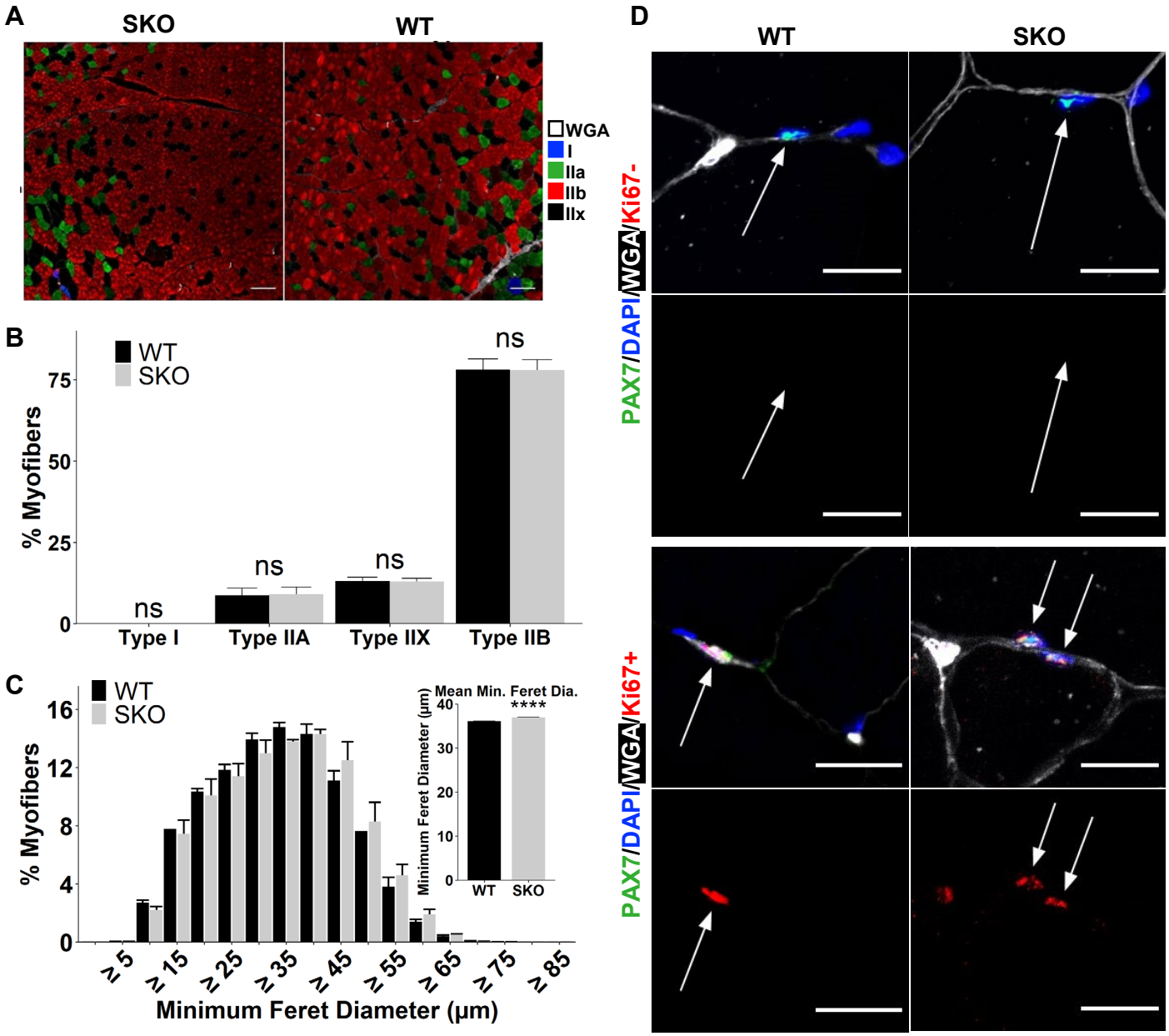
Stem Cell Reports, Volume *16*

Supplemental Information

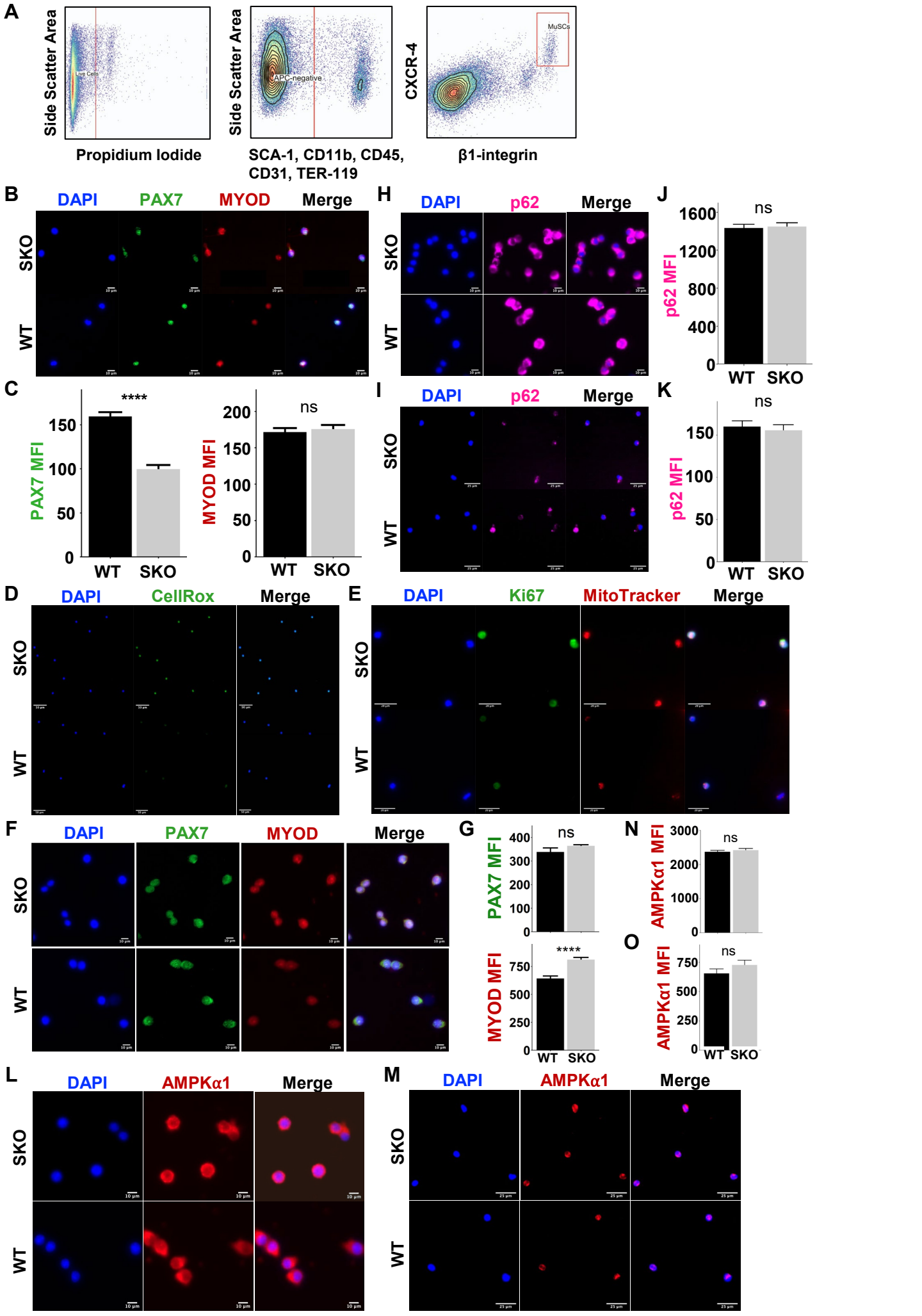
Sestrins regulate muscle stem cell metabolic homeostasis

Benjamin A. Yang, Jesus Castor-Macias, Paula Fraczek, Ashley Cornett, Lemuel A. Brown, Myungjin Kim, Susan V. Brooks, Isabelle M.A. Lombaert, Jun Hee Lee, and Carlos A. Aguilar

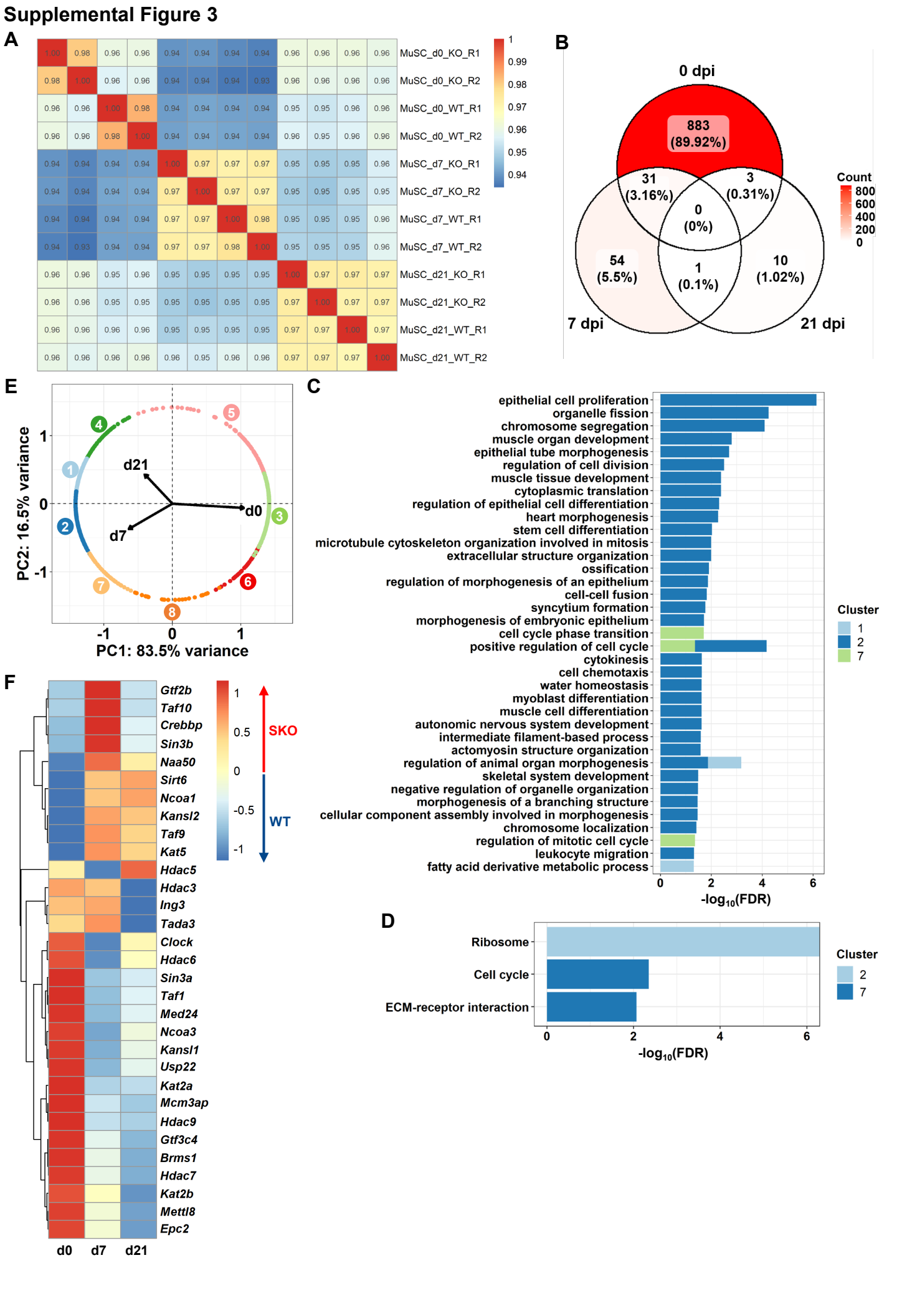
Supplemental Figure 1



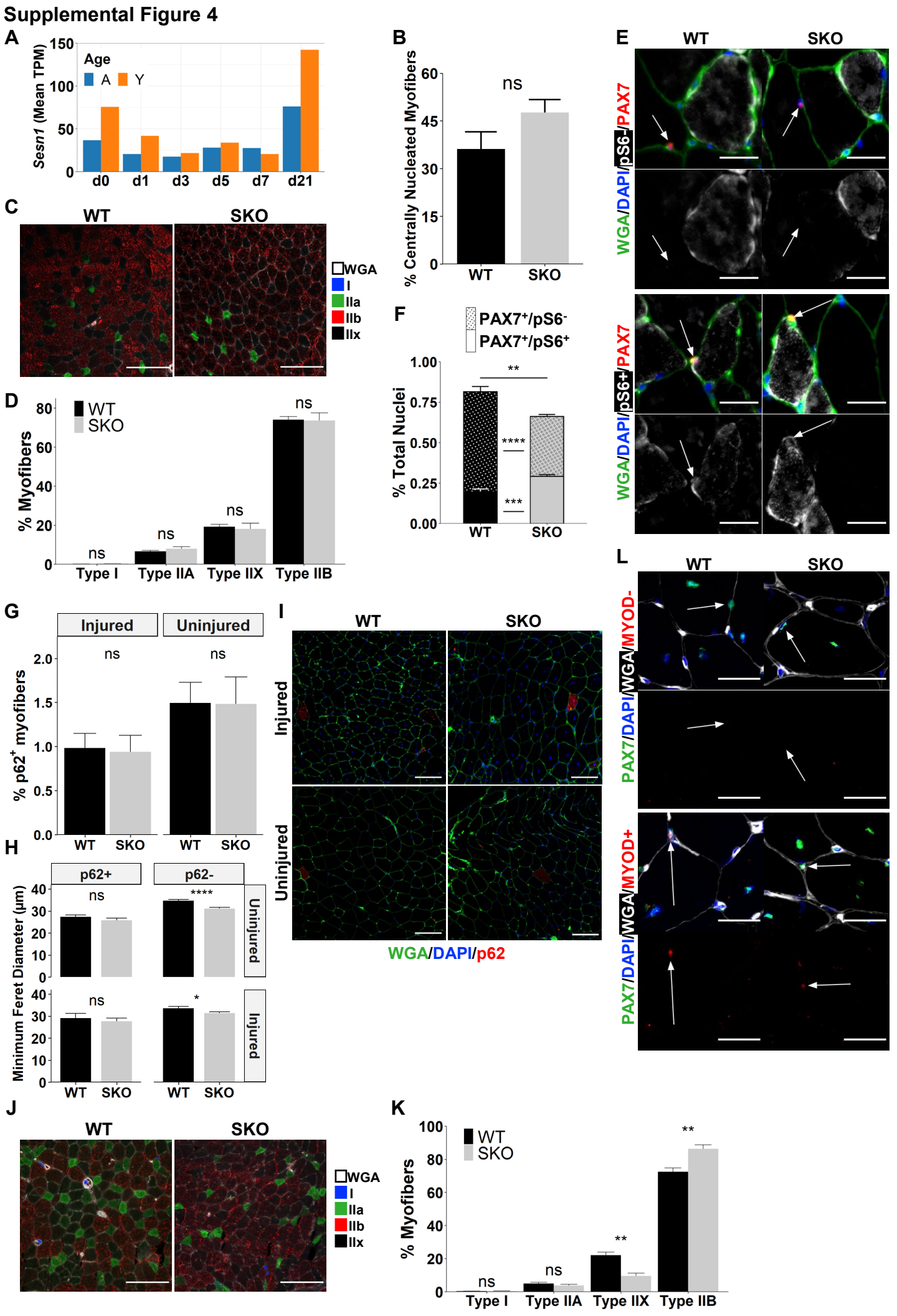
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



Supplemental Table 1

SKO MuSCs		
ID	**Name**	**Subsystem**
ACSm	Acetyl CoA synthetase	Glycolysis / Gluconeogenesis
CSm	Citrate synthase	Citric Acid Cycle
GLYATm	Glycine C-acetyltransferase	Glycine, Serine, and Threonine Metabolism
WT MuSCs		
ID	**Name**	**Subsystem**
GLUDxm	Glutamate dehydrogenase, mitochondria	Glutamate metabolism
RADH	Retinal dehydrogenase	Vitamin A metabolism
ACACtx	Hydrogen peroxide transport via diffusion	Transport, Extracellular

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Related to Figure 1.
A) Representative immunohistochemical images of fiber-type analyses in whole quadriceps muscle sections stained with antibodies against Type I (blue), Type IIa (green), and Type IIb (red) fibers. Type IIx fibers lack staining. Connective tissue was stained with wheat germ agglutinin (WGA) (white). Scale bars are 100 μm. **B)** Distribution of fiber types in the rectus femoris of the quadriceps muscle. (WT: n=3 mice, SKO: n=3 mice). Statistical comparisons are two-sided Mann-Whitney *U*-tests. **C)** Size distributions of WT and SKO myofibers across whole quadriceps muscle sections. Statistical comparisons are two-sided, unpaired Student's *t*-tests with Holm multiple testing correction for the histogram. (WT: n=3 mice, SKO: n=3 mice). Inset is mean myofiber size per condition. **D)** Representative in situ immunohistochemical images of $Ki67^-$ and $Ki67^+$ MuSCs in TA muscle sections from WT and SKO mice stained for DAPI (blue), PAX7 (green), WGA (white), and Ki67 (red). Arrows mark $PAX7^+$ cells. Scale bars are 25 μm. All data are shown as mean ± SEM. ****p<0.0001. Unmarked comparisons lack statistical significance.

Figure S2. Related to Figure 1.
A) Representative muscle stem cell (MuSC) isolation plots using FACS gating for $SCA\text{-}1^-$, $CD45^-$, $CD11b^-$, $TER\text{-}119^-$, $\beta1\text{-integrin}^+$, and $CXCR\text{-}4^+$. **B)** Representative DAPI-counterstained immunofluorescence images for PAX7 and MYOD in SKO and WT MuSCs fixed immediately after isolation. Scale bars are 10 μm. **C)** Mean fluorescence intensity (MFI) of PAX7 and MYOD in SKO and WT MuSCs fixed immediately after isolation. **D)** Representative DAPI-counterstained immunofluorescence images for CellRox (green) in SKO and WT MuSCs fixed immediately after isolation. Scale bars are 50 μm. **E)** Representative DAPI-counterstained immunofluorescence images of Ki67 (green) and MitoTracker (red) in SKO and WT MuSCs fixed immediately after isolation. Scale bars are 20 μm. **F)** Immunofluorescence staining for PAX7 (green), MYOD (red) and DAPI (blue) in SKO and WT MuSCs after 3 days of culture in activating conditions. Scale bars are 10 μm. **G)** Mean fluorescence intensity (MFI) of PAX7 and MYOD in SKO and WT MuSCs after 3 days of culture. Representative DAPI-counterstained immunofluorescence images of p62 in MuSCs **H)** after 3 days of culture in activating conditions (scale bars are 25 μm) and **I)** immediately after isolation (scale bars are 10 μm). MFI of immunofluorescence staining for p62 **J)** after 3 days of culture in activating conditions and **K)** immediately after isolation. Immunofluorescence staining for AMPKα1 in MuSCs **L)** after 3 days of culture (scale bars are 25 μm) and **M)** immediately after isolation (scale bars are 10 μm). Quantification of MFI from AMPKα1 in SKO and WT MuSCs **N)** fixed after 3 days of culture in activating conditions and **O)** immediately after isolation. All data are shown as mean ± SEM. Statistical comparisons are two-sided unpaired Student's *t*-tests. ****p<0.0001.

Figure S3. Related to Figure 2.
A) Spearman correlation heatmap between all biological replicates. **B)** Venn diagram of differentially expressed genes (padj<0.01, Log_2 Fold Change>1) in freshly isolated MuSCs at 0, 7, and 21 days post injury (dpi). **C)** Stacked bar plot of enriched GO:BP terms (FDR<0.05) per DPGP cluster from WebGestaltR analysis. **D)** Bar plot of enriched KEGG terms (FDR<0.05) per DPGP cluster from WebGestaltR analysis. **E)** Principal component analysis (PCA) plot of differentially expressed genes colored by DPGP cluster with loadings of dpi. **F)** Standardized heatmap (z-scores) of differentially expressed genes (padj<0.05, Log_2 Fold Change>0.26) from WT and SKO MuSCs in GO terms for histone deacetylase (GO:0004407) or acetyltransferase (GO:0004402) activity at 0, 7, and 21 dpi.

Figure S4. Related to Figures 3 and 4.
A) Average *Sesn1* expression in young (2-3 months) and aged (22-24 months) MuSCs at 0, 1, 3, 5, 7, and 21 days after $BaCl_2$ hindlimb (gastrocnemius and tibialis anterior) injury. **B)** Percentage of centrally nucleated myofibers in injured WT and SKO TA muscle sections. (WT: n=4 mice, SKO: n=3 mice). Statistical comparison is a two-sided Mann-Whitney *U*-test. **C)** Representative immunohistochemical images of fiber-type analyses in whole, uninjured TA muscle sections from WT and SKO mice. Sections were stained with antibodies against Type I (blue), Type IIa (green), and Type IIb (red) fibers. Type IIx fibers lack staining. Connective tissue was stained with wheat germ agglutinin (WGA) (white). Scale bars are 100 μm. **D)** Distributions of fiber types in whole TA muscle sections of uninjured SKO and WT mice (WT: n=5 mice, SKO: n=3 mice). **E)** Representative in situ immunohistochemical images of $pS6^-$ (top) and $pS6^+$ (bottom) MuSCs in whole, uninjured TA muscle sections from WT and SKO mice stained for DAPI (blue),

PAX7 (red), WGA (green), and pS6 (white). Arrows mark $PAX7^+$ cells. Scale bars are 25 μm. **F)** Quantification of $pS6^+$ (stippled) and $pS6^-$ (plain) $PAX7^+$ cells as a percentage of all nuclei in whole, uninjured TA muscle sections. Statistical comparisons are two-sided Mann-Whitney *U*-tests. (WT: n=3 mice, SKO: n=3 mice). **G)** Quantification of $p62^+$ myofibers as percentages of total myofibers in whole TA muscle sections from WT and SKO mice (WT: n=5 mice, SKO: n=4 mice). Statistical comparisons are two-sided unpaired Student's *t*-tests. **H)** Minimum Feret diameters of $p62^+$ and $p62^-$ myofibers in injured and uninjured whole TA muscle sections from WT and SKO mice (Injured - WT: n=5 mice, SKO: n=4 mice; Uninjured – WT: n=4 mice, SKO: 3 mice). Statistical comparisons are two-sided, unpaired Student's *t*-tests. **I)** Representative immunohistochemical images of p62 in whole TA muscle sections. Scale bars 100 μm. **J)** Representative immunohistochemical images of fiber-type analyses in injured whole TA muscle sections from WT and SKO mice. Sections were stained with antibodies against Type I (blue), Type IIa (green), and Type IIb (red) fibers. Type IIx fibers lack staining. Connective tissue was stained with wheat germ agglutinin (WGA) (white). Scale bars are 100 μm. **K)** Distributions of fiber types in whole TA muscle sections of injured SKO and WT mice (WT: n=5 mice, SKO: n=3 mice). Statistical comparisons are two-sided Mann-Whitney *U*-tests. **L)** Representative in situ immunohistochemical images of $MYOD^-$ (top) and $MYOD^+$ (bottom) MuSCs in TA muscle sections from WT and SKO mice stained for DAPI (blue), PAX7 (green), WGA (white), and MYOD (red). Arrows mark $PAX7^+$ cells. Scale bars are 25 μm. All data are shown as mean ± SEM. *p<0.05 ,**p<0.01, ***p<0.001, and ****p<0.0001.

Table S1. Representative significant RECON1 metabolic fluxes. Related to Figure 2.
Representative significant fluxes for metabolic subsystems in SKO (top) and WT (bottom) muscle stem cells (MuSCs).

MATERIALS & REAGENTS

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC anti-Mouse Ly-6A/E (Sca-1), clone: D7, isotype: Rat IgG2a, κ	BioLegend	108112
APC anti-Mouse CD45, clone: 30-F11, isotype: Rat IgG2b, κ	BioLegend	103112
APC anti-Mouse/Human CD11b, clone: M1/70. Isotype: Rat IgG2b, κ	BioLegend	101212
APC anti-Mouse TER-119, clone: TER-119, isotype: Rat IgG2b, κ	BioLegend	116212
PE anti-Mouse/Rat CD29, clone: HMβ1-1, isotype: Armenian Hamster IgG	BioLegend	102208
Biotin Rat anti-Mouse CD184, clone: 2B11/CXCR4 (RUO), isotype: Rat IgG2b, κ	BD Bioscience	551968
Streptavidin PE-Cyanine7	eBioscience	25-4317-82
4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI), FluoroPure™ grade	Invitrogen	D21490
M.O.M. (Mouse on Mouse) Immunodetection Kit	Vector Laboratories	BMK-2202
Mouse anti-BA-D5 antibody, isotype: MIgG2b, supernatant	Developmental Studies Hybridoma Bank	BA-D5
Mouse anti-SC-71 antibody, isotype: MIgG1, supernatant	Developmental Studies Hybridoma Bank	SC-71
Mouse anti-BF-F3 antibody, isotype: MIgM, supernatant	Developmental Studies Hybridoma Bank	BF-F3

Mouse anti-Pax7 antibody, isotype: MIgG1, κ, from supernatant	Developmental Studies Hybridoma Bank	Pax7
Alexa Fluor 488, Goat anti-Mouse IgG1, cross-adsorbed antibody	Invitrogen	A-21121
Alexa Fluor 555, Goat anti-Mouse IgM (heavy chain), cross-adsorbed antibody	Invitrogen	A-21426
Alexa Fluor 555, Goat anti-Rabbit IgG (H+L), cross-adsorbed antibody	Invitrogen	A-21428
Alexa Fluor 647, Goat anti-Mouse IgG2b, cross-adsorbed antibody	Invitrogen	A-21242
Alexa Fluor 647, Goat anti-Rabbit IgG (H+L), cross-adsorbed antibody	Invitrogen	A-21244
CF 405S WGA	Biotium	29027
CF 488A WGA	Biotium	29022
CF 640R WGA	Biotium	29026
Alexa Fluor 555, Tyramide SuperBoost Kit, Goat anti-Mouse IgG	Invitrogen	B40913
Alexa Fluor 488, Tyramide SuperBoost Kit, Goat anti-Mouse IgG	Invitrogen	B40912
Rabbit anti-Phospho-S6 Ribosomal Protein (Ser235/236) monoclonal antibody, isotype: IgG	Cell Signaling Technology	4858
Rabbit anti-SQSTM1/p62 monoclonal antibody, isotype: IgG	Cell Signaling Technology	23214
Rabbit anti-Ki67 polyclonal antibody, isotype: IgG	Abcam	ab15580
Rabbit anti-MyoD1 polyclonal antibody, isotype: IgG	Abcam	ab203383
In Situ Cell Death Detection Kit, TMR red	Roche	12156792910
Chemicals, Peptides, and Recombinant Proteins		
DMEM, high glucose, pyruvate	Life Technologies	11995065
Ham's F-10 Nutrient Mix	Life Technologies	11550043
Horse Serum	Gibco	16050122
Fetal Bovine Serum	Sigma	F2442
Fetal Bovine Serum	Life Technologies	10437028
0.25% Trypsin-EDTA (1X)	Gibco	25200-056
0.05% Trypsin-EDTA (1X)	Gibco	25300-054
Penicillin Streptomycin (10,000 U/mL Pen, 10,000 ug/mL Strep)	Gibco	15140-122
Glycine	Sigma-Aldrich	G7126-100G
Tween 20, lot# SLBS9921	Sigma-Aldrich	P9416-100mL
MEM Non-Essential Amino Acids (100X)	Gibco	11140-050
Bovine Serum Albumin (BSA), Protease-free powder	Fisher Scientific	BP9703-100
PureCol, Purified Bovine Type I Atelo-Collagen Solution, 3 mg/mL	Advanced BioMatrix	5005
Gelatin from porcine skin, type A	Sigma-Aldrich	G2500
DPBS, no calcium, no magnesium	Gibco	14190144
PBS, pH 7.4	Gibco	10010023

HBSS, no calcium, no magnesium, no phenol red	Life Technologies	14175145
Paraformaldehyde Solution, 4% in PBS	Thermo Scientific	J19943K2
Triton X-100	Acros Organics	A0376210
QIAzol Lysis Reagent	Qiagen	79306
MitoTracker Deep Red	Thermo Fisher	M22426
Dispase II (activity ≥0.5 units/mg solid)	Sigma-Aldrich	D4693-1G
Collagenase Type II (654 U/mg, non-specific proteolytic activity 487 U/mg)	Life Technologies	17101015
Propidium Iodide - 1.0 mg/mL Solution in Water	Life Technologies	P3566
Isofluorane	Vet One	502017
Corning Cell-Tak Cell and Tissue Adhesive	Fisher Scientific	C354240
Tissue-Plus OCT Compound	Fisher Scientific	23-730-571
ActinRed 555 ReadyProbes	Invitrogen	R37112
Dako Fluorescence Mounting Medium	Agilent	S302380-2
ProLong Diamond Antifade Mountant	Invitrogen	P36970
Direct Red 80	Alfa Aesar	AAB2169306
Picric Acid solution	Sigma-Aldrich	P6744-1GA
Acetic Acid, Glacial	Fisher Scientific	BP2401-500
Ricca Chemical Hematoxylin Stain Solution	Fisher Scientific	3530-16
Eosin Y Solution	EMD-Millipore	588X-75
SafeClear II Xylene Substitute	Fisher Scientific	23-044192
Permount Mounting Medium	Fisher Scientific	SP15-100
Goat Serum	Sigma-Aldrich	G9023
Deposited Data		
Sestrin Knockout RNA-Seq Dataset	This Manuscript	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE162191
Aging Muscle Stem Cell RNA-Seq Dataset	Shcherbina et al., Cell Reports (2020)	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE121589
Software and Algorithms		
DESeq2 (v.1.26.0)	Love MI, Huber W, Anders S (2014)	https://www.genomebiology.biomedcentral.com/articles/10.1186/s13059-014-0550-8
R v.4.0.0	The R Foundation for Statistical Computing	https://www.r-project.org/
WebGestaltR	Liao Y et al. (2019)	doi.org/10.1093/nar/gkz401
Flexbar	Roehr et al. (2017)	doi: 10.1093/bioinformatics/btx330.
Kallisto (v.0.46.1)	Bray et al. (2016)	doi:10.1038/nbt.3519
tximport (v.1.14.0)	Soneson C, Love MI, Robinson MD (2015)	doi.org/10.12688/f1000research.7563.2

DP_GP_cluster	McDowell et al., (2018)	https://github.com/PrincetonUniversity/DP_GP_cluster
ComplexHeatmap	Gu Z, Eils R, Schlesner M (2016)	doi.org/10.1093/bioinformatics/btw313
Escher	King ZA et al. (2015)	dx.doi.org/10.1371/journal.pcbi.1004321

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animal and Injury Models.

C57BL/6 wild-type male and female mice were obtained from Jackson Laboratory or from a breeding colony at the University of Michigan (UM). All mice were fed normal chow ad libitum and housed on a 12:12 hour light-dark cycle under UM veterinary staff supervision. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and were in accordance with the U.S. National Institute of Health (NIH). Sestrin 1 and Sestrin 2 knockout mice were previously generated[1], where we observed that Sesn1 and Sesn2 were abundantly expressed in skeletal muscle, while Sesn3 was barely detectable[2]. Sesn3 expression is also undetectable in cultured myotubes and myoblasts[2], although some was detected in heart tissue[1]. Since Sesn1/2/3 TKO mice do not breed well due to semi-sterility[2], we used Sesn1/2 DKO mice for examining the function of Sestrins in skeletal muscle stem cell (MuSC) compartments. Ablation of Sestrin1 and Sestrin2 by the corresponding genetic alleles was confirmed through previous studies[1–3]. To induce skeletal muscle injury, mice were first anesthetized with 2% isoflurane and bilaterally injected with a 1.2% barium chloride ($BaCl_2$) solution into several points of the tibialis anterior and both heads of the gastrocnemius muscles for a total of 80 µL per hindlimb[4].

Myofiber Size and Quantification

Quadriceps muscles were mounted in OCT (Fisher Scientific # 23-730-571), snap frozen in liquid nitrogen, and sectioned in a cryotome at 10 µm thickness. Sections were stained with ActinRed 555 ReadyProbes (Invitrogen #R37112) by diluting two drops of the reagent in 1 mL of 1X PBS with WGA 488 (Biotium #29022) at 5 µg/ml concentration and DAPI at 2 ug/mL concentration for one hour at room temperature. After the incubation period, the sections were washed three times for 5 minutes each in 1X PBS. Slides were then mounted with Dako fluorescence medium (Agilent #S302380-2) and coverslipped. Minimum Feret diameter distributions across whole sections were quantified in FIJI software. Error bars for each bin were plotted by calculating the mean and standard error for three technical replicates per biological replicate.

Fiber type stain and Quantification

Quadriceps muscles were mounted in OCT (Fisher Scientific # 23-730-571), snap frozen in liquid nitrogen, and sectioned in a cryotome at 10 µm thickness. Slides were first rinsed in 1X PBS three times for 5 minutes each with gentle shaking followed by a 5-minute incubation with 0.2% Triton X-100 (Acros Organics #A0376210) in 1X PBS for 5 minutes and rinsed again in 1X PBS as before. Tissue sections were then blocked in M.O.M. mouse IgG blocking reagent (Vector Laboratories #BMK-2202) overnight in a hydrated chamber. The blocking reagent was then washed three times for 5 minutes in 1X PBS, followed by a 5-minute incubation in M.O.M. diluent. Primary antibodies were then incubated at 4°C overnight in a hydrated chamber and diluted in M.O.M. diluent as follows: anti-BA-D5 (1:100), anti-SC-71 (1:500), and anti-BF-F3 (1:100). After three 5-minute 1X PBS washes, secondary antibodies and WGA were incubated for one hour at room temperature at the following concentrations: Alexa Fluor 647 Goat anti-Mouse IgG2b (Invitrogen #A-21242) at 1:300 dilution, Alexa Fluor 488 Goat anti-Mouse IgG1 (Invitrogen #A-21121) at 1:300 dilution, Alexa Fluor 555 Goat anti-Mouse IgM (Invitrogen #A-21426) at 1:300 dilution, and WGA 405 (Biotium #29027) at 100 µg/ml. Secondary antibodies and WGA were washed three times for 5 minutes in 1X PBS. Tissue sections were mounted with Dako fluorescence medium (Agilent # S302380-2) and coverslipped. Whole section images were taken using a Nikon A1 confocal microscope. Fiber type distributions of quadriceps whole section were manually quantified in FIJI. Statistical significance was calculated by performing two-sided Mann-Whitney *U*-tests across three technical replicates per biological replicate.

PAX7/Phospho-S6, Ki67, MYOD, and TUNEL Staining and Quantification

Quadriceps and TA muscles were mounted in OCT, snap frozen in liquid nitrogen, and sectioned in a cryotome at 10 µm thickness. Tissue sections were fixed with 4% PFA (Fisher Scientific #AAJ19943K2) for 10 minutes and washed three times for 5 minutes each in 1X PBS. Endogenous peroxidases were blocked with 100X H_2O_2 for 10 minutes at room temperature using the Tyramide SuperBoost Goat anti-Mouse Alexa Fluor 555 or Alexa Fluor 488 kit (Invitrogen #B40913, #B40912) followed by 1X PBS washes. Slides were incubated in citrate buffer at room temperature for 2 minutes, placed in citrate buffer at 65°C, then heated to 92°C. Tissue sections were incubated at 92°C for 11 minutes then brought back to room temperature. After 1X PBS washes, sections were blocked in M.O.M. mouse IgG blocking reagent (Vector Laboratories #BMK-2202) and 10% goat serum (Millipore Sigma #G9023) for one hour at room temperature, followed by three 5-minute 1X PBS washes. Sections were incubated with anti-Pax7 antibody (1:10) and anti-Phospho-S6 (1:50, Cell Signaling #4858), anti-Ki67 or anti-MyoD1 (1:100, ab15580 or ab203383) antibodies in M.O.M. diluent with 10% goat serum overnight at 4°C in a humidified chamber. Primary antibodies were washed three times for 5 minutes each in 1X PBS and secondary goat anti-Mouse Poly HRP antibody from the Tyramide SuperBoost Goat anti-Mouse Alexa Fluor 555 or Alexa Fluor 488 kits was added for 1 hour at room temperature, followed by 1X PBS washes. Tyramide working solution was then incubated for 10 minutes, followed by a 3-minute stop solution incubation and 1X PBS washes. Next, WGA 488 or WGA 647 (50 µg/ml, Biotium #29022, and Biotium #29026), DAPI (2µg/ml, Invitrogen #D21490), and Alexa Fluor 647 or Alexa Fluor 555 goat anti-Rabbit (Invitrogen #A-21244, #A-21428) were incubated for 1 hour at room temperature in a humidified chamber. Slides were then mounted with Dako fluorescence media and coverslipped. Whole section images were taken using a Nikon A1 confocal microscope. Phospho-S6, Ki67, MyoD1, and TUNEL satellite cells were counted by hand using FIJI. Statistical significance was calculated using two-sided Mann-Whitney *U*-tests across three technical replicates per biological replicate.

In situ cell-death detection stain:

Tissue collection, preservation and fixation were performed as described above. Tissue sections were permeabilized for 10 minutes using 0.2% Triton X-100 (Acros Organics #A0376210), followed by 1X PBS washes. Slides were incubated in citrate buffer at room temperature for 2 minutes, placed in citrate buffer at 65°C, and heated to 92°C. Tissue sections were incubated at 92°C for 11 minutes then brought back to room temperature. After 1X PBS washes, slides were immersed in Tris-HCl pH 7.5 buffer containing 3% BSA and 20% normal bovine serum for 30 minutes. After 1X PBS washes, the *In Situ* Cell Death Detection Kit (Roche #12156792910) reaction mixture was incubated for 90 minutes at room temperature. Following 1X PBS washes, endogenous peroxidases were blocked with 100X H_2O_2 for 10 minutes at room temperature from the Tyramide SuperBoost Goat anti-Mouse Alexa Fluor 488 kit (Invitrogen #B40912). After 1X PBS washes, the sections were blocked in M.O.M. mouse IgG blocking reagent (Vector Laboratories #BMK-2202) for one hour at room temperature. Sections were incubated with anti-Pax7 antibody overnight at 4°C in a humidified chamber in M.O.M. diluent at 1:10 dilution. Primary antibodies were washed three times for 5 minutes in 1X PBS and secondary goat anti-Mouse Poly HRP antibody from Tyramide SuperBoost Goat anti-Mouse Alexa Fluor 488 kit was added for 1 hour at room temperature, followed by 1X PBS washes. Tyramide working solution was then incubated for 10 minutes, followed by a 3-minute stop solution incubation and 1X PBS washes. WGA 647 (Biotium #29026) at 50 µg/ml concentration and DAPI (Invitrogen #D21490) at 2µg/ml concentration were incubated for 1 hour at room temperature. Slides were then mounted with Dako fluorescent media, and coverslipped. Whole section images were taken using a Nikon A1 confocal microscope. Satellite cells positive for TUNEL were counted manually using FIJI. Statistical significance was calculated by two-sided Mann-Whitney *U*-tests across three technical replicates per biological replicate.

p62 Staining and Quantification

TA muscles were mounted in OCT, snap frozen, and sectioned in a cryotome at 10 µm thickness. Tissue sections were re-hydrated with three washes of 1X PBS for 5 minutes each. Sections were treated with 0.2% Triton X-100 (Acros Organics #A0376210) in 1X PBS for 5 minutes followed by three washes of 1X PBS. Sections were blocked with 0.1% goat serum (Millipore Sigma #G0923) for 1 hour at room temperature followed by overnight anti-SQSTM1/p62 antibody incubation (Cell Signaling Technology #23214) at a 1:200 dilution in 0.1% goat serum at 4°C. Primary antibodies were washed 3 times with 1X PBS for 5 minutes each followed by incubation with goat anti-Rabbit Alexa Fluor 555 (Invitrogen #A27039)

at a 1:300 dilution in 0.1 % goat serum, WGA 488 (100 µg/mL, Biotium #29022), and DAPI (2 µg/mL) for 1 hour at room temperature in a humidified chamber. Slides were then mounted with ProLong Diamond (Invitrogen #P36970) and coverslipped. Whole section images were taken using a Nikon A1 confocal microscope. FIJI was used to quantify the total number of p62-positive fibers and minimum Feret diameter. Statistical significance was calculated by two-sided, unpaired Student's *t*-tests across three technical replicates per biological replicate.

Picrosirius Stain
Tissue sections were fixed in 4% PFA (Fisher Scientific #AAJ19943K2) for 15 minutes at room temperature. 4% PFA was washed two times with 1X PBS and 2 times with deionized water for five minutes each. Sections were air dried for 15 minutes at room temperature then incubated for 1 hour with Sirius Red dye (Fisher scientific #AAB2169306, Sigma #P6744-1GA) in a humidified chamber. Sirius Red dye was washed two times for 5 minutes each with acidified water (Fisher Scientific #BP2401-500) and deionized water at room temperature. Sections were then dehydrated by quick, sequential immersions in 50%, 70%, 70%, 90%, and 100% ethanol solutions, followed by two 5-minute incubations at room temperature. Coverslips were mounted with Permount (Fisher Scientific #SP15-100) and whole section images were taken using a motorized Olympus IX83 microscope.

Hematoxylin and Eosin stain
Slides were submerged in Hematoxylin (Fisher Scientific #3530-16) for two minutes followed by two sets of ten quick immersions in distilled water. Slides were then submerged in Scott's tap water and distilled water for one minute, followed by ten immersions in 80% ethanol. After a one-minute incubation in Eosin (Millipore Sigma #588X-75), the slides were immersed twice for ten repetitions in different 95% ethanol baths followed by a 100% ethanol bath. Finally, slides were submerged in SafeClear (Fisher Scientific #23-044192) for one minute, and two drops of Permount (Fisher Scientific #SP15-100) were added before placing the coverslip on top. Whole section images were taken using a motorized Olympus IX83 microscope.

Satellite Cell Isolation via Fluorescence-Activated Cell Sorting.
For tissue collection, mice were anesthetized with 3% isoflurane, then euthanized by cervical dislocation, bilateral pneumothorax, and removal of the heart. Hindlimb muscles (tibialis anterior, gastrocnemius, and quadriceps) of wild type and Sestrin double knockout mice were quickly harvested using sterile surgical tools and placed in separate plastic petri dishes containing cold PBS. Using surgical scissors, muscle tissues were minced and transferred into 50 mL conical tubes containing 20 mL of digest solution (2.5 U/mL Dispase II and 0.2% [~5,500 U/mL] Collagenase Type II in DMEM media per mouse). Samples were incubated on a rocker placed in a 37°C incubator for 90 min with manual pipetting the solution up and down to break up tissue every 30 minutes using an FBS coated 10 mL serological pipette. Once the digestion was completed, 20 mL of F10 media containing 20% heat inactivated FBS was added into each sample to inactivate enzyme activity. The solution was then filtered through a 70 µm cell strainer into a new 50 mL conical tube and centrifuged again at 350xg for 5 min. The pellets were re-suspended in 6 mL of staining media (2% heat inactivated FBS in Hank's Buffered Salt Solution - HBSS) and divided into separate FACS tubes. The FACS tubes were centrifuged at 350xg for 5 min and supernatants discarded. The cell pellets were then re-suspended in 200 µL of staining media and antibody cocktail containing Sca-1:APC (1:400), CD45:APC (1:400), CD11b:APC (1:400), Ter119:APC (1:400), CD29/B1-integrin:PE (1:200), and CD184/CXCR-4: BIOTIN (1:100) and incubated for 30 minutes on ice in the dark. Cells and antibodies were diluted in 3mL of staining solution, centrifuged at 350xg for 5 min, and supernatants discarded. Pellets were resuspended in 200uL staining solution containing PECy7:STREPTAVIDIN (1:100) and incubated on ice for 20 minutes in the dark. Again, samples were diluted in 3mL staining solution, centrifuged, supernatants discarded, and pellets re-suspended in 200uL staining buffer. Live cells were sorted from the suspension via addition of 1 µg of propidium iodide (PI) stain into each experimental sample and all samples were filtered through 35 µm cell strainers before the FACS. Cell sorting was done using a BD FACSAria III Cell Sorter (BD Biosciences, San Jose, CA) and APC negative, PE/PECy7 double-positive MuSCs were sorted into staining solution for immediate processing.

MuSC Culture and Immunofluorescence Staining:
Pax7, MyoD, P62, AMPKα1 Staining in MuSCs (Freshly Isolated and After 3 Days or Culture):

96-well plates were coated in either Cell-Tak (3.5 μg/cm^2, Fisher Scientific #C354240) for immediate attachment and fixation of cells or 0.5% gelatin (Sigma-Aldrich, #G2500) solution for 3 days of culture in myoblast growth medium (80% Ham's F10, 20% FBS, 1% Penicillin/Streptomycin solution, 20 ng/mL b-FGF) at 37.5°C and 5.0% CO_2. MuSCs were isolated as described above from two wild-type (WT) mice and two double-knockout (DKO) mice (all male, aged 3-4 months). A portion of the isolated MuSCs were resuspended in 1X PBS, seeded in Cell-Tak-coated wells at a density of 7,800 cells/cm^2, and briefly allowed to attach before fixation with methanol at -20°C for 10 minutes. The remainder of the MuSCs were resuspended in myoblast growth medium and seeded in gelatin-coated wells also at a density of 7,800 cells/cm^2. Media was replenished every 24 hours and cells were similarly fixed with methanol after 3 days of culture.

Cells were blocked and permeabilized with 0.3% Triton X-100 (Acros Organics #A0376210) and 1% BSA (Fisher Scientific #BP9703-100) in 1X PBS. Cells were then incubated overnight at 4°C with either a combination of AF488-conjugated anti-Pax7 antibody (1:100) and AF647-conjugated anti-MyoD antibody (1:50) in 0.2% BSA in 1X PBS, anti-AMPKα1 (1:1000) primary antibody in 0.2% BSA in 1X PBS, or anti-p62 (1:800) primary antibody in 1% BSA in 1X PBS. Wells stained for AMPKα1 or p62 were incubated with an AF647 goat anti-Rabbit secondary antibody for one hour at room temperature, protected from the light. All stains were performed in duplicate for each genotype and timepoint. Nuclei were stained with 1 μg/mL DAPI in 1X PBS for 10 minutes at room temperature. 20x magnification images were acquired on a Zeiss Axio Vert.A1 inverted microscope with a Colibri 7 LED light source and an AxioCam MRm camera. Images were subsequently analyzed in FIJI. ROIs were generated by thresholding on the DAPI image to identify nuclei. The average fluorescent intensities of each stain within these ROIs were recorded. The average Pax7, MyoD, AMPKα1, and p62 signals were compared between SKO and WT cells using a two-sample Student's *t*-test with the significance level set to $\alpha = 0.05$.

MitoTracker DeepRed, CellRox Green, Ki67 Staining in Freshly-Isolated MuSCs:
96-well plates were coated in 0.5% gelatin (Sigma-Aldrich, #G2500) solution prior to seeding MuSCs isolated via FACS (2 WT, 2 DKO, all female, aged 3-4 months). Cells were resuspended in myoblast growth media and seeded at a density of 7,800 cells/cm^2. Plates were incubated at 37°C for 3 hours to allow the cells to attach before replacing media with MitoTracker DeepRed (diluted to 500 nM in pre-warmed media) or CellRox Green Reagent (diluted 1:500 in pre-warmed media). Cells were incubated at 37°C with their respective live cell stains for 30 minutes before aspirating off all media and fixing in 4% PFA in 1X PBS for 10 minutes at room temperature. Cells were then permeabilized in 0.1% TritonX-100 in 1X PBS for 15 minutes at room temperature and blocked for 1 hour at room temperature in 1% BSA, 0.5% goat serum, 22.52 mg/ml glycine in PBST (0.1% Tween-20 in PBS). After 3 washes with 1X PBS, cells were then stained with PE-conjugated Ki67 antibody at a dilution of 1:50 in 1% BSA in PBST overnight at 4°C, protected from light. Afterwards, cells were washed 3 times with PBST and nuclei were stained with 1μg/mL DAPI in PBS for 10 minutes at room temperature. All stains were performed in duplicate across all samples. MitoTracker and Ki67 images were captured on a Zeiss Axio Vert.A1 inverted microscope with a Colibri 7 LED light source and an AxioCam MRm camera at a 40x magnification. CellRox images were captured on a Nikon A1si confocal microscope at a magnification of 10x. ROIs were generated by thresholding on the DAPI image to identify nuclei in FIJI, followed by measurements of average fluorescent intensity within each ROI. A two-sample t-test with the significance level set to $\alpha = 0.05$.

SKO and WT Transplantation
5 days prior to cell transplantation, the hindlimbs of young WT male mice (5 months old) were irradiated. Anesthesia was induced with 4-5% of isoflurane and maintained with 1-2% isoflurane at an oxygen flow rate of 1.5 L/min. Once anesthetized, mice were restrained in a small plastic tray placed within the irradiation chamber, with their dorsal side facing upwards, and their paws were taped down to prevent hindlimb movement during irradiation. To target irradiation to only the hindlimb muscles, a small lead plate with a 1 square-inch cut-out was positioned over the body of the mouse, with the opening lined up directly over one of the lower hindlimbs. The chamber was then closed, and an irradiation dose of 18 Gy was administered over the course of approximately 6 minutes. This was repeated for both hindlimbs. 1 day prior to cell transplantation, mice were similarly anesthetized, and TA muscles were injured with a 40 μL intramuscular injection of 1.2% $BaCl_2$ solution (w/v in sterile PBS) divided equally across 3-4 points between the proximal and distal ends of the TA. Uninjured control TAs in irradiated and non-irradiated mice received no injection

(n=2 each). On the day of transplantation, MuSCs were isolated via FACS as described above from young (2 months old) SKO and WT male mice (n=2 each). SKO and WT MuSCs were pooled between their 2 respective replicates, washed and resuspended with sterile PBS, and concentrated to 450 cells/μL. Mice were anesthetized as above, and 18,000 MuSCs were then delivered into the irradiated and injured TAs through a 40 μL intramuscular injection equally divided across 3-4 points between the proximal and distal ends of the TA (n=4 for SKO, n=4 for WT). A group of irradiated and injured TAs were given no cell transplants (n=2). Mice were allowed to recover for 14 days before harvesting the TAs, embedding in OCT and flash-freezing for cryo-sectioning. Tissue sections were stained with hematoxylin and eosin as described above to visualize muscle regeneration following the injury and transplantation. Tissue sections were also stained for the basal lamina and DAPI for quantification of centrally nucleated fibers. Sections were first air dried, then incubated with PBS for 5 minutes, and blocked with 10% normal goat serum in PBS for 1 hour. Sectioned were then incubated overnight at 4°C with a 1:200 dilution of rabbit anti-Laminin 1&2 polyclonal antibody (Abcam, ab7463) in 10% goat serum in PBS. The primary antibodies were washed off with 3x5 min washes with PBS, followed by secondary antibody incubation with a 1:500 dilution of goat anti-rabbit IgG (H+L) Alexa Fluor 647-conjugated antibody (Invitrogen #A-21245) and a 1:1000 dilution of DAPI in PBS for 1 hour. Afterwards, the sections were washed 3x5 min in PBS, and coverslips were mounted using ProLong Diamond anti-fade mounting medium. 20x images were acquired and stitched into a large-field image using a Nikon A1si confocal microscope. Centrally nucleated fibers were quantified in Fiji using the MuscleJ plugin and statistical testing and plotting was performed in R.

Preparation of RNA-Seq Libraries and Sequencing

MuSCs were FACS sorted directly into Trizol and snap frozen in liquid nitrogen. Samples were subsequently thawed, and RNA was extracted using a Qiagen miRNeasy Micro Kit as per manufacturer's instructions. The integrity of the isolated RNA was verified with a Bioanalyzer (Agilent 2100) and 1-10 ng of high-quality RNA (RIN>8) was used to produce cDNA libraries using the SmartSeq v4 protocol (Clontech) as per the manufacturer's instructions. cDNAs were prepared into sequencing libraries using 150 pg of full-length cDNA amplicons (Nextera XT DNA Library Preparation Kit, Illumina) with dual index barcodes. Barcoded cDNA libraries were pooled into a single tube and sequenced on a NextSeq (Illumina) using 76-bp single-ended reads.

RNA-Seq Data Processing and Analysis

Gene Expression Estimation

Single-end RNA-Seq data were trimmed using Flexbar (v3.5.0) and pseudo-aligned to the mouse reference genome (GRCm38.p6) using Kallisto (v.0.46.1). Reads averaged 41.75M per sample. The full Kallisto command was as follows:

kallisto quant -b 100 --single -l 300 -s 30 -i **[mm10.idx]** -t 45 -o **[output folder] [trimmed FASTQ]**

Differential Gene Expression

The estimated transcript abundances were summarized to gene-level count matrices using tximport and genes containing at least one read were retained. Differentially expressed genes in treated (KO) samples relative to untreated controls (WT) at each timepoint (days=0,7,21) were identified using DESeq2 in R with a design formula: *Count ~ group*, with group={day+treatment}, day={0,7,21}, and treatment={WT,KO}. Surrogate variable analysis was performed on the rlog-transformed count matrix using the SVA package with a null model of *rlog(Count) ~ 1* and a design matrix of *rlog(Counts) ~ group*. Contributions from the surrogate variable were quantified and removed from the rlog-transformed count matrix for downstream analyses. Pairwise Spearman correlation analysis was performed between all replicates and replicates that had $r < 0.9$ with other replicates were excluded from further analysis. Pairwise contrasts were examined to find differentially expressed genes between SKO vs WT on each day post injury. Differentially expressed genes were selected using a false discovery rate (FDR) cutoff of 0.01 and a Log_2 fold change cutoff of 1.

Time-series Clustering of Differential Genes

Genes that were differentially expressed on at least one day post injury were pooled and submitted for time-clustering using the Dirichlet Process Gaussian Process (DPGP) algorithm. To prepare inputs for the algorithm, regularized log-transformed counts were averaged across biological replicates on each day post

injury and the fold change in averaged counts was calculated between SKO vs WT. The resulting fold changes for each gene were standardized across time points using a z-score transformation. DPGP clustering was performed using the default parameters. The full command was as follows:

DP_GP_cluster.py -i **[fold change z-scores]** -o ./**[output file prefix]**

DPGP assigned each gene a unique time-dependent cluster based on similar expression dynamics, and clusters that exhibited similar temporal dynamics were manually combined into a single cluster. Log-fold z-scores were plotted as a function of time for each cluster and a heatmap of differentially expressed genes grouped by DPGP cluster was plotted using the ComplexHeatmap package in R.

Pathway Enrichment Analysis

The top 100 upregulated and downregulated genes for 0 days post injury were submitted for Gene Ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway over-representation analysis using the WebGestaltR package in R. A GO Biological Process (GO:BP) reference curated to remove redundant terms was used. Only KEGG and GO:BP terms containing between 10 and 500 genes were considered for enrichment. Genes in each DPGP cluster analyzed using the same procedure. Redundancy in the enriched terms (FDR < 0.05) was reduced using the Affinity Propagation or Weighted Set Cover algorithms in WebGestaltR. Percent coverage of all terms produced by the Weighted Set Cover algorithm was at least 95%. The resulting terms were plotted using the ggplot2 package and ComplexHeatmap packages in R.

Derivation of reaction fluxes using gene expression data and genome-scale metabolic modeling

Gene expression data from SKO and WT MuSCs were normalized (z-scores) across each gene. Normalized data were compared using an unpaired two-sided t-test in MATLAB R2018b to identify differentially expressed genes ($p<0.05$, $|z|>1.5$) between SKO and WT MuSCs before injury. Differentially expressed genes for SKO and WT MuSCs were used as input to derive reaction flux information from a human genome-scale metabolic model (RECON1)[5,6] that maximizes and minimizes flux through metabolic reactions associated with upregulated and downregulated genes, respectively, based on internal gene-protein-reaction annotations. Reaction flux data were generated using a linear optimization version of the iMAT algorithm[5,6] with the RECON1 model and the following parameters: (rho = 1E-3, kappa = 1E-3, epsilon = 1, mode = 0). Reaction fluxes in SKO MuSCs were then normalized against those in WT MuSCs and normalized (z-scores) using R. Normalized flux differences were overlaid onto maps of metabolic pathways using Escher[7] for visualization. Reaction flux differences with $|z|>3$ were considered significant.

SUPPLEMENTAL REFERENCES

1. Li R, Huang Y, Semple I, Kim M, Zhang Z, Lee JH. Cardioprotective roles of sestrin 1 and sestrin 2 against doxorubicin cardiotoxicity. Am J Physiol-Heart Circ Physiol. 2019 Apr 26;317(1):H39–48.

2. Kim M, Sujkowski A, Namkoong S, Gu B, Cobb T, Kim B, et al. Sestrins are evolutionarily conserved mediators of exercise benefits. Nat Commun. 2020 Jan 13;11(1):190.

3. Lee JH, Budanov AV, Talukdar S, Park EJ, Park HL, Park H-W, et al. Maintenance of Metabolic Homeostasis by Sestrin2 and Sestrin3. Cell Metab. 2012 Sep 5;16(3):311–21.

4. Shcherbina A, Larouche J, Fraczek P, Yang BA, Brown LA, Markworth JF, et al. Dissecting Murine Muscle Stem Cell Aging through Regeneration Using Integrative Genomic Analysis. Cell Rep. 2020 Jul;32(4):107964.

5. Duarte NC, Becker SA, Jamshidi N, Thiele I, Mo ML, Vo TD, et al. Global reconstruction of the human metabolic network based on genomic and bibliomic data. Proc Natl Acad Sci. 2007 Feb 6;104(6):1777–82.

6. Shlomi T, Cabili MN, Herrgård MJ, Palsson BØ, Ruppin E. Network-based prediction of human tissue-specific metabolism. Nat Biotechnol. 2008 Sep;26(9):1003–10.

7. King ZA, Dräger A, Ebrahim A, Sonnenschein N, Lewis NE, Palsson BO. Escher: A Web Application for Building, Sharing, and Embedding Data-Rich Visualizations of Biological Pathways. PLOS Comput Biol. 2015 Aug 27;11(8):e1004321.