

Expanded View Figures

Figure EV1. Functional validation Myf6-CTAP construct used for ChIP-seq analysis.

- A Relative expression of *Pax7*, *Myf5*, *MyoD*, *Myog*, and *Myf6* in mouse satellite cells (SC), primary myoblasts (MB), and single myofibers (SF) normalized to RPS2 as assayed by quantitative real-time PCR (RT-qPCR). $n = 3-4$ biological replicates per group, two-tailed t -test, error bars = $\pm$ SD.
- B Western blot analysis of C2C12 myoblasts infected with a retrovirus containing Myf6-CTAP.
- C Immunofluorescent staining of primary myoblasts expressing Myf6-HF for PAX7. Myf6-HF cell lines were generated from Myf6-null myoblasts infected with a retrovirus expressing Myf6-HF (scale bar = 100 μ m).
- D, E Immunofluorescent staining of primary myotubes expressing Myf6-HF for FLAG (D) and MF20 (E). Myf6-HF myotubes were generated from Myf6-null myoblasts infected with a retrovirus expressing Myf6-HF, followed by 5 days of differentiation. (scale bar = 20 μ m).
- F Dual luciferase assays showing the transcriptional activity of MYF6-CTAP on a synthetic regulatory sequence containing a trimeric canonical E-box motif (CAGCTG) spread over a 72 bp stretch of a synthetic DNA. The synthetic sequence was subcloned into the pGL4.23[luc2/minP] luciferase vector (Promega). Dual luciferase assays with Renilla/luciferase constructs were performed in Cos7 cells, and a vector containing the full-length mouse MyoD (Soleimani *et al*, 2012) was used as a positive control. Relative transcriptional activity of the MyoD and the Myf6 constructs were measured with and without co-transfection of Cos7 cells with the transcription factor E2-alpha (E47), a heterodimer of the myogenic regulatory factors (MRFs). ($n = 6$ technical replicates), bars represent mean $\pm$ SD, two-tailed t -test, asterisks (*, **, ***, ****) correspond to P -values of < 0.05 , < 0.01 , < 0.001 , and < 0.0001 , respectively.

Source data are available online for this figure.

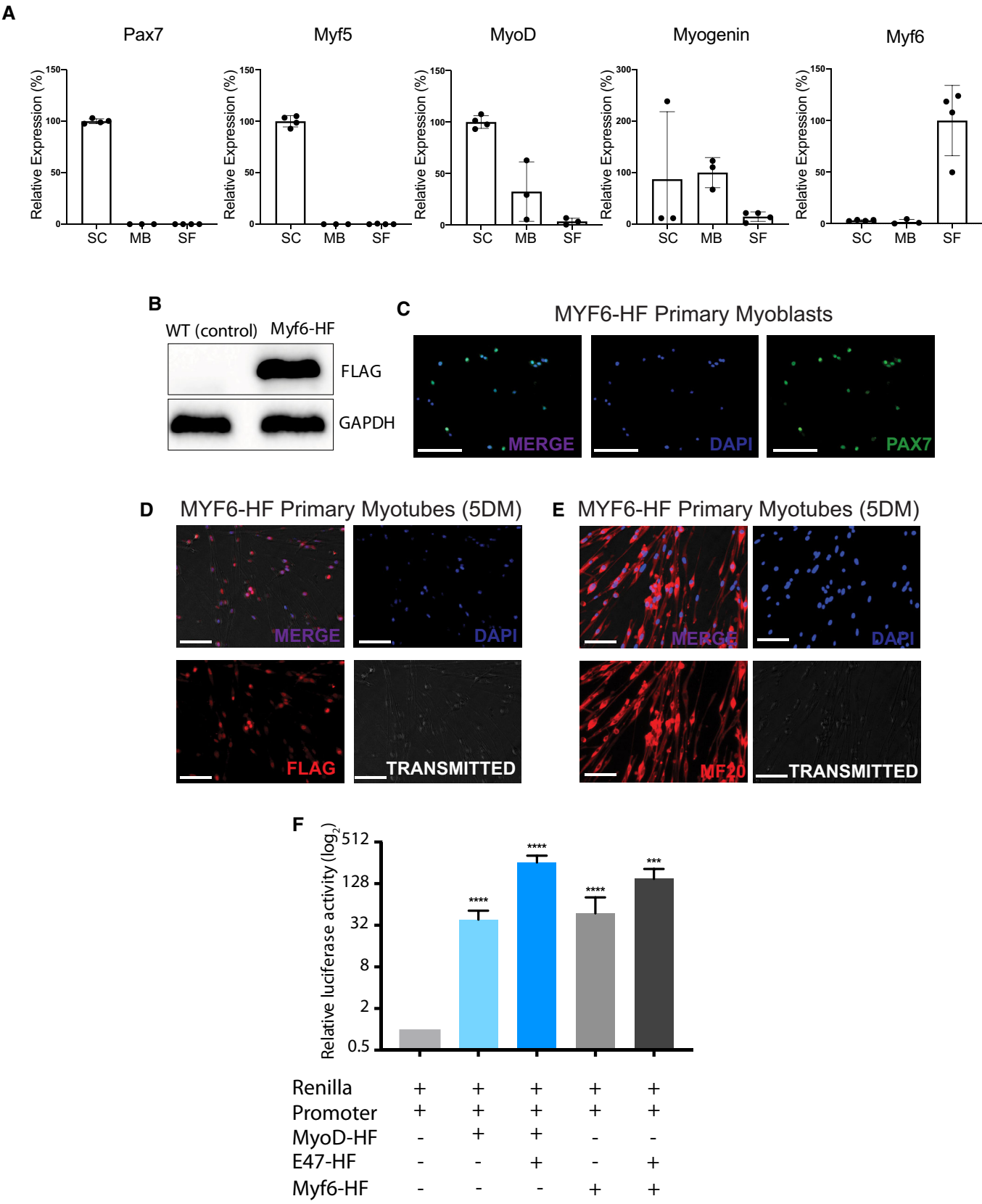


Figure EV1.

Figure EV2. Myf6 and MyoD share a vast core myogenic genetic network.

- A Enrichment of Myf6 ChIP-Seq signal on the myosin heavy chain gene cluster and location of Myf6 peaks.
- B Venn diagram showing genome-wide binding sites for MyoD and Myf6 and their overlap in differentiated muscle cells. The overlap is defined as any peaks that have any overlap between the two datasets.
- C Gene Ontology (GO analysis) using Genomic Regions Enrichment of Annotations Tool (GREAT) (McLean *et al*, 2010) of genes associated with MyoD ChIP-Seq peaks in primary myotubes (Soleimani *et al*, 2012).
- D Similar analysis as in (C) for genes associated with MyoD/Myf6 common peaks. The association rule is based on single nearest gene within 300 kb of the peaks. Within that window the number of peaks associated with more than one gene is minimal for ChIP-Seq datasets.
- E, F Snapshots of UCSC genome browser showing Myf6 (E) compared to MyoD (F) occupancy in the regulatory regions of *Egf* and *Vegfa* (GSE80588, Soleimani *et al*, 2018).
- G ChIP-qPCR for H3K27Ac compared with IgG control at the TSS of *EGF*, *VEGFA*, and a negative control region of Chromosome 17 qE5. ($n = 3$ technical replicates), two-tailed t -test, error bars = $\pm$ SD.

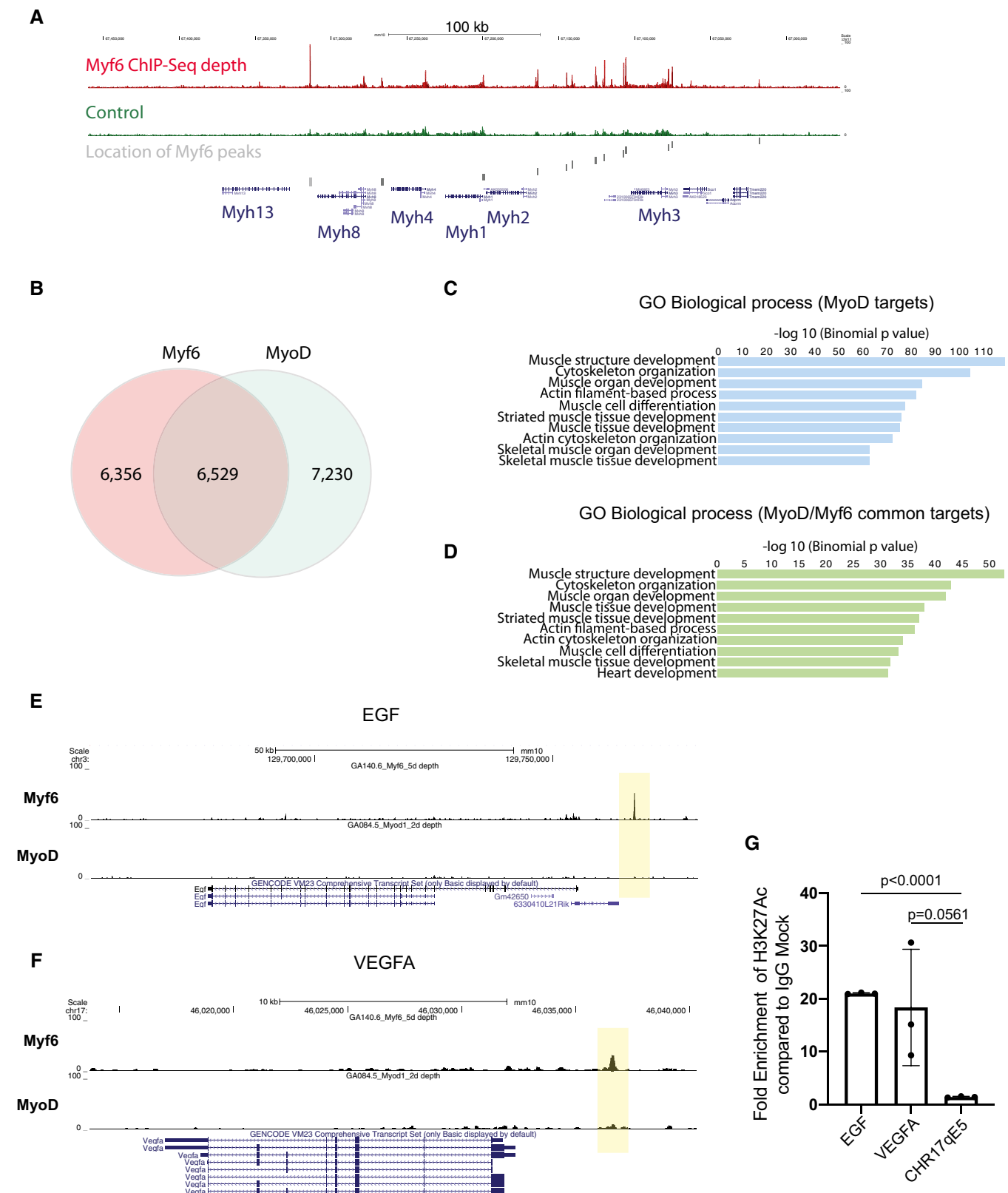


Figure EV2.



Figure EV3. Myf6 Regulates the Expression of Various Cytokine Genes.

- A Colormap of Myf5, MyoD, and Myf6 peaks within 100 kb of the Transcription Start Sites (TSS) of cytokines ranging from zero (black) to six peaks (red) occupancy. Black indicates no binding (i.e., zero peaks), red indicates up to six ChIP-Seq peaks. The onset of differentiation coincides with increased binding of MRFs to the regulatory domains of the cytokine genes.
- B Gene expression analysis of cytokines during a 5 day time course of myogenic differentiation going from cycling myoblasts in growth media (Ham's F10 supplemented with 20% Fetal Bovine Serum, 1% penicillin/streptomycin, 2.5 ng/ml basic Fibroblast Growth Factor) to terminally differentiated myocytes (2 days in differentiation media, DMEM supplemented with 5% horse serum) to the postmitotic multinucleated myotubes (5 days in differentiation media). Gene expression was assayed in biological triplicate by microarray (Soleimani et al, 2012, 2018). Expression of each gene was averaged across three replicates and normalized to mean zero and standard deviation of one. Expression values were then truncated at ± 3 for color display. Blue indicates lower than average expression, while red indicates higher than average expression.
- C Distribution of Myf6 peaks within 100 kb of the TSS of the cytokines genes, similar to the analysis shown in (A).
- D Colormap of genes that are up/downregulated in the whole muscle transcriptome (RNA-Seq) of hindlimb muscles from Myf6-KO and WT counterparts. Differential expression is Log_2 transformed, and truncated at ± 3 . Color scale goes from blue (-3 = lower than mean), to white (0 = mean) and finally to red ($+3$ = higher than mean).
- E Heat map showing the distribution of Myf6 peaks within 100 kb of the TSS of the myokine genes ranging from zero (black) to six ChIP-Seq peaks (red) occupancy.
- F Quantitative Real-Time PCR (RT-qPCR) analysis of a select set of myokine and myokine receptor genes involved in the activation of STAT3 and EGFR signaling pathways. RT-qPCR analysis was performed on the total RNA isolated from primary myoblasts and multinucleated primary myotubes derived from MuSCs isolated by fluorescence-activated cell sorting (FACS, see the Extended Materials and Methods). Myotubes were obtained by feeding > 90% confluent primary myoblasts with differentiation media (DMEM supplemented with 5% horse serum) for 5 days. ($n = 3$ technical replicates), bars = mean $\pm$ SD, two-tailed t-test, asterisks (*, **, ***, ****) correspond to P -values of < 0.05 , < 0.01 , < 0.001 , and < 0.0001 , respectively.
- G, H RT-qPCR analysis of primary myotubes for that have been depleted of Myf6 expression by siRNAs compared to controls, for vascular endothelial growth factor A (VEGFA) (G) and leukemia inhibitory factor (LIF) ($n = 3$ biological replicates), Two-tailed t-test, bars represent mean $\pm$ SD (H).
- I, J Immunofluorescence staining of WT (I) and Myf6-KO (J) EDL myofibers stained with PAX7, STAT3 and phospho-STAT3 antibodies. Live fibers were maintained in culture media for 44 h, followed by 4 h of serum starving in non-supplemented DMEM. For treated samples, 1,000 U/ml of recombinant LIF was added to the non-supplemented DMEM for 10 min before fixation. Activation status of STAT3 signaling pathways was assessed using antibodies against phospho-STAT3 (pSTAT3-Y705). Scale bar = 30 μm .
- K Quantification of the percentage of all pSTAT3⁺/PAX7⁺ (defined as PAX7⁺ cells showing low or high pSTAT3 signal) cells on WT ($n = 18$ fibers from $n = 3$ mice), Myf6-KO ($n = 18$ fibers from $n = 3$ mice), and Myf6-KO EDL myofibers treated with LIF ($n = 11$ fibers from $n = 3$ mice). Two-tailed t-test, bars represent mean $\pm$ SD.
- L Quantification of the percentage of pSTAT3^{high}/PAX7⁺ cells (defined as PAX7⁺ cells showing high pSTAT3 signal) on WT ($n = 29$ fibers from $n = 3$ mice), Myf6-KO ($n = 22$ fibers from $n = 3$ mice), and Myf6-KO EDL myofibers treated with LIF ($n = 14$ fibers from $n = 3$ mice). Two-tailed t-test, bars represent mean $\pm$ SD.
- M Activation of STAT3 signaling in MuSCs on EDL myofibers treated with LIF compared with untreated control. Myofibers were cultured for 48 h and were treated with 1,000 U/ml of recombinant LIF for 10 min before fixation and staining with antibodies for PAX7 and p-STAT3. Scale bar = 30 μm .
- N Activation of STAT3 signaling in myoblasts treated with recombinant LIF. Myoblasts were treated with 2 ng/ml of recombinant protein in growth media (Ham's F10 supplemented with 20% FBS, 2.5 ng/ml bFGF) for 15 min and lysed in RIPA lysis buffer. Western blots were performed with antibodies against STAT3 and phospho-STAT3 (pSTAT3-Y705).

Figure EV4. Myf6 is dispensable for muscle development and regeneration in adult mice.

- A Transcript expression (RPM) for myogenic factors Myf5, MyoD, and Myogenin from whole muscle RNA-Sequencing of Myf6-null compared with WT mice. ($n = 3$ biological replicates per group) Two-tailed t-test, bars represent mean $\pm$ SD.
- B, C Immunofluorescent analysis of TA muscles from WT and Myf6-KO counterparts stained with Laminin (LAMA1) and counterstained with DAPI. Scale bar = 100 μm .
- D Quantification of the number of regenerating fibers (depicted by the presence of centrally located myonuclei) between the WT and the Myf6-KO mice. Boxes represent 1st and 3rd quartile, central line represents the median, and whiskers represent the range. Two-tailed t-test.
- E Quantification of the number of myofibers per unit area (mm^2) of the TA muscles between WT and Myf6-KO counterparts. ($n = 4$ mice per group). Boxes represent 1st and 3rd quartile, central line represents the median, and whiskers represent the range. Two-tailed t-test.
- F Schematic timeline of cardiotoxin injury experiments.
- G, H Immunofluorescent analysis of TA muscle from WT and Myf6-KO animals 3 weeks after CTX injury (see extended Materials and Methods) stained with Laminin and counterstained with DAPI. Scale bar = 100 μm .
- I Quantification of the number of regenerating myofibers (centrally located myonuclei) between WT and Myf6-KO counterparts. Boxes represent 1st and 3rd quartile, central line represents the median, and whiskers represent the range. Two-tailed t-test.
- J Quantification of the number of myofibers per unit area (mm^2) between WT and Myf6-KO counterparts. ($n = 4$ mice per group) Boxes represent 1st and 3rd quartile, central line represents the median, and whiskers represent the range. Two-tailed t-test.

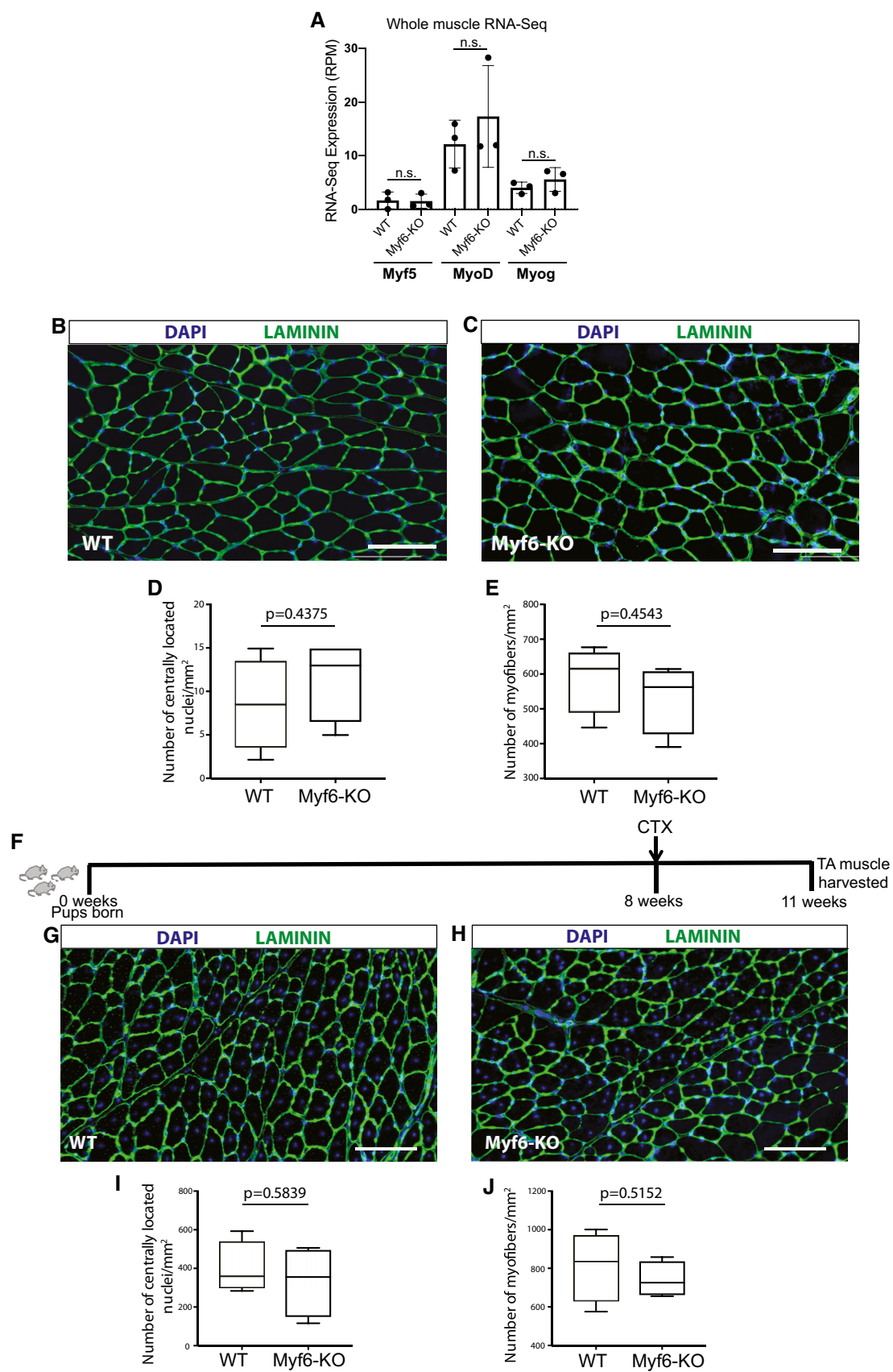


Figure EV4.

Figure EV5. Myf6-knockout mice show a reduced MuSC pool.

- A Representative FACS plots for the isolation of MuSCs from Myf6-KO and WT mice at P21. The yellow population corresponds to the ITGA7⁺/CD31⁻/SCA1⁻/CD45⁻/CD11b⁻ population of MuSCs.
- B Representative FACS plots for the isolation of MuSCs from Myf6-KO and WT mice at 3 months. The yellow population corresponds to the ITGA7⁺/CD31⁻/SCA1⁻/CD45⁻/CD11b⁻ population of MuSCs.
- C Quantification of the percentage of total FACS sorting events corresponding to the MuSC population (number of ITGA7⁺/ITGA7⁺/CD31⁻/SCA1⁻/CD45⁻/CD11b⁻ events over the total number of FACS events) at P21 ($n = 3$ mice per group). Two-tailed t -test, error bars = $\pm$ SD.
- D Quantification of the percentage of total FACS sorting events corresponding to the MuSC population (number of ITGA7⁺/ITGA7⁺/CD31⁻/SCA1⁻/CD45⁻/CD11b⁻ events over the total number of FACS events) at 3 months ($n = 3$ mice per group). Two-tailed t -test, bars represent mean $\pm$ SD.
- E Representative photographs of SA- β -galactosidase staining in Myf6-KO, WT, and WT H₂O₂-treated cultured primary myoblasts. As a positive control, primary myoblasts from WT mice were cultured and treated with 20 μ M H₂O₂ for 15 min, and left in fresh media for 48 h before fixation Scale bar = 100 μ m.
- F Quantification of the percentage of SA- β -Gal⁺ cells in Myf6-KO ($n = 33$ fields of view, myoblasts from $n = 2$ mice), WT ($n = 44$ fields of view, myoblasts from $n = 2$ mice), and WT H₂O₂-treated myoblasts. Two-tailed t -test, bars represent mean $\pm$ SD.
- G Representative photographs of staining for cleaved caspase 3 in Myf6-KO, WT, and WT strausporine-treated cultured primary myoblasts. Primary myoblasts from WT mice were cultured and treated with 3 μ M strausporine for 3 h and then fixed. Scale bar = 50 μ m.
- H Quantification of the percentage of cleaved caspase3⁺ cells in Myf6-KO ($n = 20$ fields of view, myoblasts from $n = 2$ mice), WT ($n = 20$ fields of view, myoblasts from $n = 2$ mice), and WT strausporine-treated myoblasts. Two-tailed t -test, error bars = $\pm$ SD.
- I Representative images of a co-culture assay of WT or Myf6-KO myotubes with ACTB-EGFP⁺ myoblasts. Briefly, WT and Myf6-KO myoblasts were differentiated for 2 days before adding equal numbers of ACTB-EGFP myoblasts for an additional 2 days, followed by staining for GFP, MF20, and DAPI. Arrows indicate mononuclear unfused cells. Arrowheads indicate unfused myoblasts. Scale bar = 400 μ m.
- J Quantification of the number of mononuclear cells remaining after 2 days in co-culture with WT or Myf6-KO 2DM myotubes ($n = 30$ fields of view, myoblasts from $n = 2$ mice per group). Two-tailed t -test, bars represent mean $\pm$ SD.

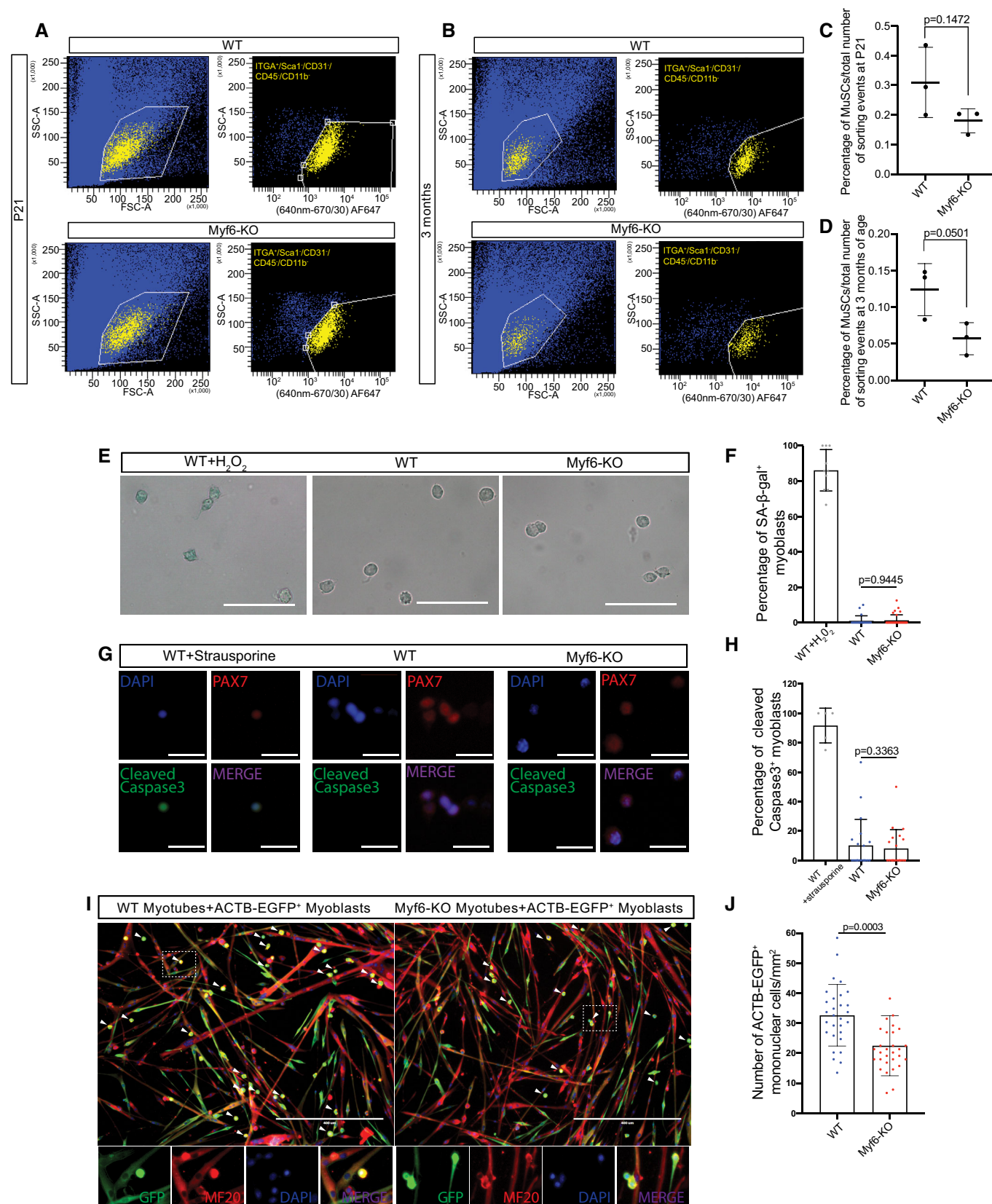


Figure EV5.