Supporting Information

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SI Materials and Methods

siRNAs. The following siRNAs were purchased and mixed before transfection: EWS-1: 5′-GCA GUU ACU CUC AGC AGA AdTdT-3′ (Sigma); EWS-2: 5′-GAG ACU AGU CAA CCU CAA UdTdT-3′ (Sigma); EWS-3: 5′-CUG ACA ACA GUG CAA UUU AdTdT-3′ (Sigma); mFbxw7-1: 5′-CAC GUU ACA GGG ACA CAC UdTdT-3′ (Sigma); mFbxw7-2: 5′-GGA AUU GUA UUC ACA CGC UdTdT-3′ (Sigma); Negative control: Scrambled Silencer (Ambion).

Primers for Mitochondria DNA Quantification. The following primers were used for SYBR Green PCR: mtND1-F: 5′-ACC ATT TGC AGA CGC CAT AA-3′, mtND1-R: 5′-TGA AAT TGT TTG GGC TAC GG-3′, Cox2-F: 5′-GCC GAC TAA ATC AAG CAA CA-3′, Cox2-R: 5′-CAA TGG GCA TAA AGC TAT GG-3′, β-Actin-F: 5′-TTG CTG ACA GGA TGC AGA AG-3′, β-Actin-R: 5′-GAA AGG GTG TAA AAC GCA GC-3′, β-globin-F: 5′-GAA GCG ATT CTA GGG AGC AG-3′ and β-globin-R: 5′-GGA GCA GCG ATT CTG AGT AGA-3′.

Primers for Gene Expression Analysis. The following primers were used for SYBR Green qRT-PCR analysis: Cox7a-F: 5′-TGG CTT CTG GTA GAT GAG CTA AA-3′, Cox7a-R: 5′-TGG CTT CTG GTA GAT GAG CTA AA-3′, Cox8b-F: 5′-CCA GCC AAA ACT CCC ACT T-3′, Cox8b-R: 5′-GAA CCA TGA AGC CAA CGA C-3′, Cytochrome *c*-F: 5′-GCT ACC CAT GGT CTC ATC GT-3′, Cytochrome *c*-R: 5′-CAT CAT CAT TAG GGC CAT CC-3′, MHC1b-F: 5′-CCT GGA GAA ACC TGC CAA GTA TGA TGA CA-3′, MHC1b-R: 5′-CTG CTT CCA CCT AAA GGG CTG-3′, MHC2a-F: 5′-AAG CGA AGA GTA AGG CTG TC-3′, MHC2a-R: 5′-CTT GCA AAG GAA CTT GGG CTC-3′, MHC2x-F: 5′-GAA GAG TGA TTG ATC CAA GTG-3′, MHC2x-R: 5′-TAT CTC CCA AAG TTA TGA GTA CA-3′, MHC2b-F: 5′-GAA GAG CCG AGA GGT TCA CAC-3′, MHC2b-R: 5′-CAG GAC AGT GAC AAA GAA CGT C-3′, Mhy8-F: 5′-GGA GAG GAT TGA GGC CCA AAA-3′, Mhy8-R: 5′-CAC GGT CAC TTT CCC TCC ATC-3′, ATP5o-F: 5′-AGG CCC TTT GCC AAG CTT-3′, ATP5o-R: 5′-TTC TCC TTA GAT GCA GCA GAG TAC A-3′, COX5b-F: 5′-GCT GCA TCT GTG AAG AGG ACA AC-3′, COX5b-R: 5′-CAG CTT GTA ATG GGT TCC ACA GT-3′, ERRα-F: 5′-CGG TGT GGC ATC CTG TGA-3′, ERRα-R: 5′-CTC CCC TGG ATG GTC CTC TTL CAD-3′, LCAD-F: 5′-CTT GCT TGG CAT CAA CAT CGC AGA-3′, LCAD-R: 5′-ATT GGA GTA CGC TTG CTC TTC CCA-3′, SCAD-F: 5′-ACC AAA GCT TGG ATC ACC AAC TCC-3′, SCAD-R: 5′-AAC CAG GAA GGC ACT GAT ACC CTT-3′, VLCAD-F: 5′-GGC CAA GCT GGT GAA ACA CAA GAA-3′, VLCAD-R: 5′-ACA GAA CCA CCA CCA TGG CAT AGA-3′, G6P-F: 5′-ACA CCG ACT ACT ACA GCA ACA G-3′, G6P-R: 5′-CCT CG AAA GAT AGC AAG AGT AG-3′, PEPCK-F: 5′-CAG GAT CGA AAG CAA GAC AGT-3′, PEPCK-R: 5′-CAG GAT CGA AAG CAA GAC AGT-3′, PGC-1α-F: 5′-AGC CGT GAC CAC TGA CAA CGA G-3′, PGC-1α-R: 5′-GCT GCA TGG TTC TGA GTG CTA AG-3′, PGC-1β-F: 5′-CTT GCT TTT CCC AGA TGA GG -3′ and PGC-1β-R: 5′-CCC TGT CCG TGA GGA ACG -3′.

Fatty Acid β-Oxidation Analysis. $Ews^{+/+}$ and $Ews^{-/-}$ brown preadipocytes were cultured overnight with low glucose DMEM containing 10% FBS, 1% free fatty acid BSA, 25 mM Hepes, 1× nonessential amino acid, 0.1% ITS, 0.1 μM dexamethasone, 20 mM Hepes, 200 mM glutamine. Next day, cells were treated with or without 50 μM etomoxir (an inhibitor of carnitine palmitoyltransferase 1) in serum free low glucose medium for 3 h and fatty acid free BSA/^{14}C-oleic acid complex (0.25 mM ^{14}C-oleic acid) was added with 1mM carnitine. After 3 h, 100 μL of 70% perchloric acid was added to culture media and fatty acid oxidation of ^{14}C-oleic acid to CO_2 trapped in filter papers and acid soluble metabolites (ASM) in media were quantified by liquid scintillation counting. Three independent experiments were performed in six replicates per experiment. The dpm (disintegrations per minute) of radioactive CO_2 and ASM were corrected by blank reads, normalized to the radioactivity of total cell lysates and converted to % values. The % values obtained from $Ews^{+/+}$ cells were set to 100% and compared with the % values of radioactive CO_2 and ASM obtained from $Ews^{-/-}$ cells. Data are represented as mean ± SEM. Student's *t* test, $^{*}P < 0.05$; $^{**}P < 0.01$.

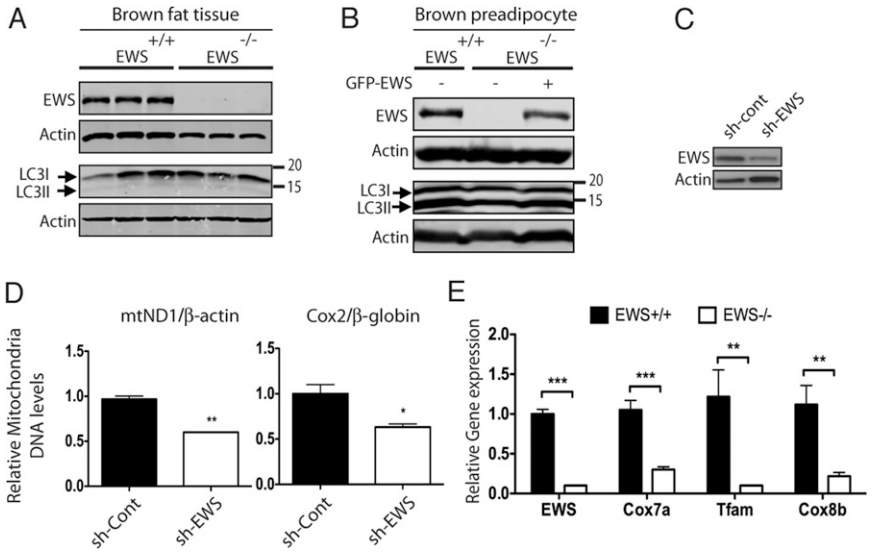

Fig. S1. Loss of *Ews* leads to a significant reduction in mitochondrial abundance and function. (*A*) *Ews* inactivation does not lead to activation of autophagy. Western blot analysis of LC3-I and –II complex, along with EWS and Actin, in iBAT of newborn pups (P0.5) and (*B*) in wild-type or EWS-KO brown preadipocytes with or without complementation of *EWS*. (*C*) Western blot analysis of EWS and Actin following depletion of *Ews* in C2C12 myoblasts using lentivirus expressing control or *Ews* shRNA. (*D*) C2C12 myoblasts were transduced with lentivirus expressing control or *Ews* shRNA and the relative amount of mitochondria DNA was analyzed as described in *Materials and Methods*. Data from three independent experiments are represented as mean ± SEM. Student's *t* test, two-tailed, $*P < 0.05$, $**P < 0.01$. (*E*) Total RNA isolated from $Ews^{+/+}$ or $Ews^{-/-}$ brown preadipocytes ($n = 2$ each) was analyzed by qRT-PCR: mitochondria respiration (Cox7a, COX8b) and DNA replication (Tfam). Data are represented as mean ± SEM. Student's *t* test, two-tailed, $**P < 0.01$, $***P < 0.001$.

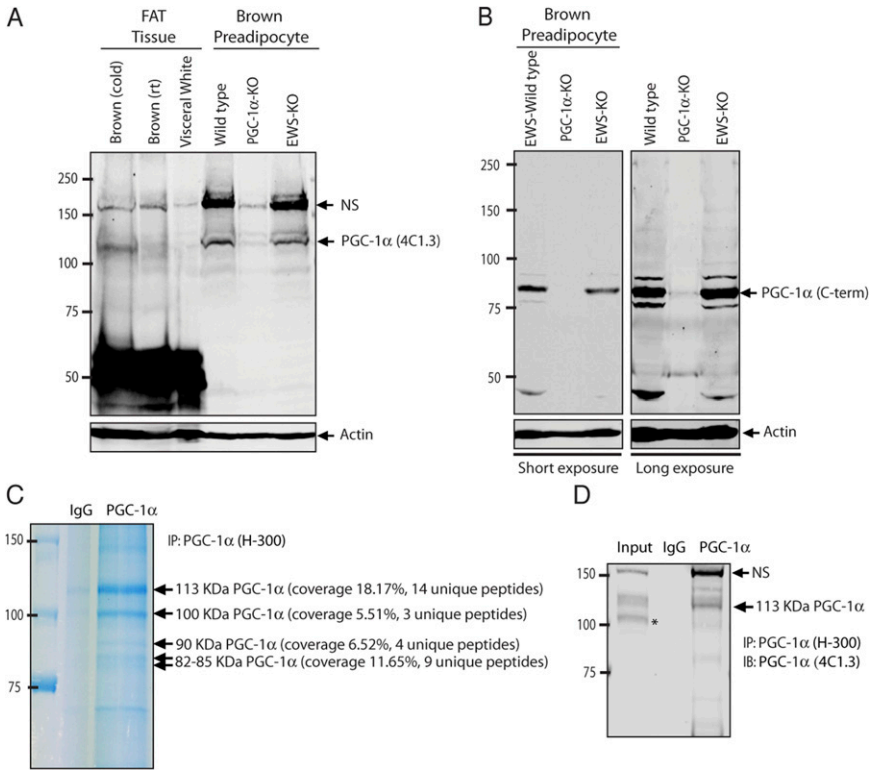

Fig. S2. Validation of different PGC-1α antibodies. We tested two PGC-1α antibodies for Western blotting analysis. (*A*) The mouse monoclonal 4C1.3 antibody detected 113kDa PGC-1α protein expressed in cold-treated BAT (cold: 4 °C for 6 h) but not in the room temperature (rt) BAT nor in visceral white fat (room temperature). The 4C1.3 antibody also detected 113kDa PGC-1α in immortalized $Ews^{+/+}$ and $Ews^{-/-}$ brown preadipocytes but not in *Pgc-1α* null brown preadipocytes. (*B*) The antibody recognizing the C terminus of PGC-1α protein (Millipore, C-term) detected 82- to 90-kDa PGC-1α proteins in $Ews^{+/+}$ and $Ews^{-/-}$ brown preadipocytes but not in *Pgc-1α* null brown preadipocytes. Actin was used as a loading control. (*C*) Colloidal Coomassie staining of HEK293-FLAG-PGC-1α cell lysates immunoprecipitated with H-300 PGC-1α antibody (Santa Cruz). Indicated bands (arrow) were excised and analyzed by mass spectrometry analysis. (*D*) PGC-1α immunoprecipitated with H-300 antibody (as in *C*) was analyzed by Western blot with 4C1.3 monoclonal PGC-1α antibody. NS indicates nonspecific band, and asterisk indicates either nonspecific or degraded PGC-1α.

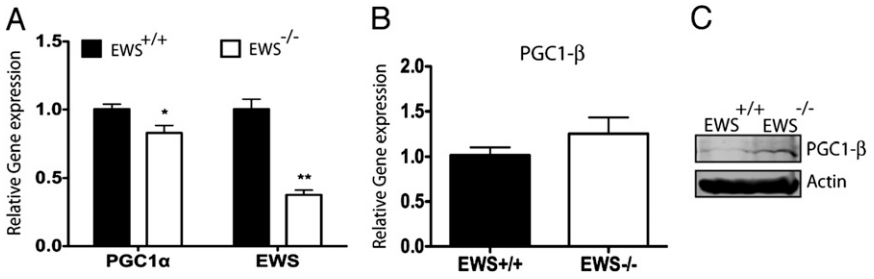

Fig. S3. *Ews* inactivation in brown preadipocytes does not alter PGC-1β transcript or protein levels. (*A*) Relative transcript levels of *Pgc-1α* and *Ews* in $Ews^{+/+}$ or $Ews^{-/-}$ brown preadipocytes were analyzed by qRT-PCR. Data from three independent experiments are represented as mean ± SEM. Student's *t* test, two-tailed, $*P < 0.05$, $**P < 0.01$. (*B*) qRT-PCR analysis of *Pgc-1β* in $Ews^{+/+}$ or $Ews^{-/-}$ brown preadipocytes. (*C*) Western blot analysis of PGC-1β (Bethyl Laboratories, A302-274A) in brown preadipocytes.

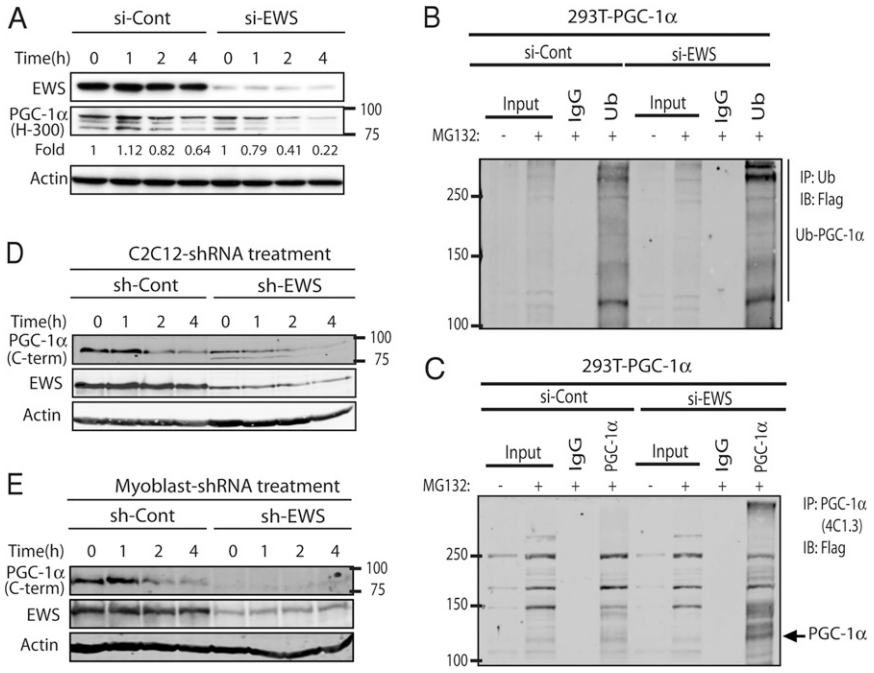

Fig. S4. *EWS* inactivation leads to enhanced degradation and ubiquitination of PGC-1α. (*A*) HEK293 cells stably expressing FLAG-PGC-1α were transfected with control siRNA or siRNA against *EWS*, and PGC-1α protein half-life was measured by cycloheximide (CHX)-chase experiment. Antibodies against EWS, PGC-1α (H-300), and Actin were used in Western blotting. Quantification of bands in each lane (time 0 set to 1) is shown below. (*B* and *C*) HEK293-FLAG-PGC-1α cells were transfected with control siRNA or siRNA against *EWS* in the presence of MG132 and cell lysates were immunoprecipitated with anti-Ubiquitin and immunoblotted with anti-Flag antibody (*B*) or immunoprecipitated with anti-PGC-1α (4C1.3) and immunoblotted with anti-Flag (*C*) antibody. (*D* and *E*) C2C12 myoblasts (*D*) or primary mouse myoblasts (*E*) transduced with lentivirus expressing control or *Ews* shRNA were analyzed for PGC-1α, EWS, and Actin expression following CHX-chase at indicated times.

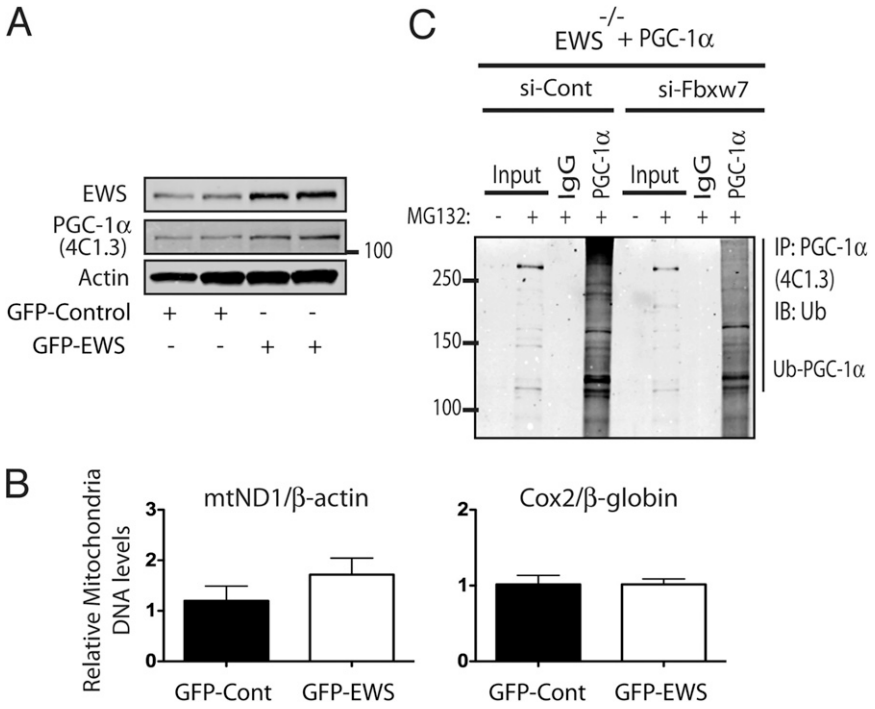

Fig. S5. (*A*) Ectopic expression of *EWS* slightly increases PGC-1α expression. Wild-type brown preadipocytes were transduced with lentivirus expressing *GFP* control or *EWS* and Western blot analysis was performed with EWS, PGC-1α (4C1.3), and Actin antibody. (*B*) Wild-type brown preadipocytes were transduced with lentivirus expressing *GFP* control or *EWS* and quantification of relative mitochondria DNA normalized to nuclear DNA was performed by SYBR Green qPCR analysis. Data from three independent experiments are represented as mean ± SEM. Student's *t* test, two-tailed. (*C*) *Fbxw7* depletion in EWS-KO cells leads to a decrease in PGC-1α ubiquitination. *Ews*$^{-/-}$ brown preadipocytes stably expressing PGC-1α were transfected with either control or Fbxw7 siRNA and treated with MG132, and cell lysates were immunoprecipitated with anti–PGC-1α (4C1.3) antibody followed by Western blot with anti-Ub antibody.

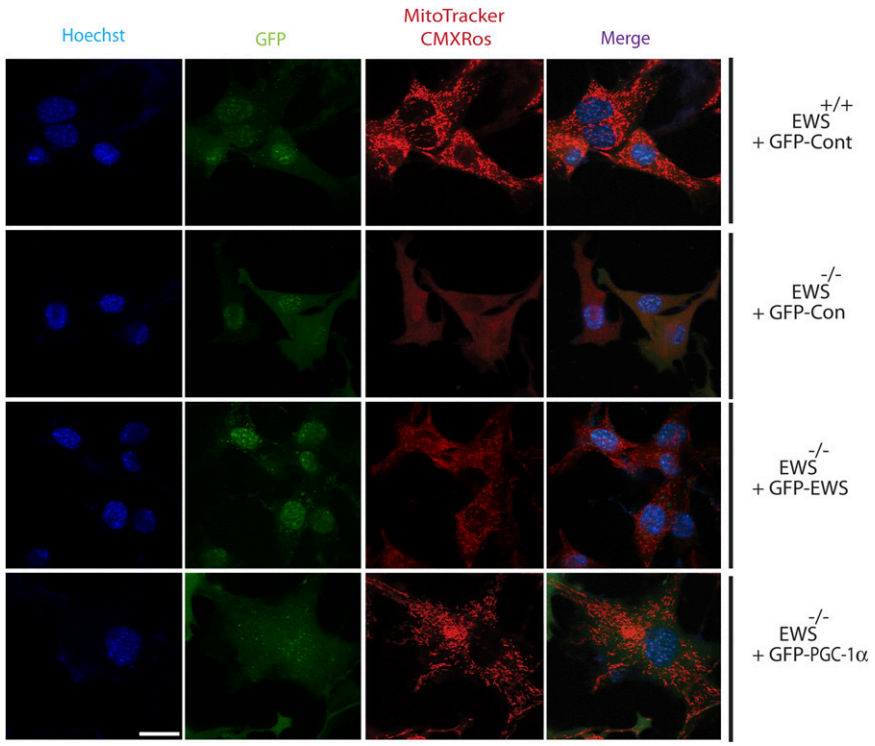

Fig. S6. Complementation of EWS or PGC-1α restores mitochondrial membrane potential in EWS-KO Cells. EWS-KO brown preadipocytes were transduced with indicated lentiviruses and stained with MitoTracker Red CMXRos and Hoechst 33342. GFP+ cells demonstrate successful transduction by lentivirus. Representative images from three independent experiments are shown. (Scale bar: 20 μm.)

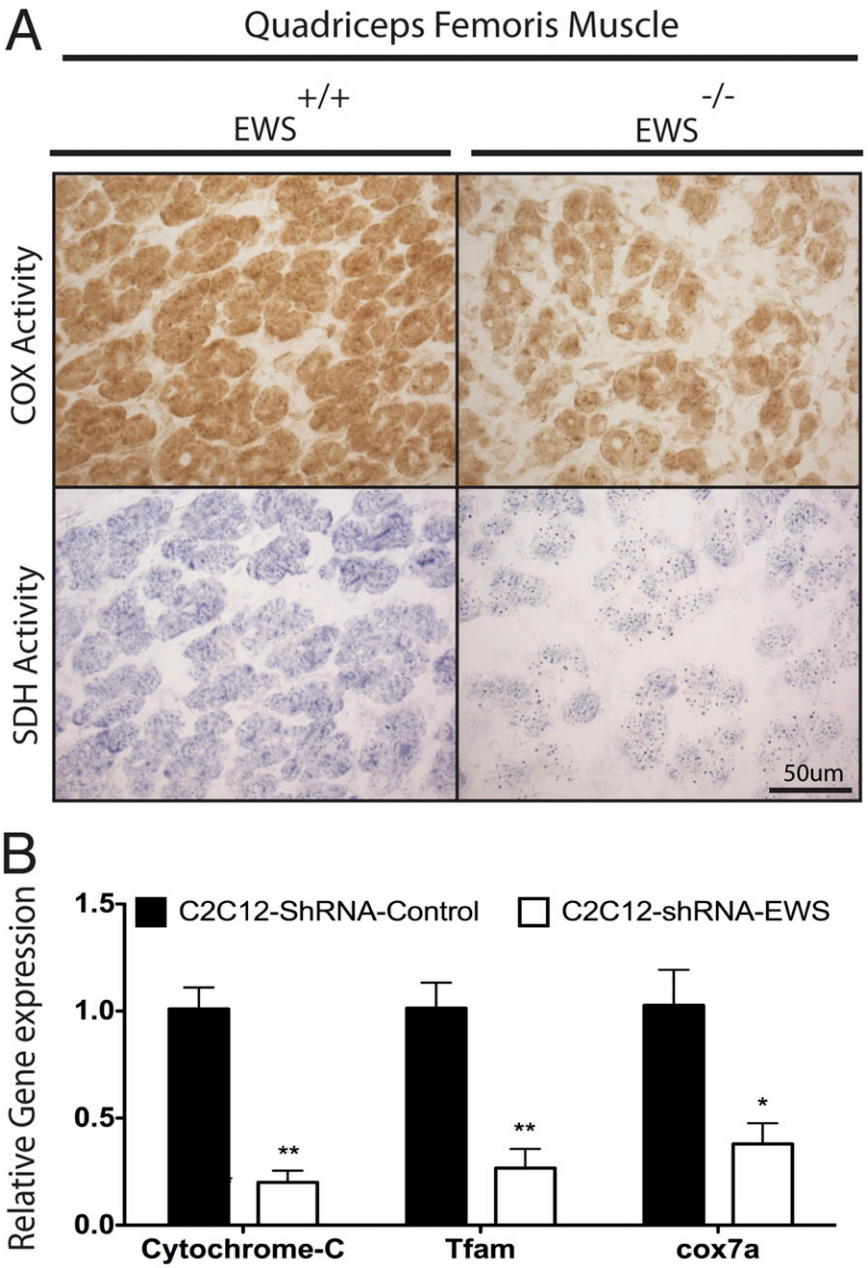

Fig. S7. *Ews* inactivation results in significant reduction in mitochondria activity in skeletal muscles. (*A*) Cross-sections of snap-frozen quadriceps muscles of $Ews^{+/+}$ and $Ews^{-/-}$ congenic newborns (P0.5) were stained for COX and SDH activity ($n = 3$ per genotype). (*B*) Depletion of *Ews* leads to a significant decrease in essential mitochondrial genes in C2C12 myoblasts. Total RNA was isolated from C2C12 cells transduced with control or *Ews* shRNA containing lentivirus and expression of essential mitochondrial genes was analyzed by qRT-PCR. Data from three independent experiments are represented as mean ± SEM. Student's *t* test, two-tailed, $*P < 0.05$, $**P < 0.01$.