P38 MAPK signaling underlies a cell autonomous loss of stem cell self-renewal in aged skeletal muscle

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SUPPLEMENTARY INFORMATION:

1. SUPPLEMENTARY FIGURES

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-Figure S4, related to Figure 3
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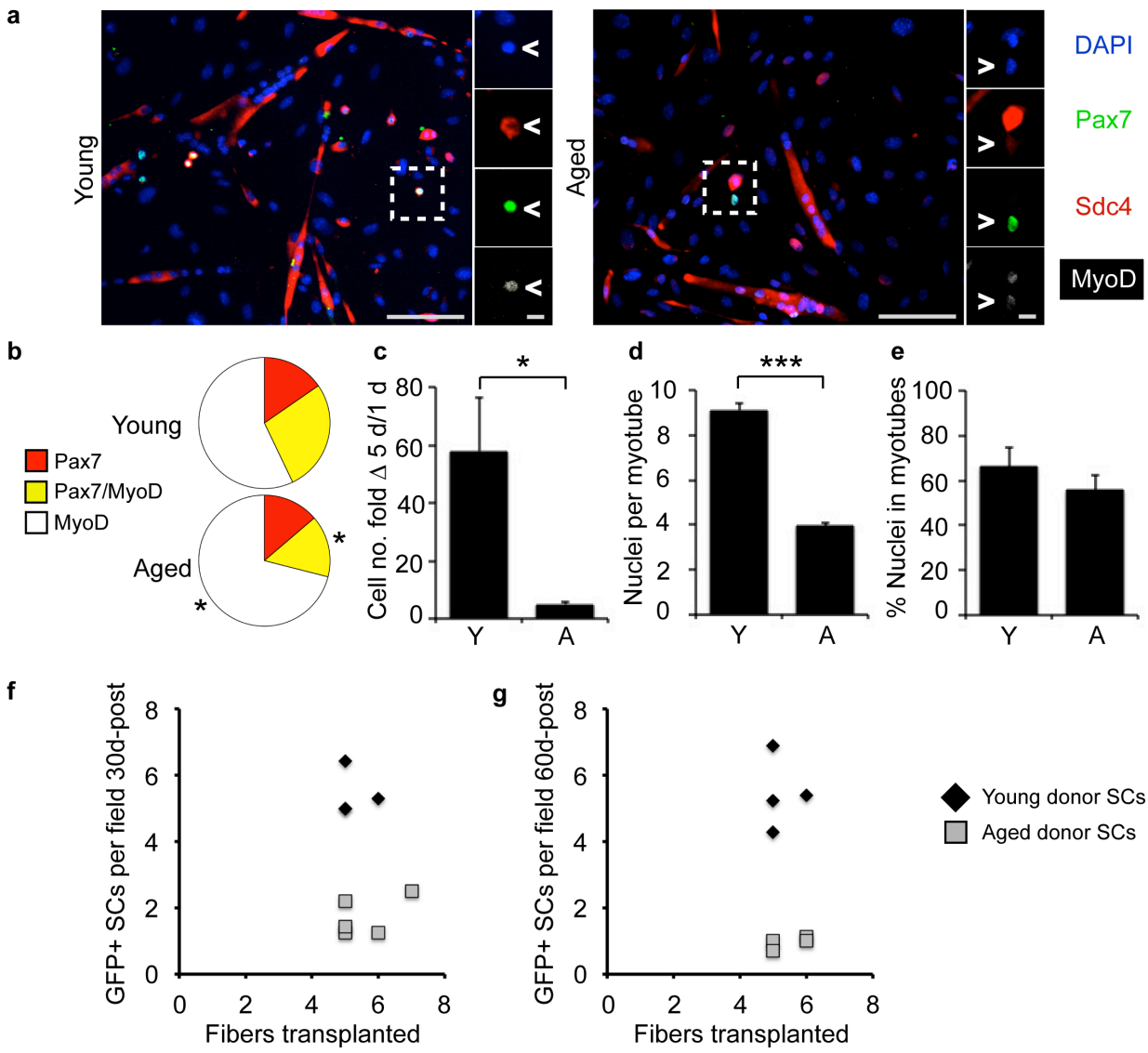
2. SUPPLEMENTARY METHODS

3. SUPPLEMENTARY TABLE

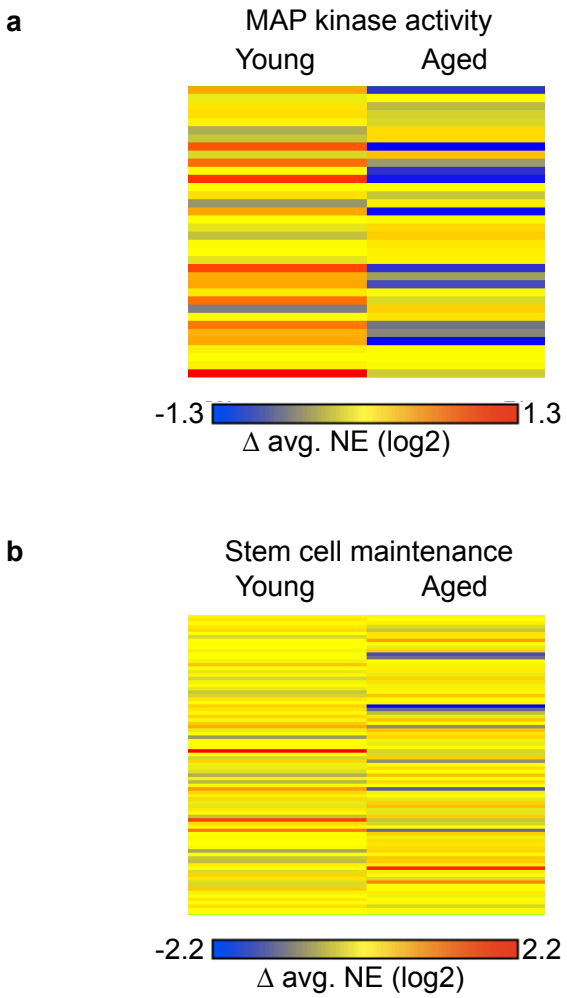
-Table S1, related to Figures 1,3

4. SUPPLEMENTARY VIDEO

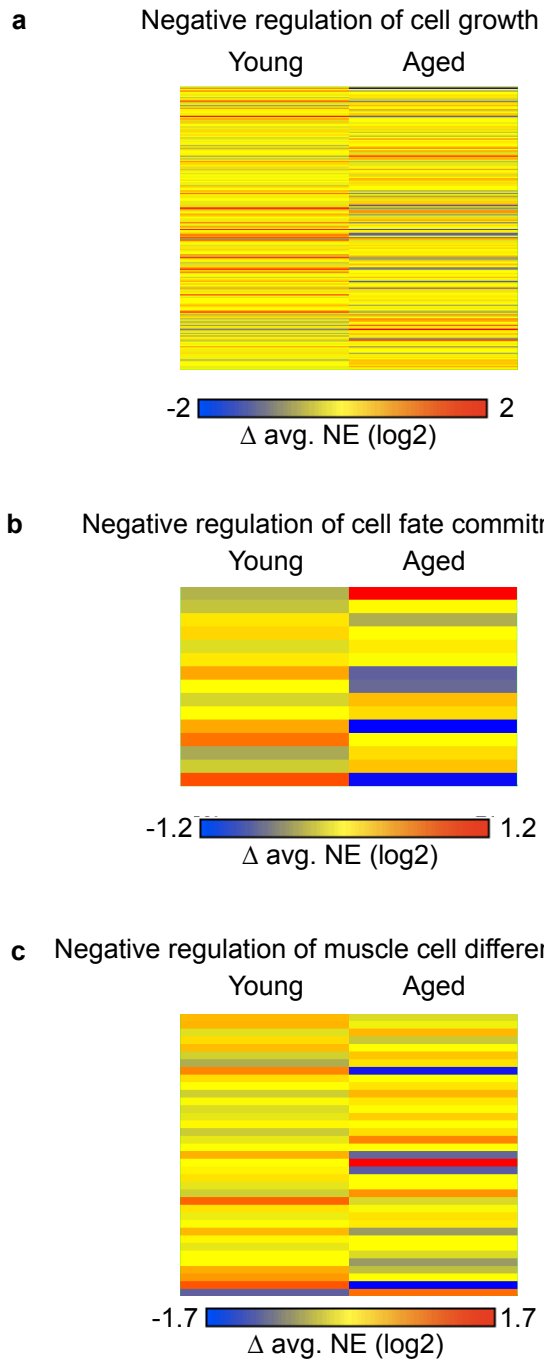
-Video S1, related to Figure 4



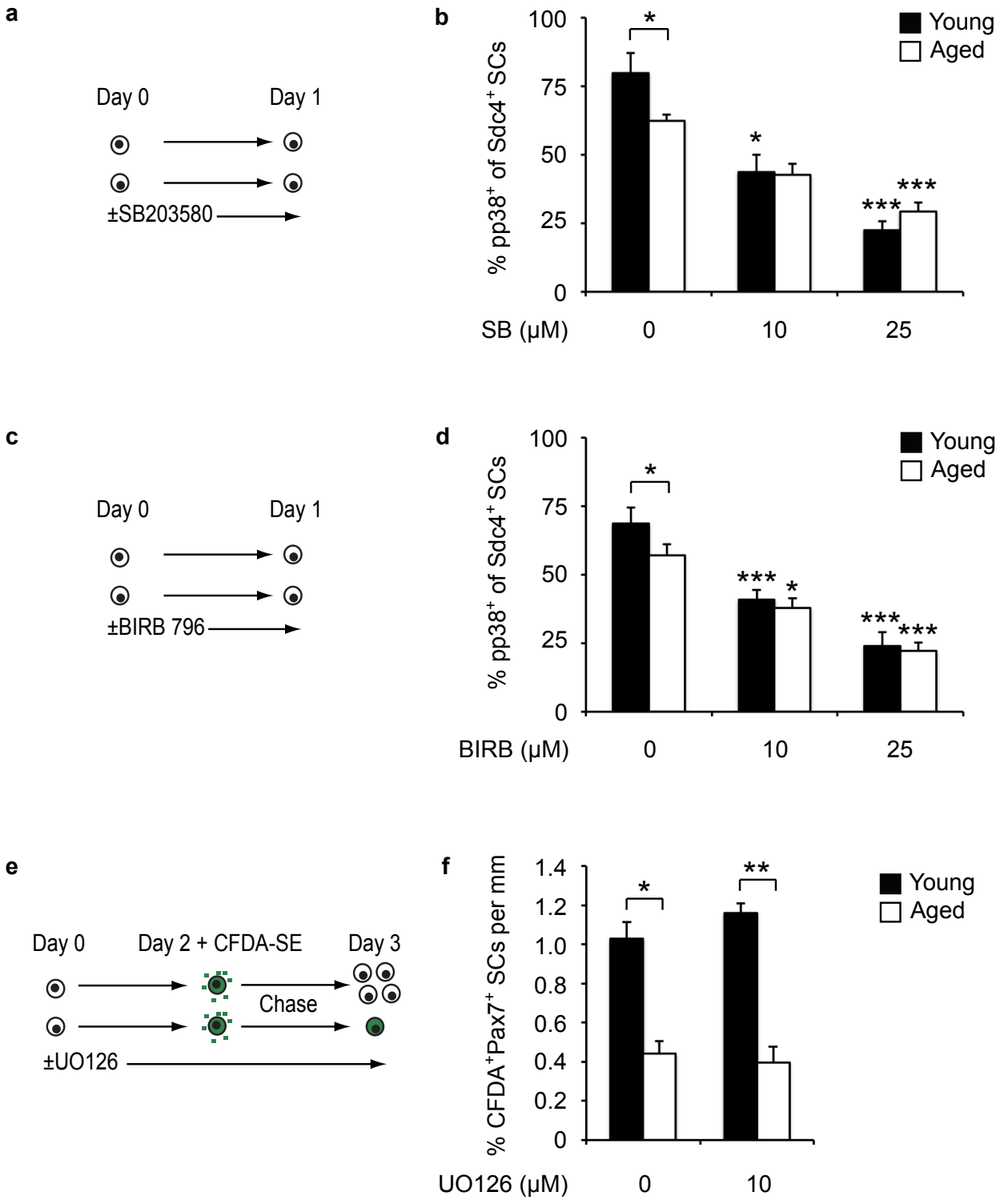
SUPPLEMENTARY FIGURE 1 (related to Fig. 1). Aged satellite cells (SCs) exhibit reduced proliferation, decreased Pax7 expression, and reduced transplantation efficiency. (**a**) Representative images of young and aged explanted Syndecan-4$^+$ (Sdc4$^+$) SCs (^) cultured five days (Sdc4$^-$ mononuclear cells are contaminating fibroblasts). Insets (boxes) show Pax7$^+$/MyoD$^+$ SCs (^). Scale bars, 50 µm (10 µm). (**b**) Pie charts depict percentages of Pax7$^+$ and MyoD$^+$ SCs in 5 d cultures. (**c**) Average fold increase in cell numbers (no.) after five days in culture. (**d**) Average nuclei per myotube after five days in culture. (**e**) Quantification of cellular differentiation as percentage of nuclei in myotubes. Mean ± s.e.m. $n = 3$ independent experiments. *$P < 0.05$ ***$P < 0.001$ by one-way ANOVA with Tukey's test. (**f,g**) (related to **Fig. 1g–h**) Plot of number of young or aged myofibers transplanted to wildtype hosts versus the number of GFP$^+$ SCs per field present (**f**) 30 d or (**g**) 60 d post-transplantation ($n = 3$–5 recipient mice).



SUPPLEMENTARY FIGURE 2 (related to Fig. 3). Transcripts related to self-renewal and MAPK signaling are decreased in aged SCs. Heat maps of transcripts from young and aged microarrays included in the GO Terms: (**a**) GO: 0004707 MAP kinase activity, and (**b**) GO: 0019827 Stem cell maintenance. Red indicates increased expression and blue indicates decreased expression.



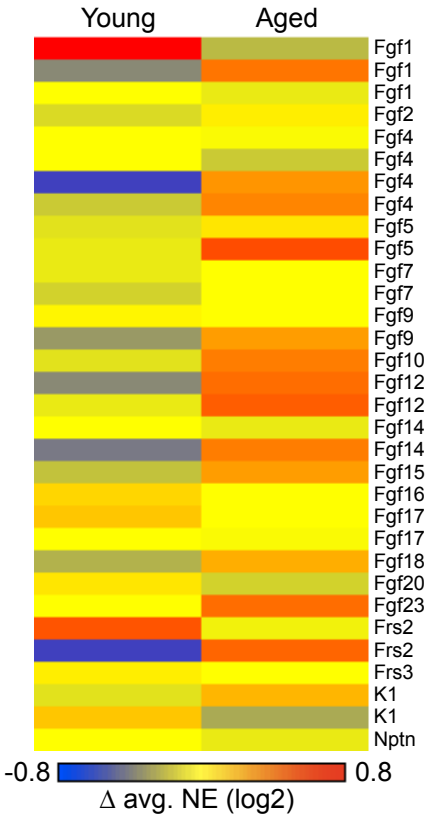
SUPPLEMENTARY FIGURE 3 (related to Fig. 3). Transcripts related to inhibition of differentiation are decreased in aged SCs. Heat maps of transcripts from young and aged microarrays included in the GO Terms: (**a**) GO:0030308 Negative regulation of cell growth, (**b**) GO:0010454 Negative regulation of cell fate commitment, and (**c**) GO: 0051148 Negative Regulation of muscle cell differentiation. Red indicates increased expression and blue indicates decreased expression.



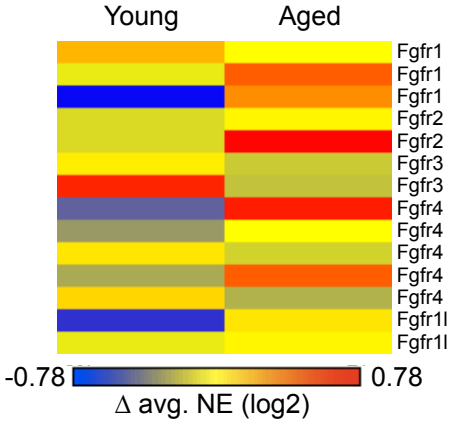
SUPPLEMENTARY FIGURE 4 (related to Figure 3). SB203580 and BIRB 796 inhibit p38αβ MAPK phosphorylation (pp38) while MEK inhibition does not affect self-renewal. (**a**) Schematic for 24 h SB203580 (SB) treatment of young and aged myofibers. (**b**) Quantification of pp38$^+$ SCs as percentage of total Sdc4$^+$ SCs after 24 h SB treatment (*P <0.05 for Young vs. Aged at 0 μM SB and for Young 0 μM SB vs. Young 10 μM SB; ***P <0.001 for

Young and Aged 0 μM SB vs. 25 μM SB by one-way ANOVA. Mean ± s.e.m., $n = 3$ experiments, ≥ 20 myofibers scored per condition). (**c**) Schematic for 24 h BIRB 796 (BIRB) treatment of young and aged myofibers. (**d**) Quantification of $pp38^+$ SCs as percentage of total $Sdc4^+$ SCs after 24 h BIRB treatment (*$P < 0.05$ for Young 0 μM vs. Aged 0 μM, Aged 0 μM vs. Aged 10 μM; ***$P < 0.0001$ for Young 0 μM vs. Young 10 μM, Young and Aged 0 μM vs. 25 μM by one-way ANOVA. Mean ± s.e.m., $n = 3$ experiments, ≥ 30 myofibers scored per condition). (**e**) Schematic for CFDA-SE retention assay concurrent with treatment of young and aged myofibers with UO126 inhibitor or DMSO control. (**f**) Quantification of self-renewal measured by average number of $CFDA^+/Pax7^+$ SCs per myofiber length. (*$P < 0.05$ for Young vs. Aged DMSO **$P < 0.01$ for Young vs. Aged at 10 μM UO126 by two-way ANOVA. Mean ± s.e.m., $n = 3$ experiments, ≥ 30 myofibers scored per condition).

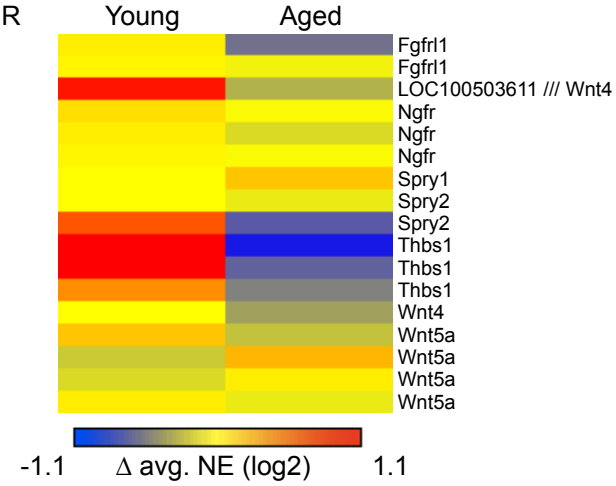
a FGFs



b FGF Receptors



c Negative regulation of FGFR signaling pathway



SUPPLEMENTARY FIGURE 5 (related to Fig.4). Transcripts related to FGF signaling are increased in aged SCs. Heat maps of transcripts from young and aged microarrays that are (**a**) FGF ligands, (**b**) FGF Receptors or (**c**) included in the GO Term 0040037 Negative regulation of FGFR signaling pathway Red indicates increased expression and blue indicates decreased expression.

SUPPLEMENTARY METHODS

Cell Culture

Satellite cells (SCs) were isolated by enzymatic digestion of whole hindlimb skeletal muscle and preplated on uncoated plates overnight at 6% O_2. Cells were then cultured on gelatin-coated 6-well plates (~10,000 cells/well) in growth medium (F12 with 0.8 mM Calcium + 15% horse serum including 1.5 nM FGF-2) at 6% O_2 for 1 to 5 d.

To isolate live myofibers for immediate use, whole hindlimb skeletal muscle was dissected, taking care to remove fat and connective tissue. Muscles were digested in F12-C and collagenase type I (Worthington LS004197) for 1.5 h. Collagenase activity was quenched by transfer of myofibers growth medium. Live myofibers were collected with a fire-polished glass pipette. Myofibers were transferred through four plates of growth medium to remove debris. Myofibers were then cultured in growth medium at 6% O_2 for 24 h or 72 h with daily medium changes. Myofibers were cultured from time of isolation in increasing concentrations of SB203580, BIRB 796, UO126, or DMSO (control) as noted. For CFDA-SE retention assays, 48 h post-isolation myofibers were treated with 10 μM CFDA-SE, then washed with growth medium and returned to growth medium for an additional 24 h.

Microscopy and Image Processing

Images of explanted cells were captured using a Nikon Eclipse E800 equipped with a Sensicam (Cooke) digital camera and Slidebook v4.1 (3i) software with a PlanFluor 20x/NA 0.50 PH1 DLL (Nikon) lens. Images of myofibers were captured with a Leica DM RXA Spinning Disk confocal microscope with EM-CCD digital camera (Hamamatsu) with Metamorph software (Molecular Devices), using HC Plan APO 20x/0.70 or HCX PL APO 40x/

0.85 CORR lenses.

Microarrays and Analysis

Microarrays were prepared and processed as described in the Online Methods. Raw data were pre-processed using Genespring software (Agilent) and analyzed for differential gene expression changes occurring over time. For heat maps, individual probeset expression (log2) was normalized to the mean relative expression (log2) of all transcript probesets to one gene across both ages. In the Affymetrix 430 v.2 mouse microarray, there may be multiple probesets corresponding to different transcripts from a single gene; this is indicated by repeated transcript names in the figures and tables. Pathway analyses were conducted using Ingenuity Pathway Analysis (IPA) software on lists of transcripts changing either 4-fold or 6-fold between young and aged datasets.

SUPPLEMENTARY TABLE 1 (related to Fig. 1,3). IPA pathway analysis of transcripts changing ≥4-fold or 6-fold between young and aged SCs.

Top Networks for transcripts changing ≥4-fold Young/Aged
1-DNA Replication, Recombination, and Repair, Cellular Response to Therapeutics, Cancer
2-Organ Morphology, Skeletal and Muscular System Development and Function, Carbohydrate Metabolism
3- Embryonic Development, Tissue Development, Organismal Survival
4-Organ Morphology, Skeletal and Muscular System Development and Function, Cellular Assembly and Organization
5-Gene Expression, Tissue Development, Cellular Development

Top Canonical Pathways	p-value
Mitochondrial Dysfunction	2.38E-06
Glycolysis I	1.12E-05
Gluconeogenesis I	1.50E-05
Calcium Signaling	3.60E-04
ILK Signaling	5.88E-04

Top Bio Functions-Molecular and Cellular Functions	p-value
Cell Cycle	8.27E-06 - 3.86E-02
Post-Translational Modification	2.48E-04 - 4.71E-02
Cell Death and Survival	6.52E-04 - 4.41E-02
Molecular Transport	7.30E-04 - 4.41E-02
Cell Morphology	8.67E-04 - 4.38E-02

SUPPLEMENTARY TABLE 1 (Cont.; related to Figs. 1,3).

Top Networks for transcripts changing ≥6-fold Young/Aged
1-Cancer, Gastrointestinal Disease, Hepatic System Disease
2-Cardiovascular Disease, Cardiovascular System Development and Function, Organ Morphology
3-Carbohydrate Metabolism, Skeletal and Muscular System Development and Function, Organ Morphology
4-Inflammatory Response, Humoral Immune Response, Protein Synthesis
5-Connective Tissue Disorders, Hematological Disease, Infectious Disease

Top Canonical Pathways	p-value
Calcium Signaling	6.37E-06
Glycolysis I	3.51E-05
Gluconeogenesis I	4.70E-04
Glycerol-3-phosphate Shuttle	2.87E-03
NRF2-mediated Oxidative Stress Response	3.38E-03

Top Bio Functions-Molecular and Cellular Functions	p-value
Carbohydrate Metabolism	4.70E-04 - 4.15E-02
Cell Cycle	5.89E-04 - 3.73E-02
Cellular Assembly and Organization	5.89E-04 - 4.31E-02
Cell Death and Survival	1.41E-03 - 4.31E-02
Molecular Transport	1.41E-03 - 4.31E-02