SUPPLEMENTAL DATA

HEXIM1 controls satellite cell expansion after injury to regulate skeletal muscle regeneration

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Inventory of Supplemental Data

SUPPLEMENTAL METHODS

Satellite cell isolation

Freshly harvested muscles were digested with 200 U/ml collagenase II (Invitrogen) in Dulbecco's modified Eagle's medium (DMEM) (HyClone) for 60 minutes at 37°C with gentle agitation. Digested muscles were dissociated into single myofibers by trituration using Pasteur pipettes. Single myofibers were further digested with 5 U/ml collagenase II and 2 U/ml dispase (Roche) in DMEM for 60 minutes at 37°C with gentle agitation. Digested myofibers were filtrated through 40 μm mesh (BD Falcon) and centrifuged at 500 g for 4 minutes to pellet satellite cells. Satellite cells were washed twice with GM before culturing or with staining buffer before flow cytometric analysis.

Histology and immunofluorescence

For the analysis of tissue sections, cryostat sections were cut at a thickness of 10 μm and only the 120 sections corresponding to the mid-belly of TA muscle were analyzed. H&E staining were performed following a standard protocol. Sections for immunofluorescence were permeabilized in 0.5% Triton X-100 (Millipore) in PBS for 15 minutes, and blocked in M.O.M. blocking reagent (Vector Labs) according to the manufacturer’s instructions. Sections were incubated in a mixture of primary antibodies overnight at 4°C, washed with 0.1% Tween-20 in PBS (PBST) and then incubated in a mixture of appropriate secondary antibodies for 1 hour at 4°C, followed by incubation with DAPI for 5 minutes before being mounted using VECTASHIELD mounting media (Vector Labs). Primary and secondary antibodies for tissue sections were diluted in PBS with MOM high-protein blocking solution (Vector Labs). Muscle cross section area was measured using a Zeiss Axio scan-microscope with MetaMorph software (Molecular Devices) at 6 mid-belly sections with 60 μm distance between each other and stained by anti-Laminin

antibody. The largest number was recorded as the cross section area of this muscle. Numbers of myofibers and myonuclei were calculated based on dystrophin and DAPI staining in 6 sections per muscle. The average size of myofibers was calculated from 1000 fibers per muscle based on dystrophin and DAPI staining. Quantitation of satellite cells and their sub-populations was performed by counting positive cells in a whole section using MetaMorph software. Adjacent sections stained with isotype controls were used as control. At least 3 sections from each muscle were analyzed and the mean results of sections from each muscle were further calculated as the mean value of muscles. Quantitation of satellite cells based on single myofibers was performed by quantitating positive cells in 12 myofibers isolated from each TA muscle and measuring the total length of the 12 analyzed myofibers. Results were expressed as satellite cell number per 100 μm length of a myofiber. For the analysis of cultured cells, cells were fixed in 2% paraformaldehyde for 10 minutes, permeabilized and blocked in 0.1% Triton X-100 and 20% (v/v) normal donkey serum (Millipore) in PBS, and stained in a mixture of primary antibodies for 1 hour at room temperature. Cells were then washed with PBST and incubated in a mixture of secondary antibodies for 1 hour at room temperature, followed by incubation with DAPI and mounting as described above. Primary and secondary antibodies for the cells were diluted in PBS with 20% normal donkey serum. For vessel counting, mid-belly muscle sections were stained with isolectin (Invitrogen) and anti-dystrophin antibody (Clone: MANDYS8, Sigma) in TBS with 0.1% Tween-20 (TBST) and 2% bovine serum albumin for 1 h at room temperature. Sections were then washed twice in TBST and stained with secondary antibody and DAPI. The total number of isolectin^{+} vessels and the total number of dystrophin^{+} myofibers were measured for each cross section. The observer making the counts was blinded to the identity of the specimen.

Assays for satellite cell cultures

For the analysis of single cell-derived colonies, freshly sorted satellite cells were plated on collagen I-coated 4-well culture slides (BD BioCoat) at a density of 20 cells per well and cultured in GM with fresh bFGF added daily. Each data point was calculated from the mean value of 6 wells. The value of each well was calculated from colonies derived from approximately 20 cells isolated in one sorting experiment and seeded in this well. Rare $Pax7^{-}MyoD^{-}$ colonies (< 1%) were excluded from analysis. For myogenic differentiation assay, low passage 50-70% confluent cultures of satellite cells were switched to low-mitogen media consisting of DMEM, 10 mM HEPES, 3% horse serum (HyClone) and 1% penicillin/streptomycin/amphotericin b and cultured at 37°C for 1-7 days. BrdU incorporation assay was performed by pulse labeling cells for 1 hour in low-density cultures on culture slides at least 24 hours after seeding. HMBA was dissolved in water and added to medium at 10 mM final concentration. DRB was dissolved in DMSO and added to medium at 50 μM. IL-6 (Sigma) was added to medium at the indicated concentrations.

Cell cycle analysis

Freshly sorted satellite cells were incubated in Hank's buffered salt solution (HBSS) (HyClone) with 5 μg/ml Hoechst 33342 (Sigma), 0.5 μg/ml verapamil (Sigma) and 3% FBS for 45 minutes at 37°C. Pyronin Y (Sigma) was added at 1 μg/ml, and the cells were incubated for another 15 minutes at 37°C. The cells were then washed twice in cold HBSS and kept on ice during analysis. Both Hoechst Blue and Pyronin Y axes were displayed in linear mode. $CD45^{+}$ hematopoietic cells extracted from regenerating muscles were used as the control to define the gate of each phase of the cell cycle.

QRT-PCR

mRNA was extracted using Trizol reagent (Invitrogen). The concentration and purity of RNA were estimated by calculating the ratio of OD_{260} to OD_{280}. cDNA was generated using a reverse transcription kit (Qiagen). Equal quantities of cDNA were subjected to QRT-PCR using the SYBR-green kit (Applied Biosystems). All QRT-PCR reactions were performed using the following thermal cycler conditions: 95°C for 10 minutes followed by 40 cycles of a 3-step reaction consisting of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds.

SUPPLEMENTAL FIGURES AND LEGENDS

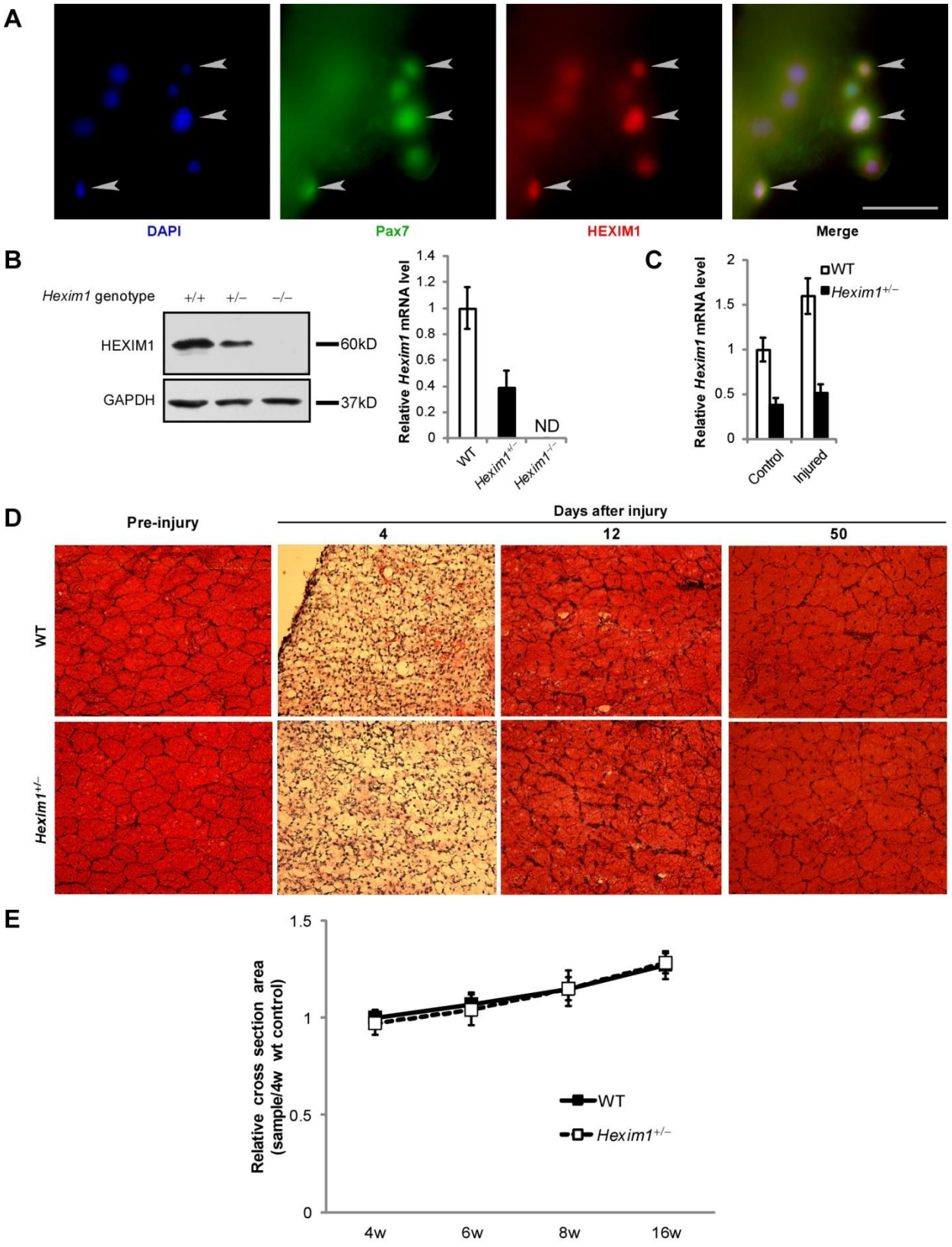

Figure S1. Characterization of HEXIM1 expression and the experimental mouse model

(A) Immunofluorescence of a single myofiber isolated from uninjured TA muscle of adult WT mice and cultured for 3 days, stained for Pax7 (green) and HEXIM1 (red). Nuclei were counterstained with DAPI (blue). Arrowheads indicate $Pax7^+$ satellite cells expressing HEXIM1. Myonuclei at the left within the myofiber also express HEXIM1. Scale bar = 30 μm.

(B) The expression of *Hexim1* mRNA and protein in E17 embryonic limb muscle tissues. ND, not detected. n = 3 embryos.

(C) *Hexim1* mRNA level in injured and contralateral control TA muscles 4 days after injury. n = 3 mice.

(D) H&E staining of transverse sections of pre-injury and regenerating (4, 12 and 50 days after injury) mid-belly TA muscles.

(E) Cross section area of TA muscles at the age of 4, 6, 8 or 16 weeks, normalized to that of the TA muscles of 4-week old WT mice. n = 3 mice per time point.

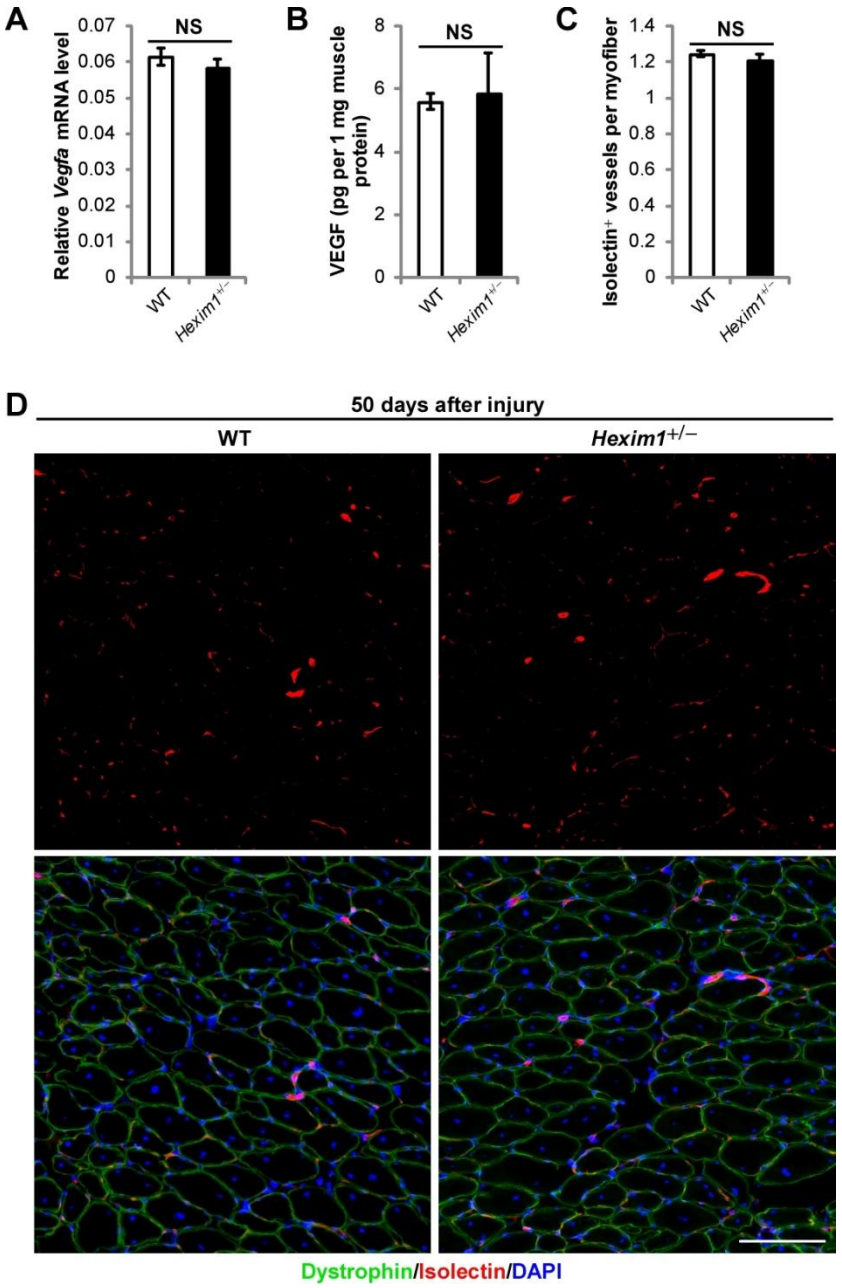

Figure S2. VEGF expression and vessel counting

(A,B) *Vegfa* mRNA (A) and VEGF protein (B) levels in TA muscles 4 days after injury. n = 3.

(C) Average numbers of isolectin^{+} vessels per myofiber in TA muscles 50 days after injury. n = 3.

(D) Muscle sections 50 days after injury stained for dystrophin (green) and isolectin (red). Nuclei were counterstained with DAPI (blue). Scale bar = 80 μm.

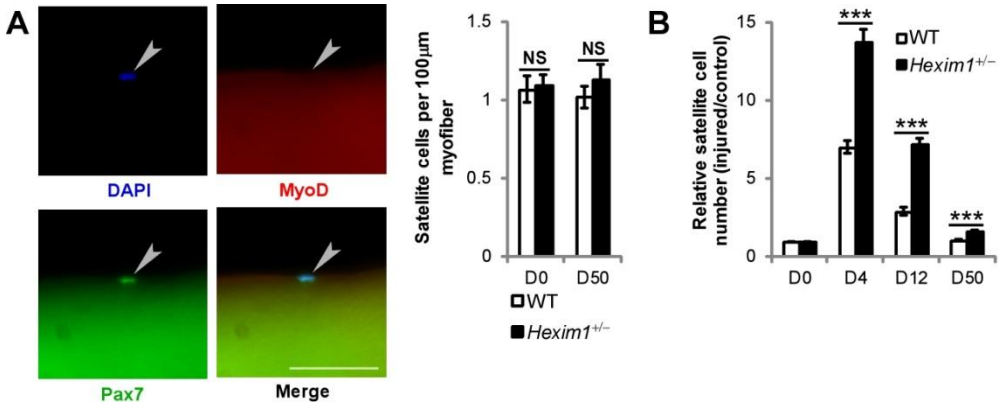

Figure S3. Supplemental satellite cell quantitation

(A) Immunofluorescence of a freshly isolated myofiber stained for Pax7 (green) and MyoD (red). $Pax7^{+}$ satellite cells were enumerated and normalized against the length of myofibers. n = 12 myofibers per mice. 3 mice were analyzed in each group.

(B) Relative satellite cell number per section normalized to that of the respective contralateral control muscle in pre-injury and regenerating muscle sections. Results were calculated from the average values of 3 muscle sections per mouse and 6 mice were analyzed. ***$p < 0.001$.

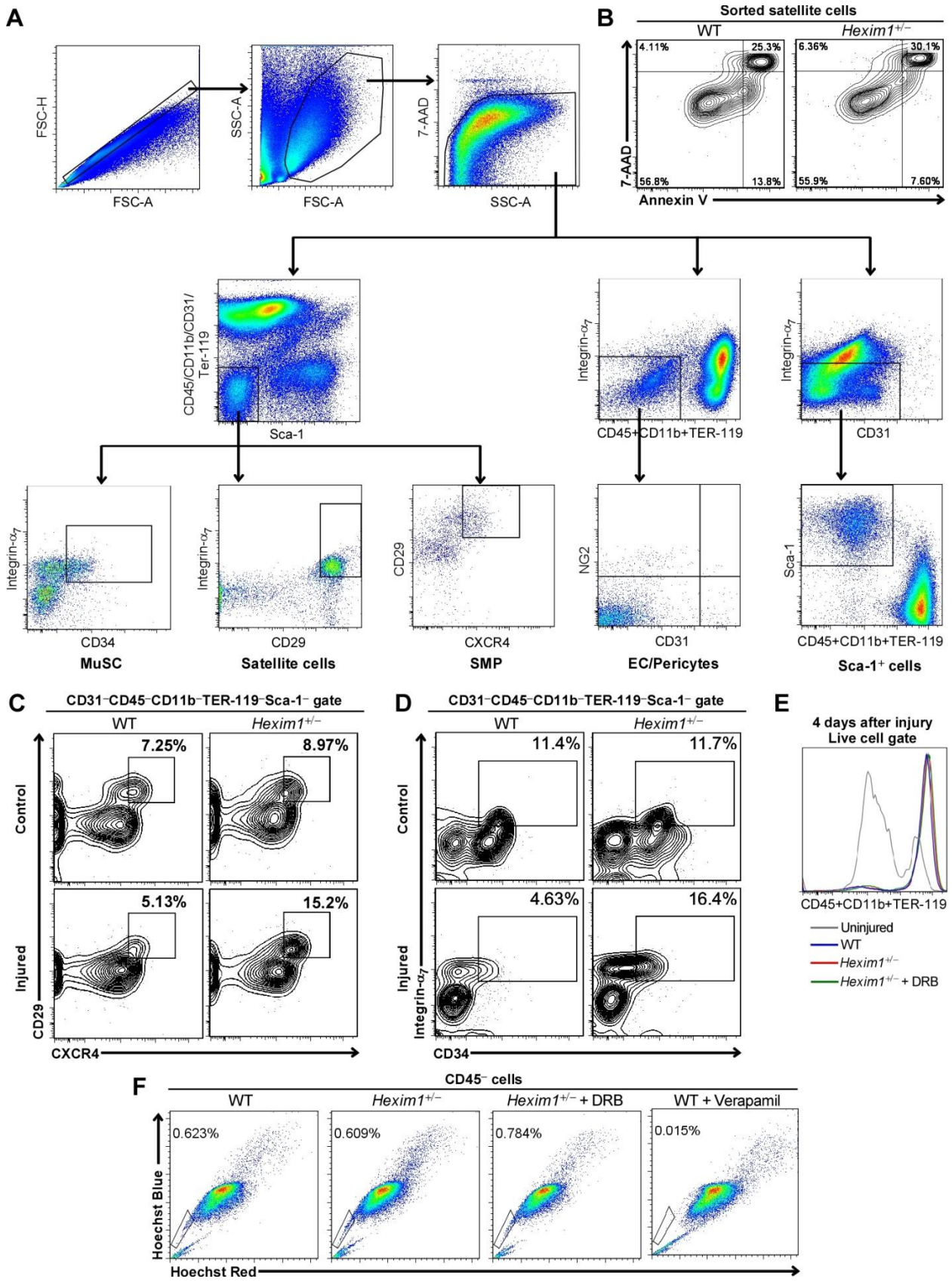
A
FSC-H
FSC-A
SSC-A
FSC-A
7-AAD
SSC-A
B
Sorted satellite cells
WT
Hexim1+/−
4.11%
25.3%
6.36%
30.1%
56.8%
13.8%
55.9%
7.60%
7-AAD
Annexin V
CD45/CD11b/CD31/Ter-119
Sca-1
Integrin-α7
CD45+CD11b+TER-119
Integrin-α7
CD31
Integrin-α7
CD34
MuSC
Integrin-α7
CD29
Satellite cells
CD29
CXCR4
SMP
NG2
CD31
EC/Pericytes
Sca-1
CD45+CD11b+TER-119
Sca-1+ cells
C
CD31−CD45−CD11b−TER-119−Sca-1− gate
WT
Hexim1+/−
Control
7.25%
8.97%
Injured
5.13%
15.2%
CD29
CXCR4
D
CD31−CD45−CD11b−TER-119−Sca-1− gate
WT
Hexim1+/−
Control
11.4%
11.7%
Injured
4.63%
16.4%
Integrin-α7
CD34
E
4 days after injury
Live cell gate
CD45+CD11b+TER-119
Uninjured
WT
Hexim1+/−
Hexim1+/− + DRB
F
CD45− cells
WT
Hexim1+/−
Hexim1+/− + DRB
WT + Verapamil
0.623%
0.609%
0.784%
0.015%
Hoechst Blue
Hoechst Red

Figure S4. Gating strategy and supplemental flow cytometric analysis

(A) Single events were gated on forward scatter area (FSC-A) versus forward scatter height (FSC-H) plot. Cells were distinguished from debris by a gate on the FSC-A versus side scatter area (SSC-A) plot. Nonviable cells were subsequently excluded by 7-AAD gating. $CD11b^{-}CD45^{-}CD31^{-}TER\text{-}119^{-}Sca\text{-}1^{-}$ cells were further analyzed for their expression of satellite cell markers (integrin-α_7 and CD29), MuSC markers (CD34 and integrin-α_7) and SMP markers (CXCR4 and CD29). $CD11b^{-}CD45^{-}$ $TER\text{-}119^{-}$ integrin-α_7^{-} cells were further analyzed for their expression of endothelia cell marker CD31 and pericytes marker NG2. $CD31^{-}$integrin-α_7^{-} cells were analyzed for the $Sca\text{-}1^{+}$ $CD11b^{-}CD45^{-}$ $TER\text{-}119^{-}$ cells.

(B) Apoptosis of satellite cells 24 hours after injury.

(C, D) Percentages of $CD29^{+}CXCR4^{+}$ (C) and $CD34^{+}$integrin-α_7^{+} cells (D) in injured and contralateral muscles 4 days after injury.

(E) Signal of hematopoietic cell markers in control and injured muscles and injured muscle with DRB treatment 4 days after injury.

(F) Percentages of side population cells in injured muscles 4 days after injury. The gate was validated by verapamil treatment, which eliminated side population cells from the plot.

All plots were representative of at least 3 independent experiments.

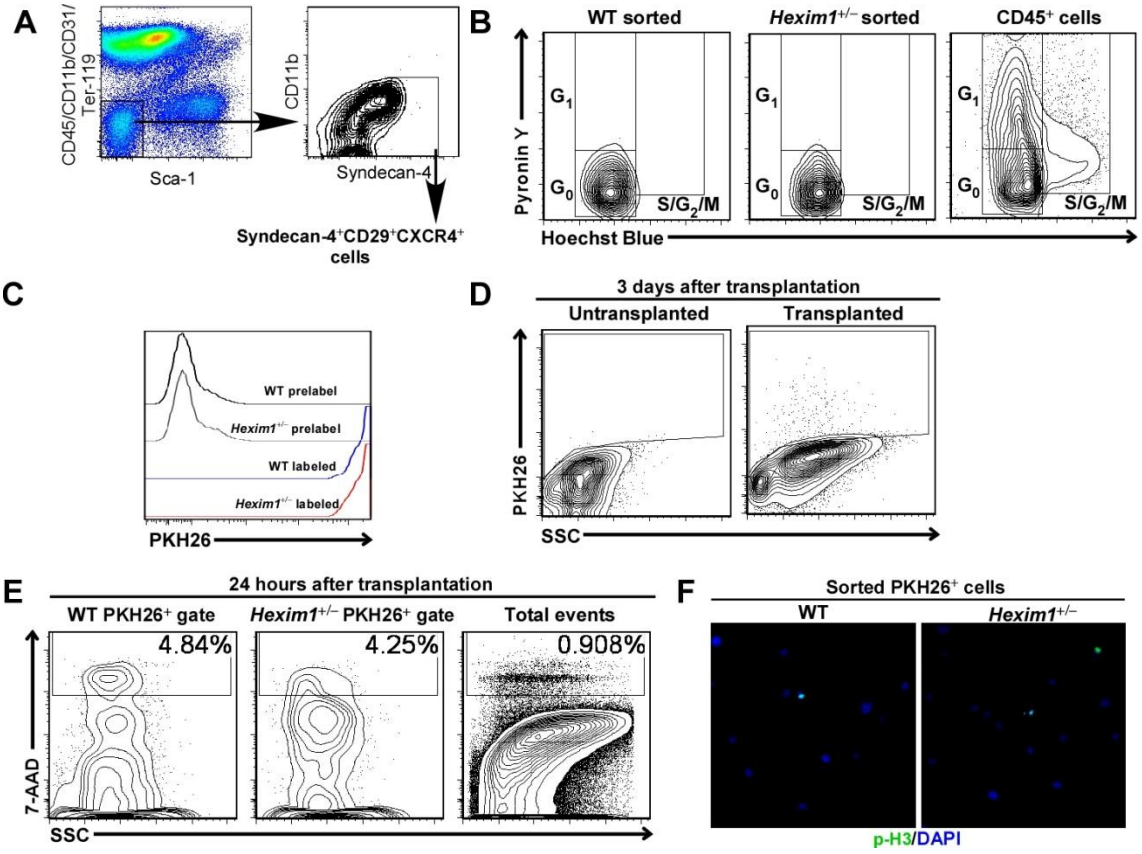

Figure S5. Sorting strategy and supplemental cell tracking data

(A) The same gating strategy as that in flow cytometric analysis shown in Figure S4A was applied to gate viable population harboring satellite cells. $CD11b^{-}CD45^{-}CD31^{-}TER\text{-}119^{-}Sca\text{-}1^{-}$ cells were subjected to an additional syndecan-4^{+} gating before proceeding to the $CD29^{+}CXCR4^{+}$ gating as shown in Figure S4A, which ensured the purify of satellite cells.

(B) Cell cycle analysis of freshly sorted satellite cells based on DNA (Hoechst Blue) and RNA (Pyronin Y) contents. Gating of each phase was drawn according to $CD45^{+}$ cells from regenerating muscles. G_0 = Hoechst $Blue^{low}$Pyronin Y^{low}, G_1 = Hoechst $Blue^{low}$Pyronin Y^{high}, $S/G_2/M$ = Hoechst $Blue^{high}$.

(C) PKH26 signals of prelabel and labeled satellite cells.

(D) Gating strategy of $PKH26^{+}$ cells in TA muscles transplanted with PKH26-labeled satellite cells 3 days ago. The contralateral TA muscle served as untransplanted control.

(E) Cell death of transplanted PKH26^{+} cells were detected by 7-AAD 24 hours after transplantation. Gate of dead cells was drawn according to total events.

(F) Sorted PKH26^{+} cells stained for p-H3 (green). Nuclei were counterstained with DAPI (blue).

All plots were representative of at least 6 independent experiments.

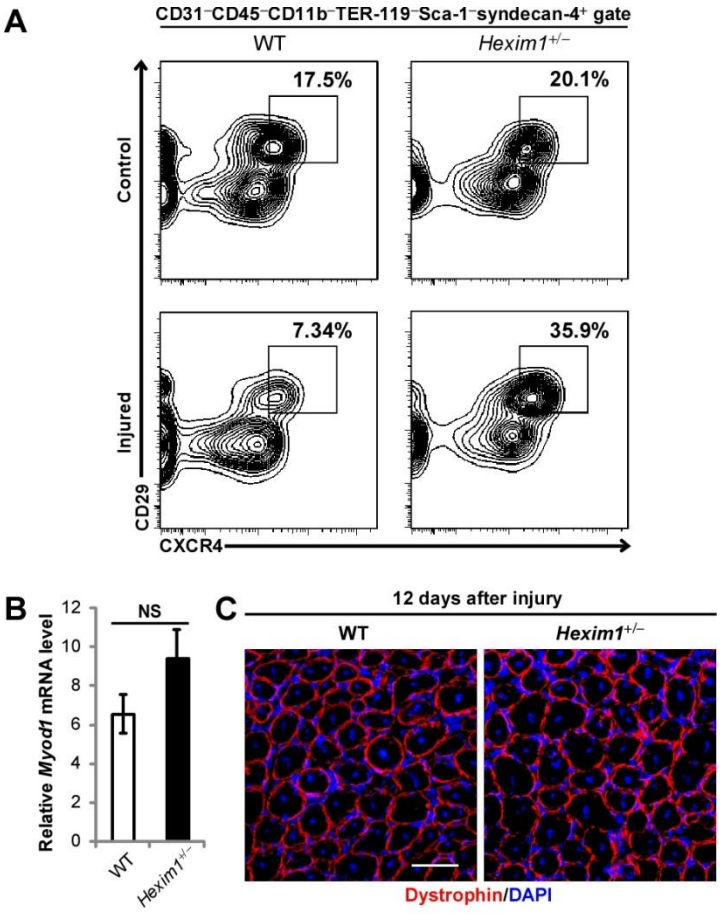

Figure S6. Supplemental proliferation and differentiation data

(A) Flow cytometry and quantitation of percentages of syndecan-$4^{+}$$CD29^{+}$$CXCR4^{+}$ cells in injured and contralateral control TA muscles 4 days after injury. n = 6.

(B) *Myod1* (MyoD) mRNA levels 4 days after injury. n = 3. NS = not significant.

(C) Sections of muscles 12 days after injury stained for dystrophin (red). Scale bar = 80 μm.

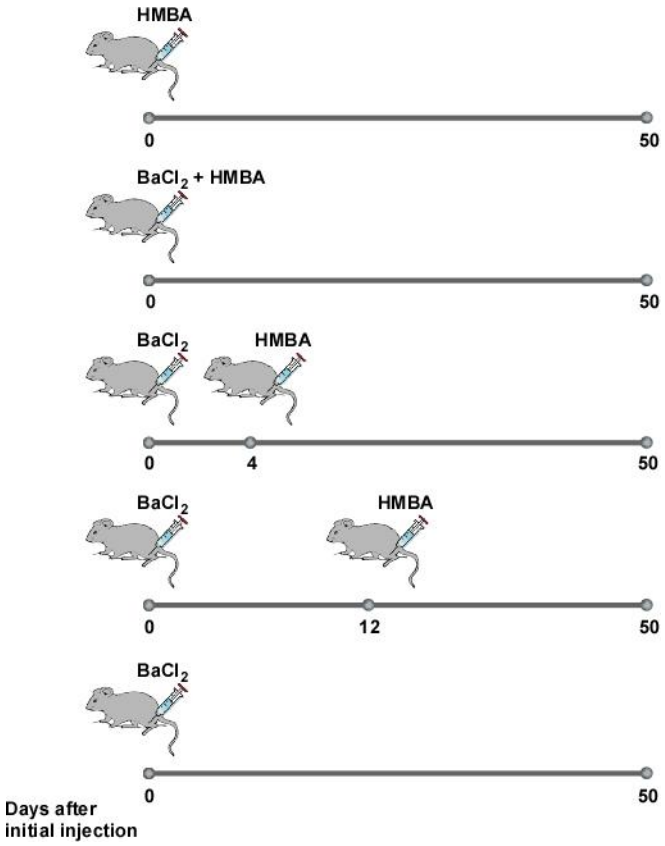

Figure S7. Scheme of HMBA treatment for skeletal muscle regeneration

50 µl 200mM HMBA was injected into the injured TA muscle either simultaneously with 1.2% $BaCl_2$ at the initial injury or with saline at the indicated time after initial $BaCl_2$–induced injury. Muscles weights and cross section areas were measured 50 days after injury.

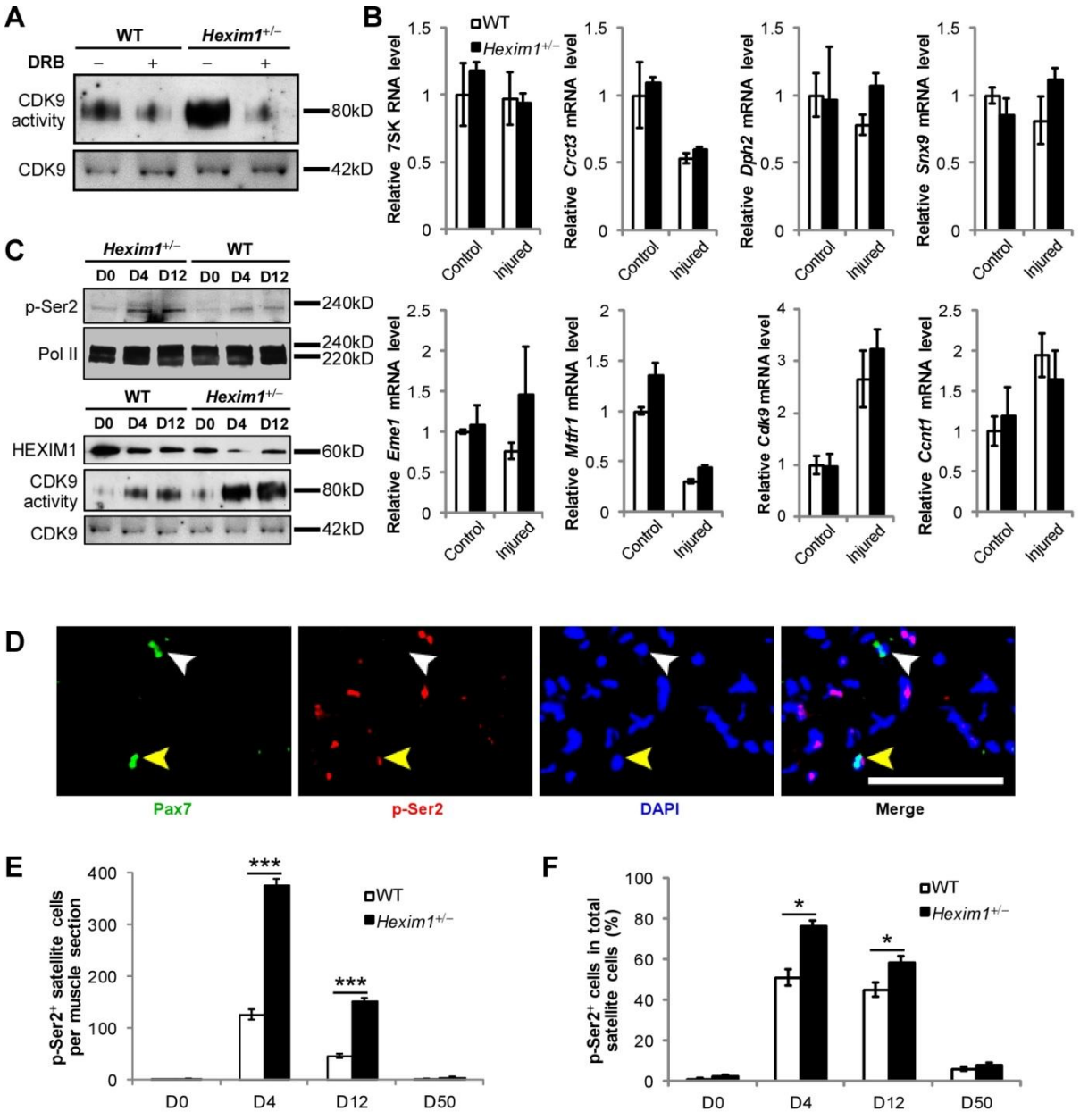

Figure S8. P-TEFb activity and the expression of P-TEFb components during regeneration

(A) CDK9 kinase activity in $CD45^-$ cells from injured muscles with or without DRB treatment 4 days after injury. CDK9 served as the control.

(B) mRNA levels of 7SK, 7SK-dependent and P-TEFb-independent genes (*Crct3*, *Dph2*, *Snx9*, *Eme1* and *Mtfr1*) and P-TEFb components *Cdk9* and *Ccnt1* (cyclin T1) in $CD45^-$ cells from TA

muscles 4 days after injury. No significant was found difference between WT and *Hexim1*$^{+/-}$ cells in all groups. n = 3.

(C) p-Ser2 level, P-TEFb-associated HEXIM1 and CDK9 activity in CD45^{-} cells from pre-injury (day 0) and injured (4 or 12 days after injury) TA muscles. Total Pol II and CDK9 served as the control for p-Ser2 and HEXIM1/CDK9 activity, respectively. Data were representative of 3 independent experiments.

(D) Sample muscle section stained for Pax7 (green) and p-Ser2 (red). White and yellow arrowheads point to P-TEFb-inactive (Pax7^{+}p-Ser2^{-}) and P-TEFb-active (Pax7^{+}p-Ser2^{+}) satellite cells, respectively. Scale bar = 80 μm.

(E, F) P-TEFb-active satellite cells per section (E) and the percentage of P-TEFb-active satellite cells in total satellite cells (F). n = 6. * $p < 0.05$; *** $p < 0.001$.

SUPPLEMENTAL TABLES

Table S1. Antibodies and reagents used

Antigen	Supplier	Format	Host	Clone	Dilution (Application)
BrdU	Millipore	Unconjugated	Mouse	BU-1	1/1000 (IF)
CD11b	eBioscience	eFluor 450	Rat	M1/70	1/200 (FC)
CD29	eBioscience	PE-Cy7	Hamster	HMß1-1	1/400 (FC)
CD31	eBioscience	eFluor 450	Rat	390	1/300 (FC)
CD31	eBioscience	PE	Rat	390	1/300 (FC)
CD34	eBioscience	eFluor 660	Rat	RAM34	1/200 (FC)
CD45	eBioscience	eFluor 450	Rat	30-F11	1/400 (FC)
CDK9	Cell Signaling	Unconjugated	Rabbit	C12F7	1/1000 (IB)
CDK9	Santa Cruz Biotechnology	Unconjugated	Mouse	D-7	1/40 (IP)
CXCR4	eBioscience	APC	Rat	2B11	1/200 (FC)
Cyclin D1	Cell Signaling	Unconjugated	Rabbit	92G2	1/1000 (IB)
Cyclin T1	Santa Cruz Biotechnology	Unconjugated	Rabbit		1/200 (IB)
Cyclin T1	Millipore	Unconjugated	Sheep		1/40 (IP)
Desmin	Cell Signaling	Unconjugated	Rabbit	D93F5	1/500 (IB)
Dystrophin	Sigma	Unconjugated	Mouse	MANDYS8	1/500 (IF)
eMHC	Developmental Studies Hybridoma Bank	Unconjugated	Mouse	F1.652	1/50 (IF)
GAPDH	Cell Signaling	HRP	Rabbit	D16H11	1/1000 (IB)
HEXIM1	Proteintech	Unconjugated	Rabbit		1/1000 (IB), 1/300 (IF)
Integrin-α_7	MBL	FITC	Mouse	3C12	1/100 (FC)
Ki67	Abcam	Unconjugated	Rabbit		1/1000 (IF)
Laminin	Abcam	Unconjugated	Chicken		1/2000 (IF)
MHC	Developmental Studies Hybridoma Bank	Unconjugated	Mouse	MF 20	1/50 (IF), 1/200 (IB)
MyoD	Santa Cruz Biotechnology	Unconjugated	Rabbit		1/200 (IF)
myogenin	Developmental Studies Hybridoma Bank	Unconjugated	Mouse	F5D	1/200 (IB)
myogenin	Santa Cruz Biotechnology	Unconjugated	Rabbit		1/200 (IF)
NG2	R&D Systems	APC	Mouse	LHM-2	1/100 (FC)
Pax7	Developmental Studies Hybridoma Bank	Unconjugated	Mouse	Pax7	1/50 (IF)
Pol II	Santa Cruz Biotechnology	Unconjugated	Rabbit		1/500 (IB)
p-H3	Cell Signaling	Unconjugated	Rabbit	D2C8	1/500 (IF)

p-Ser2	Covance	Unconjugated	Mouse	H5	1/300 (IF), 1/500 (IB)
Sca-1	BD Pharmingen	APC-Cy7	Rat	D7	1/200 (FC)
Syndecan-4	BD Pharmingen	Biotin	Rat	KY/8.2	1/200 (FC)
TER-119	eBioscience	eFluor 450	Rat	TER-119	1/200 (FC)
β-tubulin	Santa Cruz Biotechnology	Unconjugated	Mouse	D-10	1/2000 (IB)

Isotype control	**Supplier**	**Format**	**Dilution (Application)**
Am Hamster IgG	eBioscience	PE-Cy7	1/400 (FC)
Mouse IgG_1	Invitrogen	Alexa Fluor 488	1/300 (IF)
Mouse IgG_1	Invitrogen	Alexa Fluor 594	1/300 (IF)
Mouse IgG_1	eBioscience	FITC	1/100 (FC)
Mouse IgG_{2b}	Jackson ImmunoResearch	Unconjugated	1/40 (IP)
Mouse IgM	Invitrogen	Alexa Fluor 488	1/300 (IF)
Sheep IgG	Jackson ImmunoResearch	Unconjugated	1/40 (IP)
Rat IgG_{2a}	BD Pharmingen	Biotin	1/200 (FC)
Rat IgG_{2b}	eBioscience	APC	1/200 (FC)

Reagents	**Supplier**	**Concentration or Dilution (Application)**
7-AAD	BD Pharmingen	1/100 (FC)
Isolectin GS-IB_4 – Alexa Fluor 594	Invitrogen	2 μg/ml (IF)
Streptavidin-Qdot605	Invitrogen	1 μg/ml (FC)

FC = Flow Cytometry; IF = Immunofluorescence; IB = Immunoblot; IP = Immunoprecipitation; HRP = Horseradish Peroxidase

Table S2. Primers used in QRT-PCR

Gene	Forward	Reverse
18S	5′-CGGACAGGATTGACAGATTG-3′	5′-CAAATCGCTCCACCAACTAA-3′
7SK	5′-TCTGTCACCCCATTGATCGC-3′	5′-CGTATACCCTTGACCGAAGACC-3′
Ccnd1	5′-GCGTACCCTGACACCAATCTC-3′	5′-CTCCTCTTCGCACTTCTGCTC-3′
Ccnt1	5′-AACAAGCGGTGGTATTTTACTCG-3′	5′-CCTGCTGGCGGTAAGAGAG-3′
Cdk9	5′-GTACGACTCGGTGGAATGCC-3′	5′-GATGGGGAACCCCTCCTTCT-3′
Col1a2	5′-GTAACTTCGTGCCTAGCAACA-3′	5′-CCTTTGTCAGAATACTGAGCAGC-3′
Crtc3	5′-TGACTCACCTGGGGATAAGAAC-3′	5′-GTGGCACTTGAGGGACGAG-3′
Dph2	5′-AAGACCTGGACCGCGTGTA-3′	5′-ATCTTAGCTCCTGTGACTTCCT-3′
Eme1	5′-AGAGGGCGAAGAACATAGTGG-3′	5′-GCTACTGCTTAGCACTCTGACT-3′
Hexim1	5′-GAGCCACTCTTGACAGAACATC-3′	5′-TGCAAGGACGCTCTCGATTG-3′
Mtfr1	5′-CTGGGCTGGATTAAGTGCCTG-3′	5′-TGGCTGGTAAGCTGAAATTGAA-3′
Myh3	5′-AAAAGGCCATCACTGACGC-3′	5′-CAGCTCTCTGATCCGTGTCTC-3′
Myod1	5′-CCACTCCGGGACATAGACTTG-3′	5′-AAAAGCGCAGGTCTGGTGAG-3′
Pax7	5′-TCTCCAAGATTCTGTGCCGAT-3′	5′-CGGGGTTCTCTCTCTTATACTCC-3′
Snx9	5′-ACCAAGGCTCGGGTCATGTA-3′	5′-ACCAACATTCGGGTTTGTAACTG-3′
Vegfa	5′-GCACATAGAGAGAATGAGCTTCC-3′	5′-CTCCGCTCTGAACAAGGCT-3′