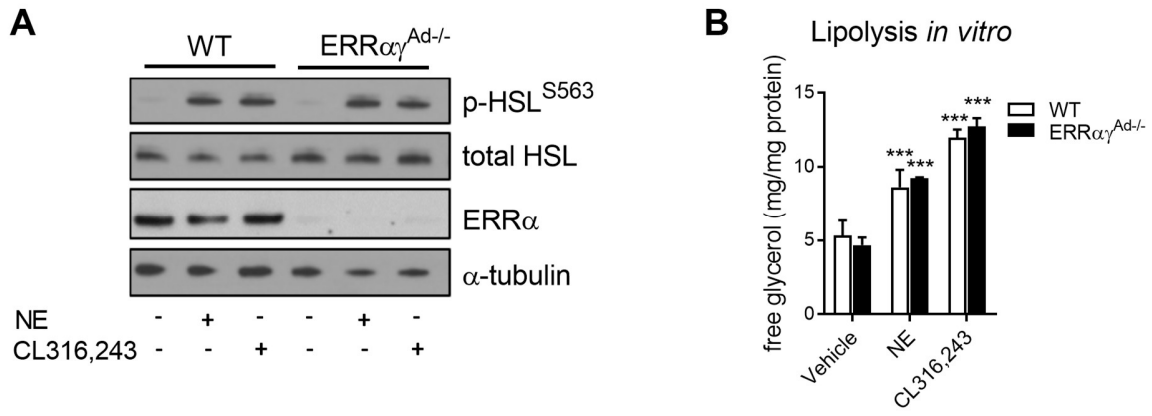

Figure S5. Related to Figure 5. Adrenergic signaling in inguinal white adipocytes from WT and ERRαγ$^{Ad-/-}$ mice. (A) Representative western blots of phosphorylated hormone-sensitive lipase (HSL)^{S563}, total HSL, ERRα, and α-tubulin (loading control) in primary inguinal adipocytes from WT and ERRαγ$^{Ad-/-}$ mice. Differentiated adipocytes were treated with either DMSO (vehicle), 100 nM norepinephrine (NE) or 50 nM CL316,243 for 10 minutes.
(B) Lipolysis (glycerol release in media) in primary inguinal adipocytes isolated from WT and ERRαγ$^{Ad-/-}$ mice. Differentiated adipocytes were treated with either DMSO (vehicle), 100 nM NE or 50 nM CL316,243 for 2 hrs. Data are mean ± SEM (n = 4). ***$p < 0.001$ vs. vehicle in the same genotype.

Figure S6

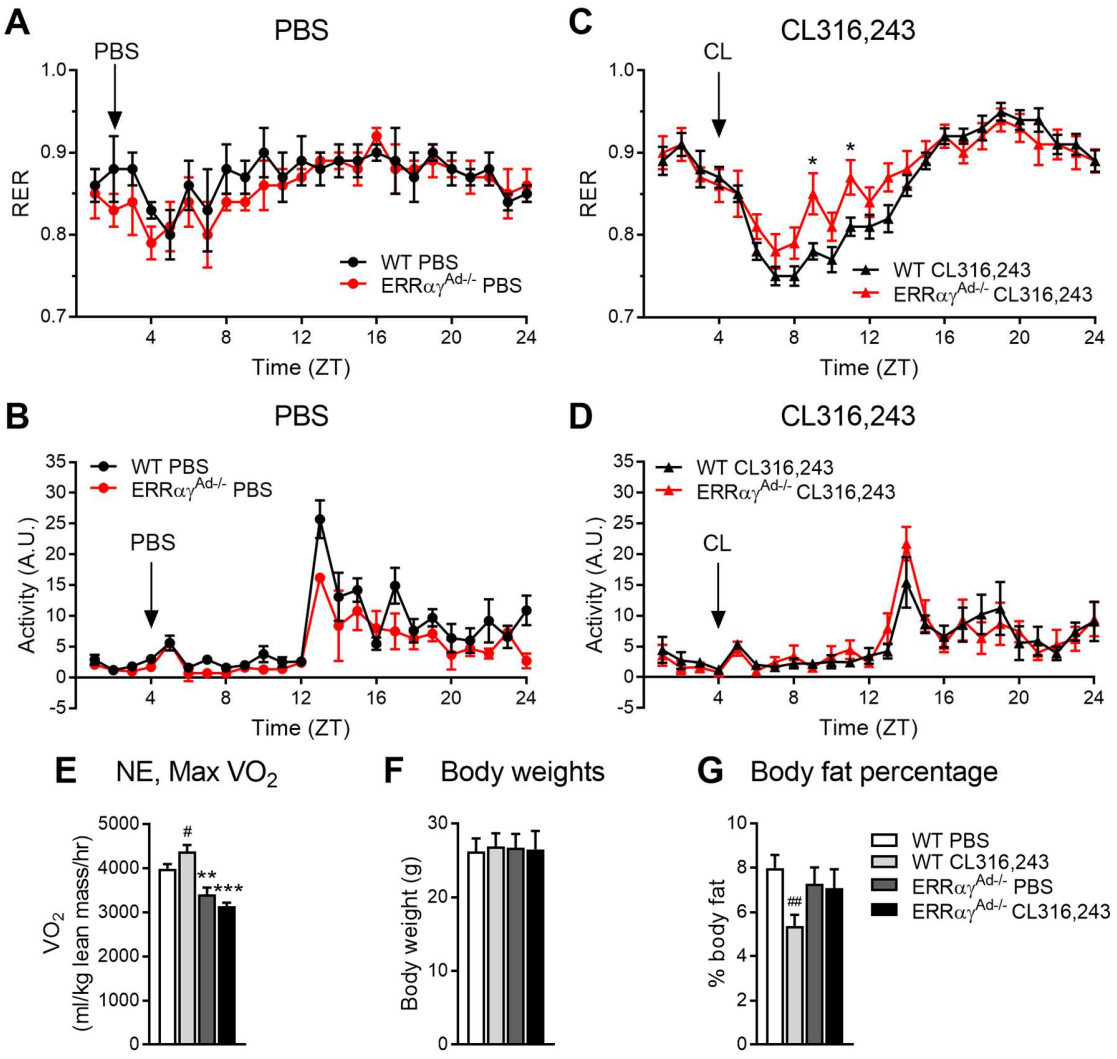

Figure S6. Related to Figure 6. Metabolic responses to adrenergic stimulation in WT and ERRαγ$^{Ad-/-}$ mice. (A,C) Average respiratory exchange ratio (RER) at 1 hour intervals of WT and ERRαγ$^{Ad-/-}$ mice injected with PBS (A) or CL316,243 (C) for ten days, on the last day of injections.
(B,D) Physical activity levels of WT and ERRαγ$^{Ad-/-}$ mice injected with PBS (B) or CL316,243 (D) for ten days, on the last day of injections.
(E) Maximum oxygen consumption (VO_2) reached following an acute norepinephrine (NE) injection in WT and ERRαγ$^{Ad-/-}$ mice, previously treated with PBS or CL316,243 for ten days. **$p < 0.01$, ***$p < 0.001$ vs. WT in the same treatment; $^{\#}p < 0.05$ vs. PBS in the same genotype.
(F) Body weights of WT and ERRαγ$^{Ad-/-}$ mice treated with PBS or CL316,243 for ten days.
(G) Body fat percentage of WT and ERRαγ$^{Ad-/-}$ mice treated with PBS or CL316,243 for ten days. $^{\#\#}p < 0.01$ vs. WT PBS. (A-G) Male mice were born and raised at thermoneutrality (30 °C), treated with PBS or CL316,243 for ten days, and euthanized at 12-14 weeks of age. NE responses were determined 2 days after the last PBS or CL316,243 injection. Data are presented as mean ± SEM (n = 5-10). ZT, Zeitgeber time. A.U., Arbitrary units.

Figure S7

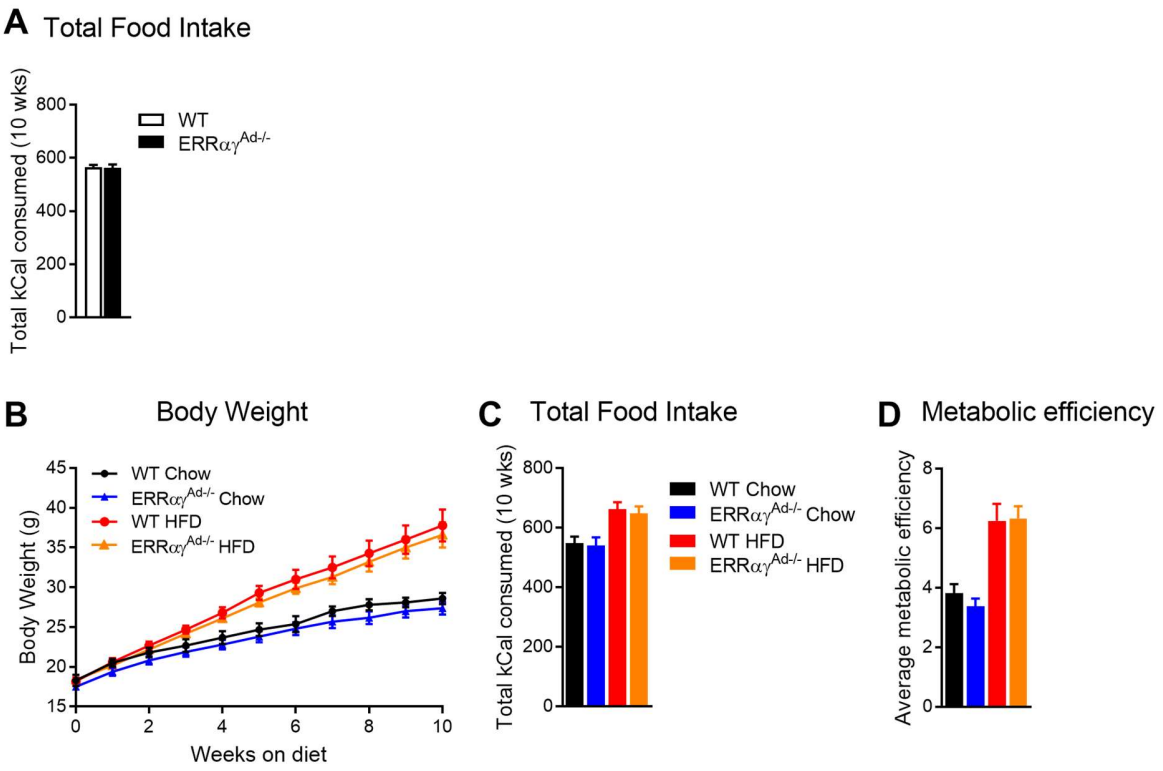

Figure S7. Related to Figure 7. Food intake and body weights of HFD-fed WT and ERRαγ$^{Ad-/-}$ mice.
(A) Total food intake (kCal) of female WT and ERRαγ$^{Ad-/-}$ mice shown in Figure 7 during 10 weeks on the 60% high fat diet (HFD). Data are the mean ± SEM consumption per mouse in group-housed mice (n = 8-25).
(B-D) Body weights (B), total food intake (C) and metabolic efficiency (D) of male WT and ERRαγ$^{Ad-/-}$ mice fed a control chow or 60% HFD for 10 weeks. Data are the mean ± SEM of singly-housed mice (n = 8-9).

SUPPLEMENTAL TABLES

Table S1. The top canonical pathways regulated (p < 0.05) by CL316,243 in ERRαγ$^{Ad-/-}$ BAT, and their ranking in WT BAT, using a 1.5-fold cut-off. Related to Figure 4.

	ERRαγ$^{Ad-/-}$ mice				WT mice			
Canonical pathway	**rank**	**down regulated**	**up regulated**	**-log(p-value)**	**rank**	**down regulated**	**up regulated**	**-log(p-value)**
Fatty Acid β-oxidation I	1	0/26 (0%)	21/26 (81%)	8.28E+00	4	0/26 (0%)	16/26 (62%)	6.80E+00
LPS/IL-1 Mediated Inhibition of RXR Function	2	12/114 (11%)	32/114 (28%)	7.03E+00	23	16/114 (14%)	32/114 (28%)	3.32E+00
Complement System	3	14/23 (61%)	2/23 (9%)	6.83E+00	13	13/23 (57%)	1/23 (4%)	4.11E+00
Antigen Presentation Pathway	4	17/24 (71%)	1/24 (4%)	5.58E+00	14	11/24 (46%)	1/24 (4%)	3.90E+00
Allograft Rejection Signaling	5	12/19 (63%)	0/19 (0%)	4.60E+00	9	11/19 (58%)	0/19 (0%)	4.23E+00
Interferon Signaling	6	11/27 (41%)	0/27 (0%)	4.16E+00	331	9/27 (33%)	1/27 (4%)	2.95E-01
Mitochondrial L-carnitine Shuttle Pathway	7	1/12 (8%)	6/12 (50%)	4.07E+00	103	1/12 (8%)	5/12 (42%)	1.17E+00
Valine Degradation I	8	0/17 (0%)	15/17 (88%)	4.01E+00	7	0/17 (0%)	13/17 (76%)	5.81E+00
Ubiquinol-10 Biosynthesis (Eukaryotic)	9	1/10 (10%)	6/10 (60%)	3.46E+00	39	0/10 (0%)	6/10 (60%)	2.20E+00
FXR/RXR Activation	10	11/49 (22%)	10/49 (20%)	3.25E+00	16	10/49 (20%)	15/49 (31%)	3.85E+00
γ-linolenate Biosynthesis II (Animals)	11	1/11 (9%)	4/11 (36%)	3.22E+00	89	2/11 (18%)	3/11 (27%)	1.30E+00
OX40 Signaling Pathway	12	15/37 (41%)	0/37 (0%)	3.02E+00	24	14/37 (38%)	0/37 (0%)	3.13E+00
Fatty Acid β-oxidation III (Unsaturated, Odd Number)	13	0/4 (0%)	4/4 (100%)	2.86E+00	48	0/4 (0%)	3/4 (75%)	2.05E+00
Fatty Acid Activation	14	0/8 (0%)	4/8 (50%)	2.84E+00	112	0/8 (0%)	3/8 (38%)	1.09E+00
LXR/RXR Activation	15	17/64 (27%)	8/64 (13%)	2.76E+00	10	13/64 (20%)	16/64 (25%)	4.20E+00

Table S2. The top canonical pathways regulated (p < 0.05) by ERRs in PBS or CL316,243-treated mice, using a 1.5-fold cut-off. Related to Figure 4.

	PBS				CL316,243			
Canonical pathway	**rank**	**down regulated**	**up regulated**	**-log(p-value)**	**rank**	**down regulated**	**up regulated**	**-log(p-value)**
Oxidative Phosphorylation	1	76/85 (89%)	2/85 (2%)	4.50E+01	1	78/85 (92%)	2/85 (2%)	4.20E+01
Mitochondrial Dysfunction	2	105/142 (74%)	16/142 (11%)	4.40E+01	2	107/142 (75%)	17/142 (12%)	3.74E+01
TCA Cycle II (Eukaryotic)	3	17/19 (89%)	2/19 (11%)	1.23E+01	3	17/19 (89%)	1/19 (5%)	1.09E+01
Glycolysis I	4	12/17 (71%)	2/17 (12%)	5.60E+00	4	12/17 (71%)	2/17 (12%)	5.71E+00
Gluconeogenesis I	5	10/17 (59%)	4/17 (24%)	5.60E+00	5	10/17 (59%)	3/17 (18%)	4.72E+00
FXR/RXR Activation	6	10/49 (20%)	20/49 (41%)	4.99E+00	19	11/49 (22%)	20/49 (41%)	2.78E+00
Isoleucine Degradation I	7	9/13 (69%)	1/13 (8%)	4.20E+00	11	8/13 (62%)	3/13 (23%)	3.54E+00
Ketolysis	8	5/5 (100%)	0/5 (0%)	3.75E+00	13	5/5 (100%)	0/5 (0%)	3.34E+00
Ketogenesis	9	6/7 (86%)	1/7 (14%)	3.73E+00	15	5/7 (71%)	1/7 (14%)	3.26E+00
Fatty Acid β-oxidation I	10	15/26 (58%)	4/26 (15%)	3.10E+00	9	16/26 (62%)	4/26 (15%)	3.54E+00
2-ketoglutarate Dehydrogenase Complex	11	3/4 (75%)	1/4 (25%)	3.00E+00	22	3/4 (75%)	1/4 (25%)	2.68E+00
LXR/RXR Activation	12	17/64 (27%)	26/64 (41%)	2.97E+00	30	19/64 (30%)	23/64 (36%)	2.26E+00
Valine Degradation I	13	9/17 (53%)	1/17 (6%)	2.97E+00	7	11/17 (65%)	5/17 (29%)	3.84E+00
Antigen Presentation Pathway	14	2/24 (8%)	15/24 (63%)	2.85E+00	196	1/24 (4%)	16/24 (67%)	6.16E-01
Glutaryl-CoA Degradation	15	9/15 (60%)	2/15 (13%)	2.71E+00	17	10/15 (67%)	2/15 (13%)	2.88E+00

Table S3. Primer sequences used for Q-PCR. Related to Figures 1, 2, 3 and 5.

Esrra	TGCTCAGCTCTCTACCCAAAC	GGACAGCTGTACTCGATGCTC
Esrrb	CCGGCCACCAATGAATGT	ATCCAGCCGTCGCTTGTACT
Esrrg	ATGCCCAAGAGACTGTGCTT	CTTCTTTCAGCATGCCCACT
Cyclophillin A / Ppia	CAAGACTGAATGGCTGGATG	ATGGGGTAGGGACGCTCTCC
Aco2	TCTCTAACAACCTGCTCATCGG	TCATCTCCAATCACCACCCACC
Idh3A	AGGACTGATTGGAGGTCTTGG	ATCACAGCACTAAGCAGGAGG
Sdhb	TACCGATGGGACCCAGACA	CGTGTGCACGCCAGAGTAT
Cycs	CACGGCTCTCCCTTTCTCAAG	ACAGTTGCCTCCTGGTGGTTA
Minos1	GACACGGTCGTGAAGCTAGG	AGATACGGAGCCTGAAAGTCA
Immt	AGCAAGAACAAGTTGAGATGGAG	GTGCCTTGACAGCCTGAACT
Chchd3	TGAAGAGGAGCGCATGAAG	GGCCAGCTGCTCTTTGTAGA
Chchd10	TGCCTTCAGTGGGGGAAAT	CAGGGCAGGGAGCTCAGAC
Apoo	TAATGCCCTTTTGGGGTTG	CCCACAGATCTCTGAATTACCC
Apool	TGCTACCCAGCTCAGTCAGTAA	TTGAAAAATTTGCTGGCTTGTA
Mftp1	TAATCCACCCCATCGACAG	TCCACTGACGGGTACAGCTT
Prdm16	ACAGGCAGGCTAAGAACCAG	CGTGGAGAGGAGTGTCTTCAG
Zic1	AACCTCAAGATCCACAAAAGGA	CCTCGAACTCGCACTTGAA
Lhx8	CCAAAAGAGCTCGGACCAG	GTTGTCCTGAGCGAACTGTG
Pparg	AGGCCGAGAAGGAGAAGCTGTTG	TGGCCACCTCTTTGCTCTGCTC
Fabp4	TGTGTGATGCCTTTGTGGGAACC	CTTCACCTTCCTGTCGTCTGCGG
Cox7a1	GACAATGACCTCCCAGTACAC	GCCCAGCCCAAGCAGTATAAG
Cs	AGAGGCATGAAGGGACTTGTGTA	TGTTCCTCTGTGGGCATCTGT
Cpt1b	CTCCGAAAAGCACCAAAACAT	AGGCTCCAGGGTTCAGAAAGT
Elovl6	CAGCAAAGCACCCGAACTA	AGGAGCACAGTGATGTGGTG
Ppara	AAGGCTATCCCAGGCTTTGC	TTTAGAAGGCCAGGCCGATCTC
Mcad	AAGCCACGAAGTATGCCCTG	CCATAGCCTCCGAAAATCTG
Acat1	TATCTCACGGCAGGAACAGG	TGCTCCATCGTTCAGTGTGC
Acaca	GACTCCAGGACAGCACAGATCAT	TGCCTGGAACCTCTTTGATTG
Fasn	ACACAGTCATCGGAGGTACGC	AGGATCTGACGAACTTCCACA
Scd1	TGCACTTGGGAGGCCTGTA	TGAGCCCCGGCTGTGAT
Plpn	TGCTGGATGGAGACC	ACCACCGGCTCCA
Ucp1	TGGAGGTGTGGCAGTGTTCAT	TGACAGTAAATGGCAGGGGAC
Dio2	CTGCGCTGTGTCTGGAAC	GGAGCATCTTCACCCAGTTT
Nrf1	CCACGTTGGATGAGTACACG	CTGAGCCTGGGTCATTTTGT
Gabpa	CCGCTACACCGACTACGATT	ACCTTCATCACCAACCCAAG
Ppargc1a	GGAGCCGTGACCACTGACA	TGGTTTGCTGCATGGTTCTG
Ppargc1b	AGTGGGTGCGGAGACACAGAT	AAAGCTCCACCGTCAGGGACT
Gadd45g	TTCGTGGATCGCACAATGACT	GGACTTTGGCGGACTCGTAGA
Tfam	CAAAGGATGATTCGGCTCAG	AAGCTGAATATATGCCTGCTTTTC
Polrmt	CTCCTCCCACATGATGCTGAC	AATTGCTCGCGGCATACCT
Tfb2m	TTTGGCAAGTGGCCTGTGA	CCCCGTGCTTTGACTTTTCTA
Sirt3	TTTCTTTCACAACCCCAAGC	ACAGACCGTGCATGTAGCTG
Endog	CCACCAATGCGGACTACC	AGGCATTCTGGTTGAGGTGT
Cidea	AAACCATGACCGAAGTAGCC	AGGCCAGTTGTGATGACTAAGAC
Pck1	ATCTTTGGTGGCCGTAGACCT	GCCAGTGGGCCAGGTATTT
Pdk4	GTTCCTTCACACCTTCACCAC	CCTCCTCGGTCAGAAATCTTG
Gk	TGAAGTCAATTGGTTGGGTTACA	ATGCAGCCAGTGGCTTATGAA
Slc6a8	GAGACTTGGACACGCCAGAT	AAAATGGGGATTCCTCCAAC
Phospho1	AAGCACATCATCCACAGTCCCTC	TTGGTCTCCAGCTGTCATCCAG
Phospho2	AGGTGAAGGACAGCCCTTTG	ATGCAGCAAAGGAACAAAGAC
Gatm	TCACGCTTCTTTGAGTACCG	TCAGTCGTCACGAACTTTCC
Gamt	TGGCACACTCACCAGTTCA	AAGGCATAGTAGCGGCAGTC
Mt-CoxII (mitochondrial DNA)	TCTCCCCTCTCTACGCATTCTA	ACGGATTGGAAGTTCTATTGGC
Rip140 (genomic DNA)	TCCCCGACACGAAAAAGAAAG	ACATCCATTCAAAAGCCCAGG

TRANSPARENT METHODS

Animals

Mice with floxed ERR alleles, kindly provided by Johan Auwerx, have been backcrossed to the C57BL/6JBom background (NNT WT background) (Taconic Biosciences) for at least 10 generations. The floxed ERR alleles have been described previously (Gan et al., 2013; LaBarge et al., 2014). To generate mice with adipose-specific deletions of ERRs, ERR floxed mice were crossed to ones expressing CRE recombinase driven by the adiponectin promoter (Eguchi et al., 2011). ERR floxed littermates that did not carry the CRE transgene were used as controls and are referred to as WT. Mice were born and housed at 30 °C, on a 12-hour light-dark cycle, and fed a standard chow breeder diet (5058, Picolab) or a 60 % high fat diet (D12492, Research Diets) where specified. All animal procedures were approved by the Institutional Animal Care and Use Committees of The Scripps Research Institute and the Johns Hopkins University Medical School. Both male and female mice were used, in separate and gender-matched experiments. No gender specific differences were observed. Levels of mRNA and mtDNA were quantitatively similar in male and female tissues, and data were pooled together. For other experiments, male or female data are shown, as specified in the figure legend. For body weight gain under HFD, data from both female and male mice are shown.

CL316,243 administration

At 10-11 weeks of age, mice were given an intraperitoneal (i.p.) injection of CL316,243 (1 mg/kg) or equivalent volume of PBS at ~ Zeitgeber time (ZT) 4 each day for 10 days. Mice were either euthanized 24 hours after the last CL316,243 dose for tissue collection or subjected to CLAMS, as described later. For mice on the HFD, CL316,243 (1 mg/kg) or PBS injections were administered after 18 weeks on a HFD, at 22 weeks of age, every other day for 4 weeks (total of 14 injections). For determination of the acute adrenergic signaling response in BAT, mice were injected with CL316,243 (1 mg/kg) or equivalent volume of PBS and euthanized 45 minutes later.

Cold exposure

Mice were implanted with IPTT-300 temperature transponders (Bio Medic Data Systems) at ~10 weeks of age, and cold exposure experiments were performed at 12-14 weeks of age. Mice were individually caged with minimal bedding and restricted access to food. Mice were either transferred to 4 °C or remained at 30 °C, and body temperature was monitored every 30 minutes for a total of 2.5 hours; at that time ERR$\alpha\gamma^{Ad-/-}$ mice became hypothermic and all cold-exposed mice were euthanized.

Metabolic, telemetry and body composition measurements

Whole body metabolism was determined in individually housed mice using the Comprehensive Lab Animal Monitoring System (Columbus Instruments). Mice were acclimated to the metabolic cages for at least 3 days before measurements. Oxygen consumption and carbon dioxide production were measured continuously for 48 hours, starting at ZT0 (7am) on the tenth day of CL316,243 injections. Body temperature and physical activity were measured via telemetry using TA-F10 transmitters (Data Sciences International), which were implanted into the abdominal cavity of 8 week old mice followed by at least 2 weeks of recovery. Receiver platforms were placed adjacent to each individual metabolic cage, and data were collected simultaneously with the metabolic data. For measurement of NE-induced thermogenesis,

oxygen consumption and carbon dioxide production were measured 2 days after the last CL316,243 (or PBS) injection, for 20 minutes prior and 60 minutes after an i.p. NE injection (1 mg/kg). Mice were euthanized the day after NE injection, and body composition was determined by MRI (EchoMRI LLC).

Glucose Tolerance Test

Mice were fasted for 12 hours and blood glucose concentrations were determined by collecting a small amount of blood from the tip of the tail (~ 5 µl). Mice were then given an i.p. injection of glucose (1 mg/kg), and blood glucose concentrations were determined at 20, 40, 60 and 90 minutes following the glucose injection.

Analysis of gene expression and mitochondrial DNA

Total RNA was isolated from BAT and ingWAT depots using TRIzol Reagent (Life Technologies). Following separation using chloroform, the upper aqueous phase containing RNA was mixed with equal volume of 70 % EtOH, and transferred to an RNeasy spin column (QIAGEN). RNA isolation was completed using the RNeasy mini kit according to manufacturer's protocol (QIAGEN). cDNA was synthesized using SuperScript II Reverse Transcriptase, and qPCR was performed using specific primers for each gene (Table S3) with HotStart-IT SYBR green qPCR master mix (Affymetrix). Relative mRNA expression was normalized to cyclophilin (*Ppia*) mRNA as a reference gene. Total DNA was extracted from the remaining interphase and lower phenol-chloroform phase, and mitochondrial DNA content was determined by measuring the mitochondrial gene CoxII (*Mt-Co2)*, normalized to genomic DNA Rip140 (*Nrip1*) via qPCR.

RNA sequencing

RNA libraries were prepared using 1 µg of total RNA and the TruSeq total RNA Sample Preparation Kit version 2.0 Kit (Illumina, San Diego, CA) following the manufacturer's instructions. Approximately 16 million 75bp single-reads were generated for each sample by the NextSeq Analyzer (Illumina) at The Scripps Research Institute (TSRI) DNA Sequencing Facility, and processed and analyzed by the core. More than 80% of the reads for each library were effectively mapped to the mouse genome reference mm10. Annotation was performed using Partek software (Partek Inc., St Louis, MO). Data were filtered to include only transcripts for which the average number of reads was >40 reads in one condition (WT or ERR$\alpha\gamma^{Ad-/-}$, PBS- or CL316,243-treated), to minimize noise. The R Bioconductor package EdgeR program was used to determine significantly changed transcripts (DE) between groups (Robinson et al., 2010). For heat maps, data was log transformed and clustered using Cluster 3 (Stanford University), and the visual contrast was set to 2. Minimum and maximum values for each heat map are in the figure legends. The differentially expressed genes were subjected to Ingenuity Pathway Analysis (Qiagen, Hilden, Germany) to decipher the major biological pathways, networks, and diseases emphasized by the significantly deregulated genes (with a p-value < 0.05).

Western Blot Analysis

Protein was extracted from BAT and ingWAT using RIPA buffer (150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM Tris pH 7.5) with protease and phosphatase inhibitors [1 µM phenylmethylsulfonyl fluoride, 4 µl/ml protease inhibitor cocktail P8340 (Sigma), 1 mM NaF, 1 mM Na_3VO_4]. Protein concentration was determined using the Pierce BCA protein assay kit

(Thermo Scientific). A total of 1-20 μg of protein were subjected to SDS-PAGE and transferred to nitrocellulose membrane. Blots were probed using the following antibodies: ERRα (ab76228, Abcam), ERRγ [polyclonal serum from rabbits immunized with peptides SNKDRHIDSSC and CSSTIVEDPQTK (ERRγ amino acids 25-35 and 104-115, respectively) and purified for binding to SNKDRHIDSSC], Ucp1 (ab10983, Abcam), OxPhos complex cocktail (45-8099, Life Technologies), RAN (610340, BD Biosciences), Phospho-HSL (ser563; 4139, Cell Signaling), HSL (4107, Cell Signaling), phospho-p38 MAPK (Thr180/Tyr182; 9211, Cell Signaling), p38 MAPK (9212, Cell Signaling), phospho-ATF2 (Thr71; 9221, Cell Signaling), ATF2 (20F1; 9226, Cell Signaling), PGC-1α [rabbit polyclonal, affinity purified (Olson et al., 2008)], PGC-1β [rabbit polyclonal, affinity purified (Lai et al., 2008)], and α-tubulin (GTX27291, GeneTex). Protein abundance was quantified using ImageJ and normalized to RAN protein or ponceau.

Morphological Analysis of BAT

For histological and electron microscopy analyses, BAT samples were processed as described previously (Villena et al., 2007). For histological analysis, interscapular BAT was dissected clean from white adipose tissue and muscle, washed in PBS, fixed overnight in 10% formalin, dehydrated, and embedded in paraffin for sectioning. Eight- to 10-um sections were stained with hematoxylin/eosin. For transmission electron microscopy, dissected and cleaned interscapular BAT was cut into small pieces (~1mm^3), and fixed overnight with 2.5% glutaraldehyde / 2% paraformaldehyde in 0.1M phosphate buffer (pH7.4). Samples were then washed in 0.1M sodium cacodylate buffer and postfixed for 5h with 1% OsO_4 in cacodylate buffer. Samples were dehydrated in a graded ethanol series and embedded in Epon/Araldite resin overnight. Ultrathin sections were obtained, contrasted with uranyl acetate/lead citrate, and examined with a Philips CM100 microscope.

Adrenergic signaling and Lipolysis in primary adipocyte cultures

Primary brown and white adipocytes were isolated from the interscapular and inguinal adipose depots, respectively, from WT and ERRαγ$^{Ad-/-}$ neonate mice. Briefly, adipose tissue depots were minced and digested by shaking for 40 min at 37 °C in isolation buffer containing NaCl (61.5 mM), KCl (2.5 mM), $CaCl_2$ (0.65 mM), glucose (2.5 mM), HEPES (50 mM), pen/strep (50 U/ml, 50 μg/ml), 2% w/v BSA, and 1.5 mg/ml collagenase type I (Worthington). Cells were filtered through a 70 μm cell strainer and plated in DMEM (Gibco, Invitrogen Life Technologies) with 25 mM glucose, 20 mM HEPES, 20% Fetal Bovine Serum (Gemini Bio-Products) and pen/strep (50 U/ml, 50 μg/ml). Differentiation was induced when cells reached confluency. Brown adipocytes were induced with DMEM, 10% FBS, 20 nM insulin, 1 nM triiodothyronine (T3), 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX), 2 μg/ml dexamethasone (Dex), and 0.25 mM indomethacin. Inguinal white adipocytes were induced with DMEM, 10% FBS, 200 nM insulin, 0.5 mM IBMX and 0.4 μg/ml Dex. After 2 days, media was replaced with DMEM, 10% FBS, 20 nM insulin and 1 nM T3 for brown adipocytes, and DMEM, 10% FBS and 200 nM insulin for white adipocytes. On day 4 of differentiation, cells were washed once and then incubated for 18 hours in starvation media (DMEM, 1% FFA-free BSA). Cells were then treated with either 100 nM norepinephrine, 50 nM CL316,243 or vehicle for 10 minutes for signaling studies or 2 hours to measure glycerol release. For signaling, protein was isolated in RIPA buffer, and western blotting was performed as described above. To measure glycerol release, Free Glycerol Reagent Kit (F6428, Sigma) was used following the manufacturer's instructions. Data were normalized to protein content in each well.

Lipolysis in BAT explants

BAT was isolated from WT and ERRαγ^{Ad}-/- mice and sliced into ~10 mg pieces. Each piece was placed in a separate well of a 96-well plate and incubated in DMEM containing 2% FFA-free BSA for 1 hour at 37 °C, 5% CO2 and 95% humidified atmosphere. Tissues pieces were then transferred to a new plate containing fresh DMEM (2% FFA-free BSA) with either vehicle or 1 μM CL316,243, and incubated for a further 2 hours. Glycerol content in the media was measured using the Free Glycerol Reagent Kit (Sigma) and normalized to the total protein content of each tissue piece.

Statistical Analyses

Statistical analyses were performed using a student's t-test when comparing just two datasets, with the level of significance set to $P < 0.05$. For more than two datasets, a two-way ANOVA (Graphad Software) was used, followed by a Tukey's multiple comparisons test, with the adjusted P-value set to $P < 0.05$. All data are presented as mean ± SEM unless otherwise specified.

Data availability

The RNA-sequencing gene expression data can be found at GEO: GSE104285.

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