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Supplemental Information

Short-Term Calorie Restriction

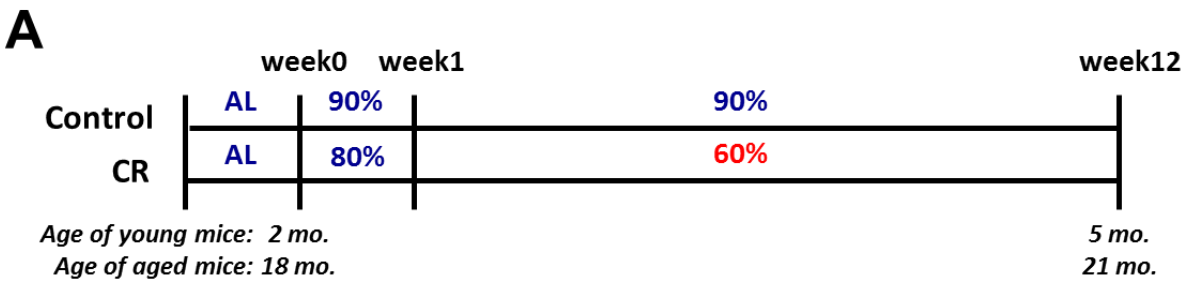
Enhances Skeletal Muscle Stem Cell Function

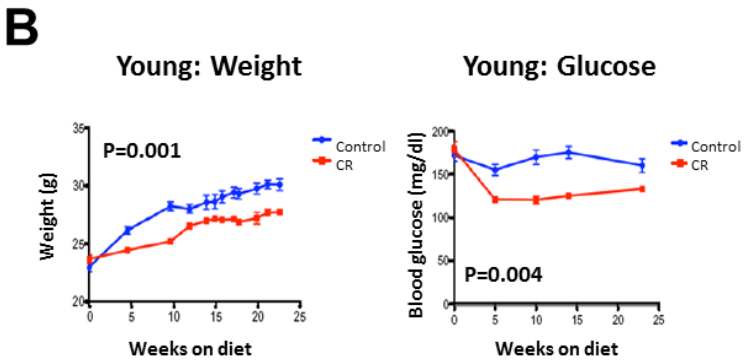
Massimiliano Cerletti, Young C. Jang, Lydia W.S. Finley, Marcia C. Haigis, and Amy J. Wagers

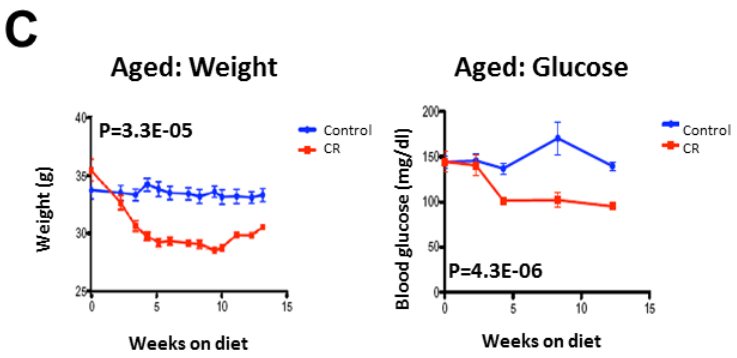
Supplemental Inventory

SUPPLEMENTAL FIGURES

FIGURE S1







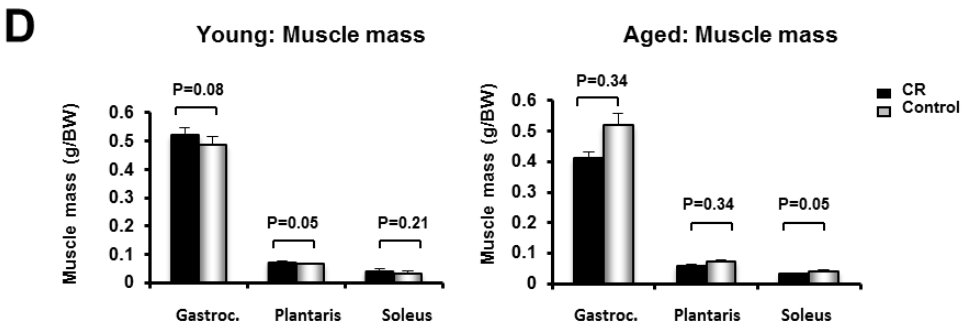


Figure S1. Physiological parameters of CR treatment. (**A**) Calorie restriction (CR) timeline. Animals were fed *ad libitum* (AL) until 2 months (young mice) or 18 months (aged mice) of age, and during this time, their baseline food intake was determined. Mice were then switched to a CR diet for the next 12 weeks (1 week at 20% restriction (80% of baseline calorie intake) and 11 weeks at 40% restriction (60% of baseline calorie intake)). Control mice were maintained on a 90% diet for the entire 12 weeks. Analysis of muscle stem cell frequency and function was performed after 12 weeks of CR or control diet, when animals were 5 months or 21 months of age. (**B, C**) Mean (±SEM) body weight (g) and blood glucose levels (mg/dL) of young and aged C57BL/6 mice on control or CR diet. P values reflect significance at the latest time point indicated. **(D)** Muscle mass, normalized for body weight (g/Body Weight) was determined for the gastrocnemius, plantaris and soleus muscles of young or aged mice at the time of sacrifice (12 weeks after initiation of CR (black bars) or control (grey bars) diet). Data are presented as mean (±SEM) and compiled from analysis of n=7 mice in each group. Related to Figure 1.

FIGURE S2

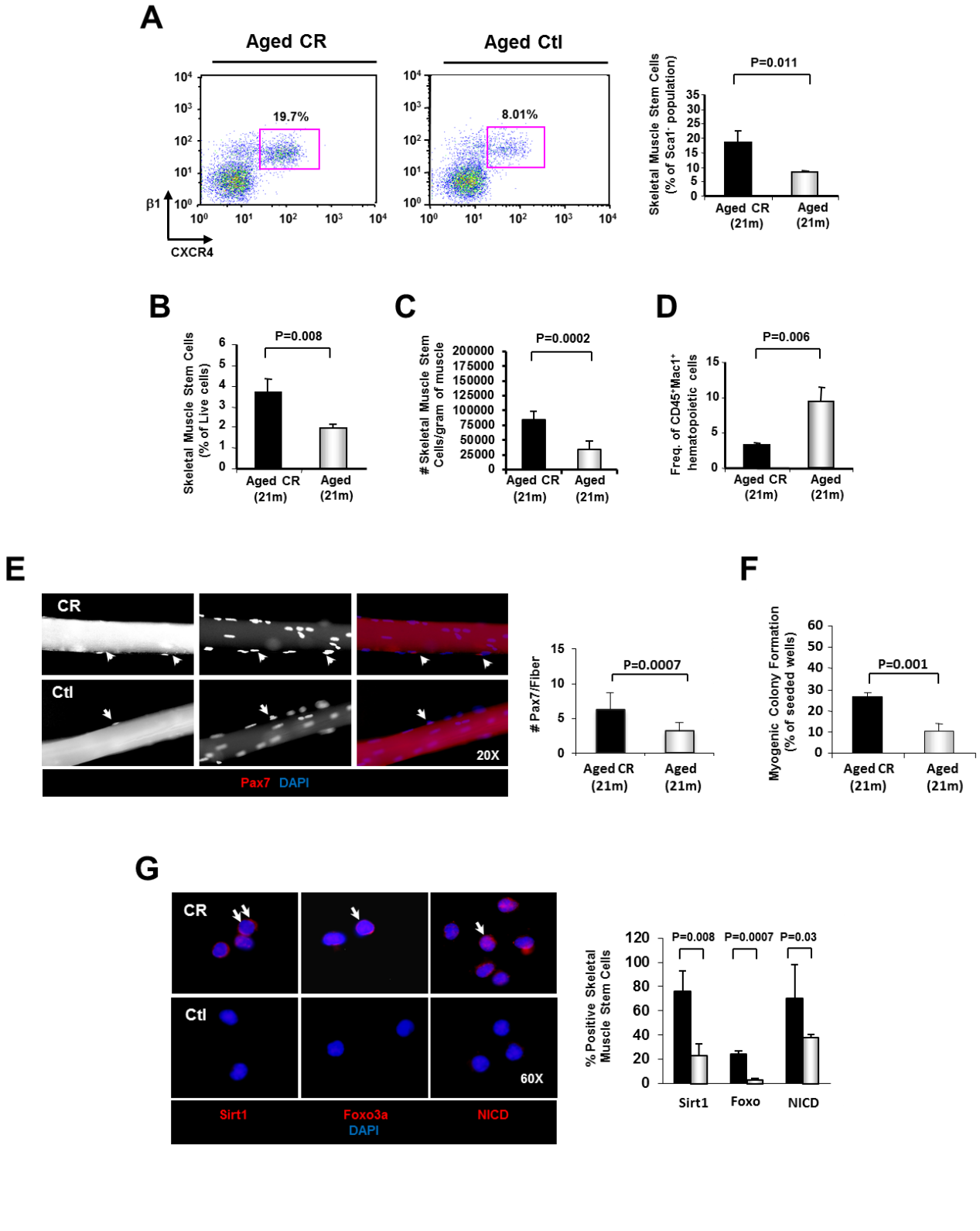

A
Aged CR
Aged Ctl
19.7%
8.01%
β1
CXCR4
Skeletal Muscle Stem Cells (% of Sca1- population)
P=0.011
Aged CR (21m)
Aged (21m)
B
Skeletal Muscle Stem Cells (% of Live cells)
P=0.008
C
# Skeletal Muscle Stem Cells/gram of muscle
P=0.0002
D
Freq. of CD45+Mac1+ hematopoietic cells
P=0.006
E
CR
Ctl
20X
Pax7 DAPI
# Pax7/Fiber
P=0.0007
F
Myogenic Colony Formation (% of seeded wells)
P=0.001
G
CR
Ctl
60X
Sirt1
Foxo3a
DAPI
NICD
% Positive Skeletal Muscle Stem Cells
P=0.008
P=0.0007
P=0.03
Sirt1
Foxo
NICD

Figure S2. Skeletal muscle stem cell frequency and function are enhanced in aged CR-treated muscle. (**A, B**) Flow cytometric analysis of satellite cells isolated from aged CR-treated and control (Ctl) C57BL/6 mice (21 mo. at time of sacrifice, Figure S1A). (**A**) Representative FACS plots showing gating of $CD45^-Sca1^-Mac1^-CXCR4^+\beta1\text{-integrin}^+$ satellite cells are shown at left; plots depict CXCR4 and β1-integrin staining of $CD45^-Sca1^-Mac1^-$ cells previously gated also by scatter and vital dyes (see (Cerletti et al., 2008)). The percent (mean ± SD) of $CD45^-Sca1^-Mac1^-$ myofiber-associated cells expressing the $CXCR4^+\beta1\text{-integrin}^+$ satellite cell profile is indicated for each group at right. (**B**) Satellite cell frequency (mean ± SD) was determined by flow cytometry as in (**A**) for aged C57BL/6 mice receiving CR (black bars) or control (grey bars) diet (n=4 mice per condition, all 21 months of age at analysis). (**C**) Yield of $CD45^-Sca1^-Mac1^-CXCR4^+\beta1\text{-integrin}^+$ satellite cells per gram of muscle (mean ± SD) following FACS sorting from aged CR or aged Ctl mice. (**D**) Frequency (mean ± SD) of $Sca\text{-}1^-CD45^+MAC1^+$ inflammatory cells in muscle was determined by flow cytometry (CR-treated, black bars; Ctl, grey bars; n=8 mice per condition) in aged CR or aged Ctl mice. (**E**) Images of $Pax7^+$ satellite cells identified by staining single isolated myofibers from aged CR-treated or control mice (left). Numbers of $Pax7^+$ satellite cells per fiber were quantified from stained muscle fibers (data represent mean ± SEM; more than 20 myofibers analyzed from each of 4 individual mice in each group (total of 80-160 fibers per experimental condition)). (**F**) Clonal myogenesis assays of double-sorted satellite cells from aged CR-treated or Ctl mice. Data are presented as mean ± SD and represent the percent of wells seeded with 1 satellite cell that contained a myogenic colony at day 5-6. (**G**) Immunofluorescence analysis of Sirt1, Foxo3a and Notch intracellular domain (NICD) in aged CR-treated and Ctl satellite cells. The percentage of positive-staining satellite cells is shown at right. Data were compiled from analysis of 100 cells per group from 4-7 individual mice (CR-treated, black bars; Ctl, grey bars). Data were considered statistically significant at $p<0.05$ and all p-values were calculated by Student's t-test. Related to Figure 1.

Supplemental Experimental Procedures

Mice Aged C57BL/6 mice (16 months when shipped) were obtained from the National Institutes of Aging (NIA); young C57BL/6 mice (2 months) and *mdx* mice (C57BL/10ScSn-Dmd^{mdx}, 8 weeks old) were purchased from Jackson Laboratories, Bar Harbor, ME, USA. GFP transgenic mice (C57BL/Ka-β-actin-EGFP, (Okabe et al., 1997; Wright et al., 2001)) were bred at the Joslin Diabetes Center. Calorie restriction in mice was performed according to established protocols (Pugh et al., 1999). Mice were housed individually and fed AIN-93M diet (BioServ, NJ) *ad libitum* until baseline food intake could be calculated. Mice were then randomly divided into two groups: control mice were fed 90% and CR mice were fed 80% of their *ad libitum* food intake (3g/day). After one week, CR mice were switched to AIN-93M 40% CR diet and fed 60% of their *ad libitum* intake (2.3 g/day) until sacrifice. All procedures were approved by Institutional Animal Care and Use Committees at Harvard University or the Joslin Diabetes Center.

Muscle stem cell isolation Single myofibers and myofiber-associated cells were prepared from intact limb muscles (extensor digitorum longus, gastrocnemius, quadriceps, soleus, tibialis anterior, and triceps brachii), essentially as described (Cerletti et al., 2008; Conboy et al., 2003; Sherwood et al., 2004). After isolation, myofiber-associated cells were stained for FACS to identify satellite cells, which are CD45$^-$Sca-1$^-$Mac-1$^-$CXCR4$^+$β1-integrin$^+$ (Cerletti et al., 2008; Sherwood et al., 2004). FACS data were collected using DIVA (Becton Dickinson (BD), Franklin Lakes, NJ) or Summit (Cytomation, Fort Collins, CO) software and analyzed offline using Flowjo software (Tree Star, Inc., version 8.6.1, Ashland, OR). Antibodies used for flow cytometry included: 30-F11 (1:200, anti-mouse CD45, phycoerythrin (PE) or allophycocyanin (APC) conjugate (eBioscience, San Diego, CA)); M1/70 (1:200, anti-mouse CD11b, PE conjugate, (eBioscience); or 1:800, anti-mouse CD11b, APC conjugate, (eBioscience)); D7 (1:800, anti-

Sca-1, Ly-6A/E, APC conjugate (eBioscience)), β1-integrin (1:200, anti-mouse CD29, purified, (BD Pharmingen, San Jose, CA; or 1:400, anti-mouse/rat CD29, PE conjugate (Biolegend, San Diego, CA); CXCR4 (1:100, biotinylated anti-mouse CD184 (BD Pharmingen)), Streptavidin (1:100, Cy7-PE conjugate (eBioscience)), anti-armenian hamster IgG, fluorescein isothiocyanate (FITC) conjugate (1:100, eBioscience). Live cells were identified as calcein blue positive (1:1000, Invitrogen, Carlsbad, CA) and propidium iodide negative (PI, 1μg/mL). Antibody incubations were performed in staining media (SM = Hank's Buffered Salt Solution (Gibco) + 2% donor bovine calf serum), on ice for 20 min. Satellite cells were always double-sorted to maximize purity. Satellite cell yields were determined as the total number of $CD45^-$ $Sca1^-Mac1^-CXCR4^+\beta1\text{-integrin}^+$ cells sorted per gram of muscle tissue harvested. Satellite cell frequencies were determined by analysis of flow cytometry data using FlowJo (Treestar, Inc., Ashland, OR).

Single myofiber isolation Single myofibers were isolated from tibialis anterior (TA) muscles as described in (Wada et al., 2002). Briefly, muscle was dissected and clamped to minimize fiber length variability and fixed in 4% paraformaldehyde (PFA) for 3 hours at room temperature. Muscles were washed and mechanically dissociated into myofiber bundles using fine forceps. Myofiber bundles were blocked in Vector M.O.M immunodetection kit (Vector Laboratories, Burlingame, CA) for 2 hours at room temperature and incubated with Pax7 antibody (1:200 DSHB, University of Iowa) overnight at 4°C. After 3 washes in PBS, myofiber bundles were incubated in goat anti-mouse AlexaFluor 594 secondary antibody (1:500, Invitrogen) for 1 hour at room temperature. After 3 washes in PBS, fiber bundles were mechanically dissociated into single fibers using fine forceps under dissecting microscope and mounted on slides with Vectashield mounting medium with DAPI (Vector Laboratories). Single fibers were quantified under Zeiss Imager M1 Fluorescence microscope (Carl Zeiss, Thornwood, NY).

Clonal myogenesis assay Satellite cells from young and aged mice were clone-sorted into collagen/laminin coated 96-well plates using an automated cell deposition unit (ACDU). Collagen/laminin coating was accomplished by brief exposure (<10 seconds) of wells to PBS containing collagen (1μg/ml, Sigma) and laminin (10μg/ml, Invitrogen). Coated wells were allowed to air-dry for up to 1 hour prior to addition of satellite cell culture medium (F10 (1.1 g/L D-glucose) or DMEM (4.5 g/L D-glucose) containing 20% horse serum and 5ng/ml bFGF (Sigma, St. Louis, MO)). Where indicated, clonal cultures were seeded instead into DMEM (no glucose) containing galactose (4.5 g/L) alone or with etomoxir (200μM, Sigma). In all cases, individual satellite cells were cultured 5-6 days, with fresh bFGF added daily, and wells containing myogenic colonies were scored by brightfield microscopy at day 5 or 6.

Mitochondrial assay The rate of oxygen consumption and glycolysis was measured using a Seahorse Bioscience extracellular flux analyzer (XF24). FACS sorted freshly isolated satellite cells were seeded in 24-well plates at a density of 5 X 10^3–2 X 10^4 cells per well. Basal and maximal oxygen consumption rates (OCR) were calculated by comparing values with or without the presence of mitochondrial uncoupler, FCCP (5μM). Glycolysis was measured simultaneously by determination of extracellular acidification rate (ECAR).

Western blot analysis Protein levels were analyzed in whole cell lysates obtained from FACS-sorted satellite cells using RIPA buffer, and protein samples were resolved by SDS polyacrylamide gel. Gels were analyzed by Western blotting with antibodies for cytochrome c (BD Pharmingen), prohibitin (Abcam), and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1000. After incubation in horseradish peroxidase (HRP) conjugated secondary antibodies (1:2000) (Santa Cruz Biotechnology) at room temperature for 90 min, the

immunoblots were developed with an enhanced chemiluminescence (ECL) plus system (Amersham: Piscataway, NJ), and then visualized and scanned on a Typhoon 9400 (Amersham: Piscataway, NJ) or X-ray film. Quantification of immunoblots was performed with ImageQuant Software (Sunnyvale, CA).

Immununocytofluorescence Mitotracker Green (500nM, Invitrogen), SOD2 (1:250, Enzo Life Sciences, Farmingdale, NY), Sirt1 (1:200, monoclonal anti-Sirt1, Millipore, Billerica, MA), Foxo3a (1:200, rabbit anti-Foxo3a, Cell Signaling, Denvers, MA), NICD (1:200, rabbit anti-activated Notch1, Abcam), and Pax7 staining were performed on freshly isolated satellite cells. After fixation in 4% PFA for 30 minutes at room temperature, satellite cells were incubated with normal goat serum for 30 minutes, primary antibody for 90 minutes, and secondary antibody (1:200, AlexaFluor 594 or AlexaFluor 488, Invitrogen) for 60 minutes (with 3-4 washes in PBS following each antibody incubation). For immunostaining with primary antibodies raised in mouse, the M.O.M immunodetection kit (Vector Laboratories) was used. Slides were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories) to mark nuclei. Fluorescence images were collected using a Zeiss Imager M1 Fluorescence microscope (Carl Zeiss, Thornwood, NY).

Muscle Injury Tibialis anterior (TA) muscles of anesthetized C57BL/6 mice (5 month-old) were cryo-injured one week before harvest. Harvested muscle was snap-frozen in liquid nitrogen cooled isopentane and sectioned using Microm HM550 cryostat (Thermo Scientific). H&E staining was performed on 8-10 μm tissue cryo-sections for quantification of newly-formed (centrally nucleated) regenerative myofibers.

Muscle stem cell transplantation 5000 or 8000 double sorted satellite cells were injected into the tibialis anterior (TA) muscle of anesthetized recipient mice, injected one day previously with 25 μl (0.03 mg/ml) *Naja mossambica mossambica* cardiotoxin (Sigma) to facilitate engraftment (Cerletti et al., 2008). The next day, double-sorted satellite cells, in 5–10 μl SM were injected directly into the pre-injured muscles. Muscles were harvested 4 weeks after transplant and analyzed for engraftment using epiflourescence (for GFP) or immunofluorescence (for dystrophin staining) microscopy. Dystrophin staining (polyclonal anti-dystrophin (1:50; Abcam) of fixed (4% paraformaldeyhyde), frozen muscle sections (5–8 μm thick) was performed as in (Cerletti et al., 2008). Fluorescence images were acquired using Olympus BX60 Standard fluorescence microscope (Olympus Optical CO, LTD)

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