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Supplemental Information

Distinct Phases of Postnatal Skeletal Muscle Growth Govern the Progressive Establishment of Muscle Stem Cell Quiescence

Francesca Gattazzo, Béatrice Laurent, Frédéric Relaix, Hélène Rouard, and Nathalie Didier

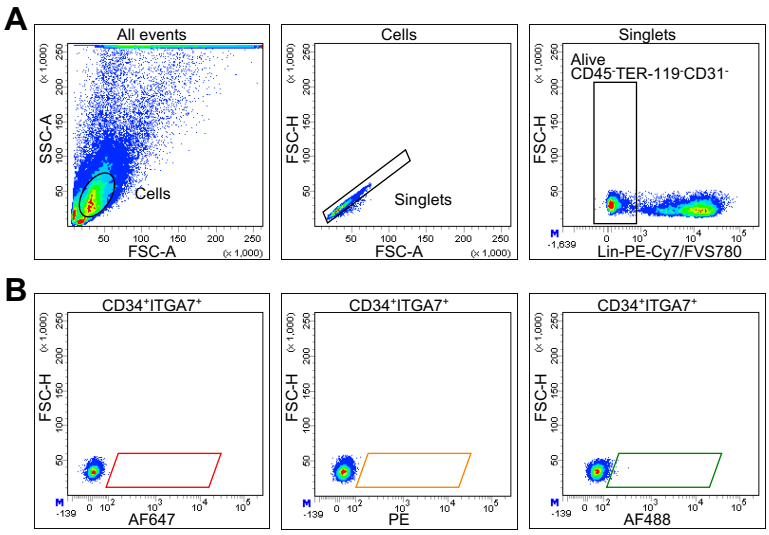

Figure S1. Characterization and purification of CD34$^+$ITGA7$^+$ myogenic fraction from postnatal and adult hind-limb muscles. Related to Figure 1. Strategy for determining the proportion of PAX7+, MYOD+, MYOG+ cells among the CD45$^-$TER-119$^-$CD31$^-$SCA-1$^-$CD34$^+$ITGA7$^+$ fraction (referred to as CD34$^+$ITGA7$^+$ fraction). Representative density scatterplots showing the gates used to analyse CD34$^+$ITGA7$^+$ fraction from P7 mouse muscles. (**A**) Debris, doublets, Lin+ cells (namely, CD45$^+$TER-119$^+$CD31$^+$ cells) and dead cells were excluded from the analysis. (**B**) Fluorescence Minus One controls (FMO) gates were used to determine the positivity for PAX7+, MYOD+, MYOG+ cells among the CD34$^+$ITGA7$^+$ fraction.

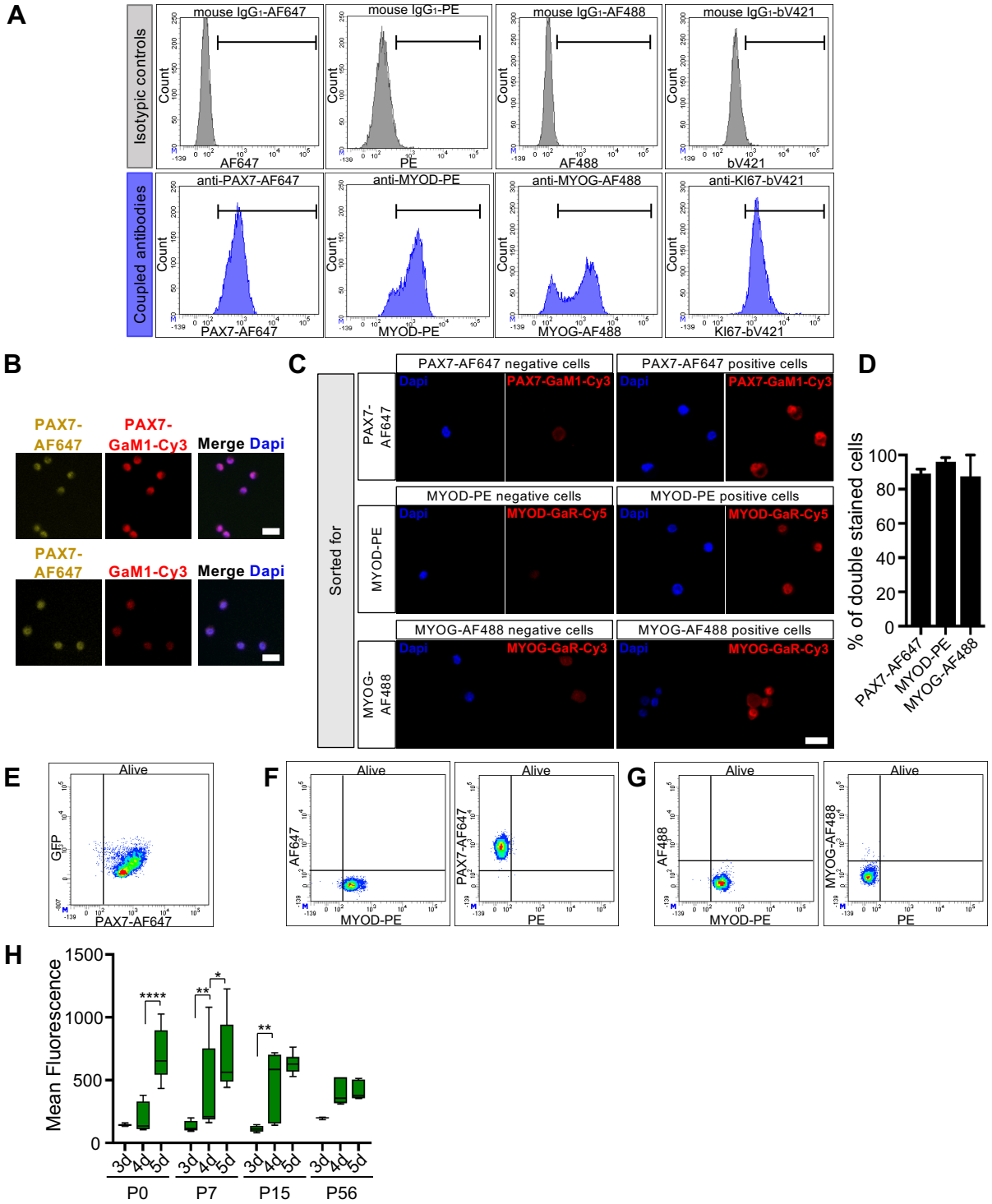

Figure S2. Validation of the coupled antibodies used for flow cytometry analysis of cultured myogenic cells. Related to Figure 2. (**A**) Cells were stained with the coupled isotypic control immunoglobulin or the coupled antibody at the same concentration. (**B-D**) Cells immunostained with coupled antibodies were FACS sorted and counterstained with distinct primary antibodies. See Supplemental Experimental Procedure. (**B**) Images showing the different intensity of the signal when cells immunostained with coupled anti-PAX7-AF647 (#sc-365843) were counterstained with primary mouse anti-PAX7 (#sc-81648) and secondary GaM1-Cy3 antibodies or with secondary GaM1-Cy3 antibody only. Only cells with a strong signal intensity were considered as double stained for PAX7. (**C**) Representative images of FACS sorted cells counterstained for PAX7, MYOD and MYOG. Scale bar, 25 µm. (**D**) Percentage of double stained cells. Data are shown as mean ± SEM of replicates (n=1 experiment). (**E**) Density scatter plot showing the correlation between endogenous PAX7 expression revealed with coupled anti-PAX7-AF647 antibody and GFP transgene expression in cultured cells derived from *Tg:Pax7-nGFP* mouse muscles. (**F-G**) Fluorescence Minus One (FMO) controls used to position the gates on Figure 2. (**H**) Mean level of expression of MYOG in mononucleated cells cultured for 3, 4 and 5 days determined by flow cytometry. Values are the mean ± SEM of 3 independent experiments. Two-Way ANOVA analysis, with **p<0.05, **p<0.01, ****p<0.0001*.

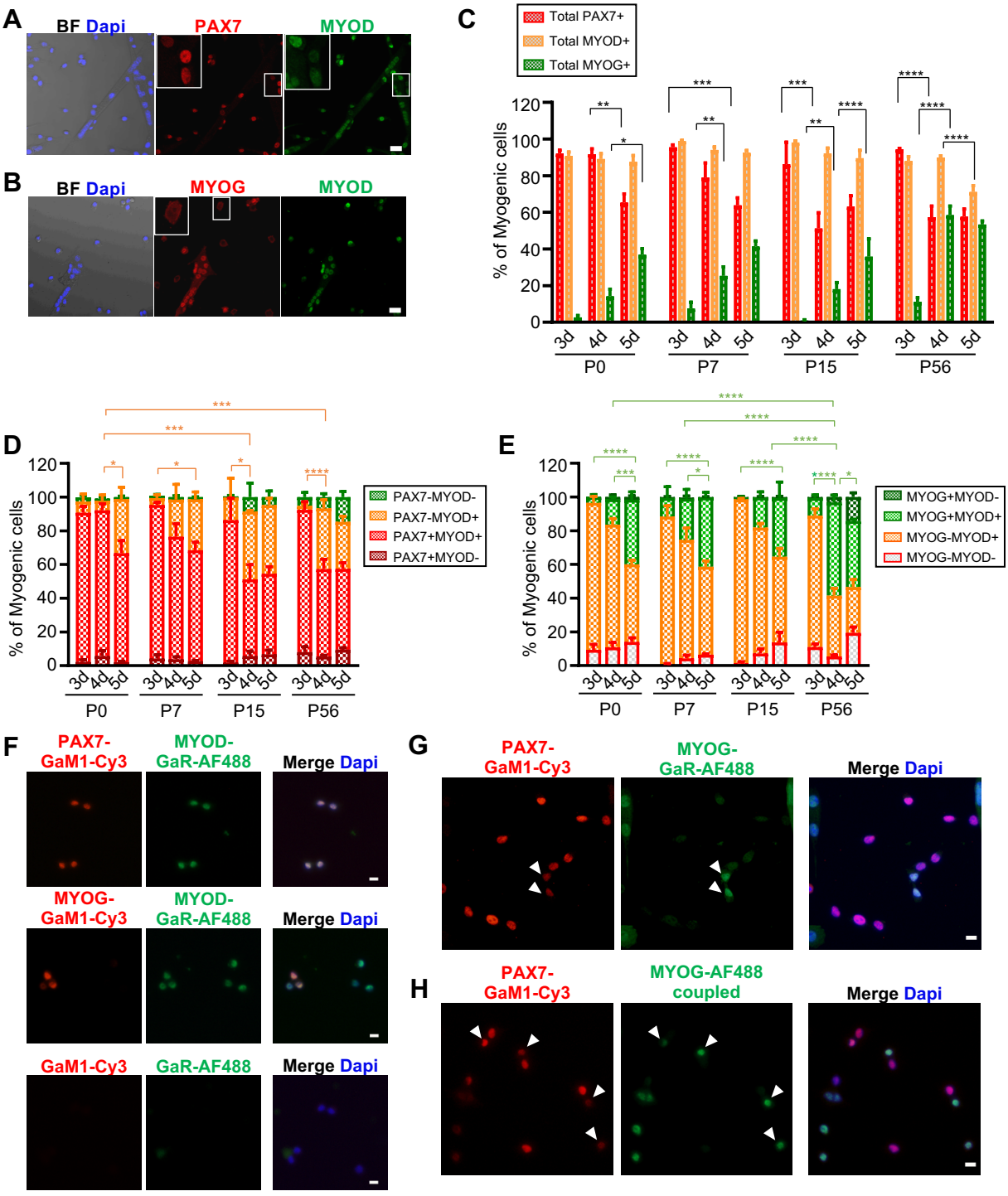

Figure S3. Analysis of the *in vitro* behavior of myogenic cells of the CD34^{+}ITGA7^{+} fraction by immunofluorescence. Related to Figure 2. (**A, B**) Representative images of cells stained for (**A**) PAX7 (red) and MYOD (green) and (**B**) MYOD (green) and MYOG (red). Nuclei were stained with DAPI. Magnification inlets show the variability of intensity of fluorescence signal. Scale bar, 20 µm. Graphs showing, (**C**) the quantification of the proportion of total PAX7+, MYOD+ and MYOG+ cells on the total myogenic population, (**D**) the distribution of PAX7 and MYOD, (**E**) the distribution of MYOD and MYOG on the total myogenic population. Values are represented as the mean ± SEM from 3 independent experiments (n=3 mice per group). Only mononucleated cells were quantified. Minimum 7 fields (20x) per well were quantified. (**F**) Representative images of cells immunostained for PAX7 (red) and MYOD (green) or MYOD (green) and MYOG (red) and the corresponding negative controls incubated with the secondary antibodies only. (**G, H**) Representative images of P56 cells cultured for 5 days in GM showing the co-expression of PAX7 and MYOG. Cells were immunostained for PAX7 (red) (#sc-81648) and MYOG (green) using (**G**) a rabbit anti-MYOG antibody (#sc-576), or (**H**) a coupled mouse anti-MYOG-AF488 antibody (#sc-52903). See Supplemental experimental procedures. Arrowheads point to double positive PAX7+MYOG+ cells. Scale bar, 20 µm.

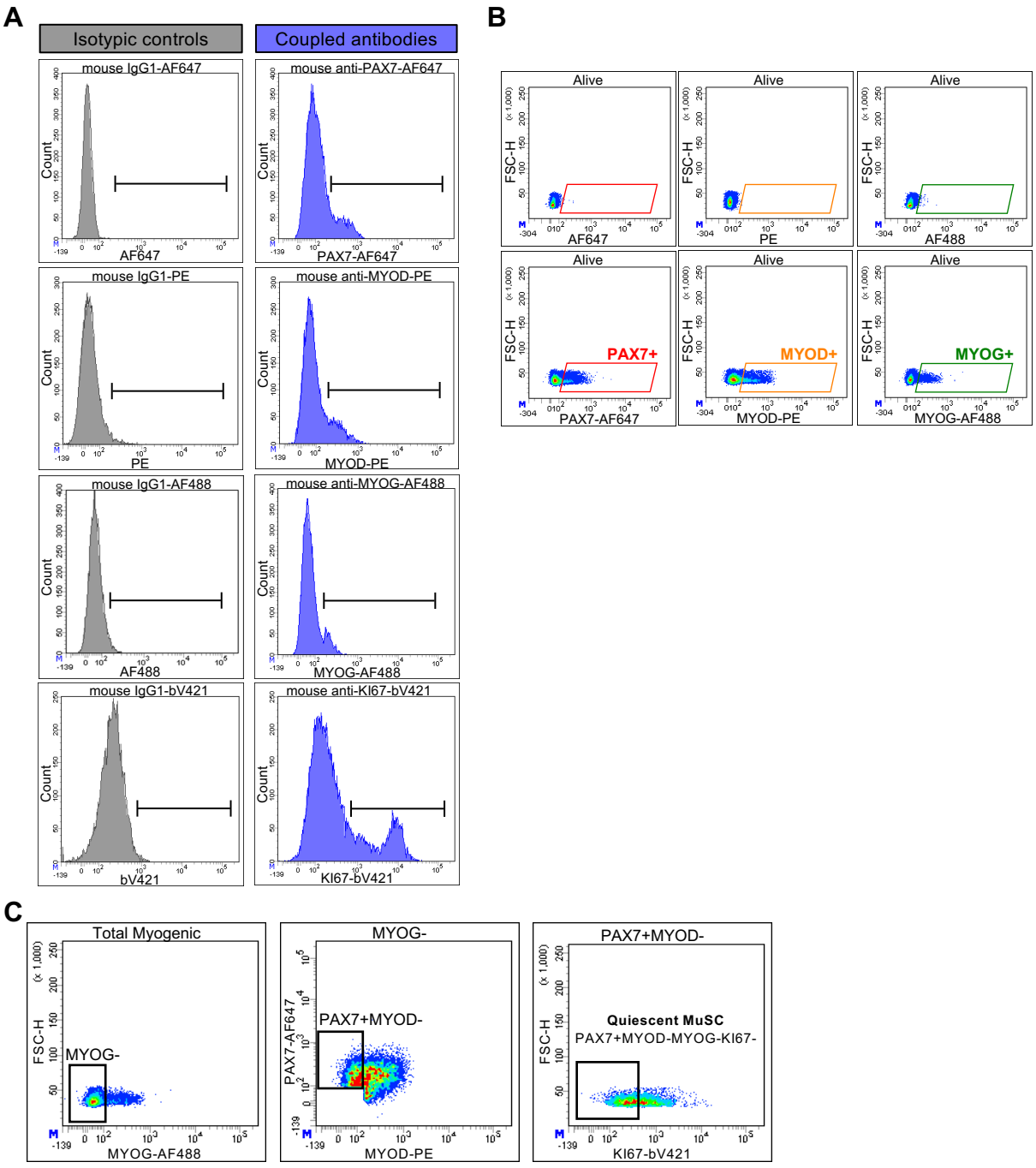

Figure S4. Validation of the *in vivo* flow cytometry analysis. Related to Figure 3. (**A**) Specificity of mouse coupled antibodies used for the *in vivo* analysis by flow cytometry. Mononucleated cells from P7 digested muscles were stained with either the coupled isotypic control immunoglobulin, either the mouse coupled antibody. (**B**) FMO controls used to position the gates for PAX7, MYOD and MYOG positivity. (**C**) Representative density scatterplots showing the gating strategy used to select quiescent MuSC defined as the PAX7+MYOD-MYOG-KI67- fraction.

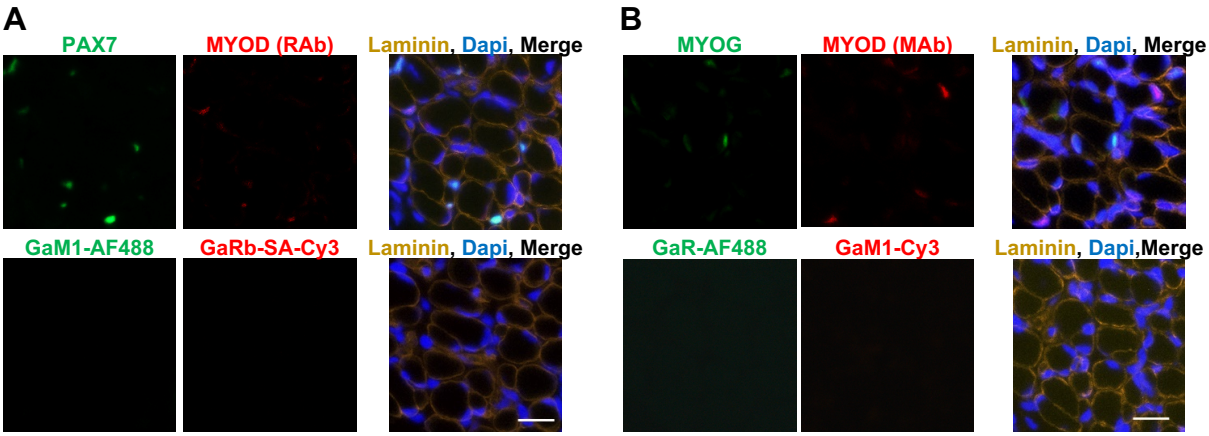

Figure S5. Validation of the immunostaining protocol on muscle sections. Related to Figure 4. Representative tibialis anterior (TA*)* muscle cross-sections of P3 mouse immunostained for (**A**) PAX7 (green), MYOD (red) (upper panel), or corresponding secondary antibodies only: GaM1-AF488 for Goat anti-Mouse IgG1-AF488 and GaRb-SA-Cy3 for Goat anti-Rabbit biotinylated-Streptavidin-Cy3 (bottom panel), (**B**) MYOG (green), MYOD (red) (upper panel), or corresponding secondary antibodies only: GaR-AF488 for Goat anti-Rabbit-AF488 and GaM1-Cy3 for Goat anti-Mouse IgG1-Cy3 (bottom panel). Laminin was immunostained (yellow) in each panel. Nuclei were stained with DAPI. Scale bar, 20 μm.

Supplemental Tables

Table S1. List of antibodies used for sorting and analysis of MuSC by flow cytometry. Related to Figure 1.

Protein	Catalog	Dilution**	Brand
rat anti-mouse CD45-PECy7	#552848	1 µg/300 µl	BD Biosciences
rat anti-mouse TER-119-PECy7	#557853	1 µg/300 µl	BD Biosciences
rat anti-mouse CD31-PE-Cy7	#561410	1 µg/300 µl	BD Biosciences
rat anti-mouse CD34-bV421	#562608	1 µg/300 µl	BD Biosciences
rat anti-mouse SCA-1-FITC	#553335	1 µg/300 µl	BD Biosciences
rat anti-mouse SCA-1-PE	#553108	1 µg/300 µl	BD Biosciences
mouse anti-mouse ITGA7-AF700	#FAB3518N	0.2 µg/300 µl	R&D Systems
mouse anti-mouse ITGA7-FITC	#NBP154412	1 µg/300 µl	Novus

**Amount of antibodies used for the immunostaining of mononucleated cells from digested hind-limb muscles of one adult mouse.

Table S2. Mean fold increase number of mononucleated cells between 3 and 5 days of culture. Related to Figure 1.

	Mean fold increase 5d *vs* 3d
P0	8.7
P7	3.9
P15	3.8
P56	4.0

Table S3. List of antibodies used for nuclear factors immunostaining for flow cytometry analysis. Related to Figure 2.

Protein	Catalog	Dilution*	Brand
mouse anti-PAX3/7-AF647 (B-5)	#sc-365843	20 ng/100 µl	Santa Cruz
mouse anti-MYOG-AF488 (5FD)	#sc-52903	20 ng/100 µl	Santa Cruz
mouse anti-MYOD-PE	#554130	80 ng/100 µl	BD Biosciences
mouse anti-KI67-bV421	#562899	4.5 µl/100 µl	BD Biosciences
mouse IgG1-AF647	#sc-24636	20 ng/100 µl	Santa Cruz
mouse IgG1-AF488	#sc-3890	20 ng/100 µl	Santa Cruz
mouse IgG1-PE	#550617	80 ng/100µl	BD Biosciences
mouse IgG1-bV421	#562438	4.5 µl/100 µl	BD Biosciences

*Amount of antibodies used for the immunostaining of 2.10^5 mononucleated cells from digested muscles.

Table S4. List of antibodies used for IF staining. Related to Figure 2.

Protein	Catalog	Final Dilution	Brand
mouse IgG1 anti-PAX7	#sc-81648	1:100	Santa Cruz (same as DSHB Ab)
mouse IgG1 anti-MYOD (MAb)	#554130	1:100	BD Biosciences
mouse IgG1 anti-MYOG 5FD	#sc-52903	1:100	Santa Cruz
rabbit anti-MYOD M-318 (RAb)	#sc-760	1:100	Santa Cruz
rabbit anti-MYOG M-225	#sc-576	1:100	Santa Cruz
rabbit anti-KI67 D3B5	#9129	1:150	Cell Signaling
rabbit anti-Laminin	#L9393	1:200	Sigma
Secondary antibodies	**Catalog**	**Final Dilution**	**Brand**
Goat anti-Mouse IgG1-AF488 (GaM1-AF488)	#115-545-205	1:800	Jackson Immunoresearch
Goat anti-Mouse IgG1-Cy3 (GaM1-Cy3)	#115-165-205	1:800	Jackson Immunoresearch
Goat anti-Rabbit-AF488 (GaR-AF488)	#111-545-144	1:800	Jackson Immunoresearch
Goat anti-Rabbit-Cy3 (GaR-Cy3)	#111-165-144	1:800	Jackson Immunoresearch
Goat anti-Rabbit-Cy5 (GaR-Cy5)	#A10523	1:800	Jackson Immunoresearch
Goat anti-Rabbit IgG biotin (GaRb)	#111-065-144	1:1000	Jackson Immunoresearch
Streptavidin-Cy3 (SA-Cy3)	#016-160-084	1:1250	Jackson Immunoresearch
Goat anti-mouse immunoglobulin G (IgG) Fab fragment	#115-007-003	1:100	Jackson Immunoresearch

Table S5. Statistical analysis of the global repartition of myogenic cells and PAX7/MYOD and MYOD/MYOG repartition on Total Myogenic cells. Related to Figures 3E, 3F and 3G.

Two Way ANOVA **Fig. 3E**	**Total PAX7+**	**Total MYOD$^+$**	**Total MYOG$^+$**	
P0 *vs* P3/P4	ns	ns	ns	
P0 *vs* P5/P6	*,**	ns	ns	
P0 *vs* P7-P10	**,***	ns	ns	
P0 *vs* P15	*	ns	ns	
P0 *vs* P21, P28	ns	ns	ns	
P0 *vs* P49	ns	****	ns	
P0 *vs* P56	***	****	**	
P7/P8 *vs* P10/P15	ns	ns	ns	
P10 *vs* P15	ns	ns	ns	
P10 *vs* P21	ns	*	**	
P15 *vs* P21	ns	ns	ns	
P7 *vs* P49/P56	****	****	**/****	
P10 *vs* P49/P56	****	****	***/****	
P15 *vs* P49/P56	****	****	ns/***	
P21 *vs* P49/P56	****	****	ns	
P21 *vs* P28	ns	**	ns	
P28 *vs* P49	*	****	ns	
P28 *vs* P56	****	****	*	
P49 *vs* P56	ns	ns	ns	
Fig. 3F	**PAX7$^+$ MYOD$^-$**	**PAX7$^+$ MYOD$^+$**	**PAX7$^-$ MYOD$^+$**	**PAX7$^-$ MYOD$^-$**
P0 *vs* P3/P4	ns	ns	ns	ns
P0 *vs* P5/P6	ns	****	**,***	ns
P0 *vs* P7/P8	ns	**,ns	ns	ns
P0 *vs* P10/15	ns	****,*	*	ns
P0 *vs* P21	ns	ns	*	ns
P0 *vs* P28	ns	ns	ns	ns
P0 *vs* P49/P56	****	****	ns,**	ns
P3/P4 *vs* P6	ns	***,**	ns	ns
P3/P4 *vs* P49/P56	****	****	****	ns
P6 *vs* P49/P56	****	ns	****	ns
P7/P8/P10 *vs* P15	ns	ns	ns	ns
P7 *vs* P21	ns	ns	ns	*
P7/P10 *vs* P49	****	ns	***,****	*
P7/P10 *vs* P56	****	*,ns	****	****
P8 *vs* P49/P56	****	**,****	****	ns,*
P10 *vs* P21	ns	*	ns	ns
P15 *vs* P21	ns	ns	ns	ns
P15/P21 *vs* P28	**	ns	**	ns
P15/P21 *vs* P49	****	*,****	****	ns
P15/P21 *vs* P56	****	***,****	****	ns
P28 *vs* P49	****	***	ns	ns
P28 *vs* P56	****	****	***	ns
P49 *vs* P56	*	ns	ns	ns
Fig. 3G	**MYOG$^+$ MYOD$^-$**	**MYOG$^+$ MYOD$^+$**	**MYOG$^-$ MYOD$^+$**	**MYOG$^-$ MYOD$^-$**
P0 *vs* P3-P15	ns	ns	ns	ns
P0 *vs* P21	ns	ns	*	ns
P0 *vs* P28	ns	ns	ns	ns
P0 *vs* P49	ns	ns	****	****
P0 *vs* P56	ns	*	****	****
P3 *vs* P7	*	ns	ns	ns
P3 *vs* P10	*	ns	**	ns
P6 *vs* P49/P56	ns	ns	****	****
P7/P8/P10 *vs* P15	ns	ns	ns	ns
P7 *vs* P21	*	ns	***	ns

P8 *vs* P49/P56	ns,**	ns	****	****
P10 *vs* P21	*	ns	****	ns
P7/P10 *vs* P49	*	ns	****	****
P7/P10 *vs* P56	****	ns,*	****	****
P15 *vs* P21	ns	ns	ns	ns
P15 *vs* P28	ns	ns	ns	**
P21 *vs* P28	ns	ns	****	**
P15/P21 *vs* P49/P56	ns	ns	****	****
P28 *vs* P49	ns	ns	****	****
P49 *vs* P56	ns	ns	ns	*

Table S6. Statistical analysis of the distribution of cycling and non-cycling myogenic populations. Related to Figure 3I.

Two Way ANOVA	**Quiescent MuSC**	**Cycling MuSC**	**Cycling PAX7+MYOD+**	**Non-Cycling PAX7+MYOD+**	**Cycling MYOD+**	**Non-Cycling MYOD+**	**MYOG+**
P0 *vs* P3/P4	ns	ns	ns	ns	ns	ns	ns
P0 *vs* P5	ns	ns	**	ns	****	ns	ns
P0 *vs* P6	ns	ns	****	ns	ns	****	ns
P0 *vs*	ns	ns	**	ns	ns	ns	ns
P0 *vs* P10	ns	ns	***	ns	**	ns	ns
P0 *vs* P21	ns	**	**	ns	ns	****	ns
P0 *vs* P28	****	ns	**	ns	ns	ns	ns
P0 *vs* P49	****	ns	****	ns	ns	ns	**
P0 *vs* P56	****	****	****	ns	ns	ns	****
P3/P4 *vs* P49	****	**,*	****	ns	ns	ns	ns,**
P3/P4 *vs* P56	****	****	****	ns	*,**	ns	**,****
P5 *vs* P6	ns	ns	ns	ns	*	****	ns
P5/P6 *vs* P49	****	*,ns	*,ns	ns	****,ns	ns,****	**,ns
P5 *vs* P56	****	****	**	ns	****	ns	****
P6 *vs* P7/P10	ns	ns	ns	ns	ns	****	****
P6 *vs* P15	ns	ns	ns	ns	ns	***	ns
P6 *vs* P21	ns	**	ns	*	ns	ns	ns
P6 *vs* P56	****	****	ns	ns	ns	****	**
P7 *vs* P8	ns	ns	ns	ns	ns	ns	ns
P7/P8 *vs* P15	ns	ns	ns	ns	ns	ns	ns
P7 *vs* P21	ns	ns	ns	ns	ns	***	****
P7/P8 *vs* P49	****	ns	ns	ns	ns	ns,*	****
P7/P8 *vs* P56	****	ns	ns	ns	**,ns	ns,***	****
P10 *vs* P15	ns	ns	ns	ns	ns	ns	ns
P10 *vs* P21	ns	ns	ns	*	*	****	****
P10 *vs* P49	****	ns	ns	ns	****	ns	****
P10 *vs* P56	****	*	ns	ns	****	ns	****
P15 *vs* P21	ns	ns	ns	ns	ns	**	*
P15/P21 *vs* P28	****/**	ns	ns	ns	ns	ns,****	ns
P15 *vs* P49	****	ns	ns	ns	*	ns	***
P15 *vs* P56	****	ns	ns	ns	**	**	****
P21 *vs* P49	****	ns	ns	***	ns	****	ns
P21 *vs* P56	****	ns	*	****	ns	****	**
P28 *vs* P49	****	ns	ns	ns	ns	ns	ns
P28 *vs* P56	****	ns	ns	ns	ns	ns	****
P49 *vs* P56	****	ns	ns	ns	ns	ns	ns

Table S7. Statistical analysis of the repartition of MYOD+ and KI67+ cells among total PAX7+ cells. Related to Figure 5F.

Two Way ANOVA	Quiescent MuSC	Cycling MuSC	Non-cycling PAX7+MYOD+	Cycling PAX7+MYOD+
P0 *vs* P3/P4	ns	ns	ns	ns
P0 *vs* P5	***	ns	ns	***
P0 *vs* P6	****	ns	ns	****
P0 *vs* P7-P15	****	ns	ns	***,****
P0 *vs* P21	****	****	*	****
P0 *vs* P28	****	ns	ns	****
P0 *vs* P49	****	***	**	*****
P0 *vs* P56	****	****	***	*****
P3/P4/P5 *vs* P49/P56	****	****	ns	****
P4 *vs* P5	ns	ns	ns	**
P5 *vs* P6	*	ns	ns	***
P6 *vs* P7	ns	***	ns	*
P6 *vs* P8	ns	****	***	**
P6 *vs* P49/P56	****	****	ns	ns
P7 *vs* P8	ns	ns	ns	ns
P7/P8 *vs* P15	ns	ns	ns	ns
P7 *vs* P21	ns	*	***	ns
P7 *vs* P49	****	*	ns	***
P7 *vs* P56	****	****	*	****
P8 *vs* P49	****	ns	****	****
P8 *vs* P56	****	****	****	****
P10 *vs* P15	ns	ns	*	ns
P10 *vs* P21	ns	****	****	ns
P10 *vs* P49	****	***	ns	****
P10 *vs* P56	****	****	ns	****
P15 *vs* P21	ns	ns	ns	ns
P15 *vs* P28	**	ns	ns	ns
P15/P21 *vs* P49	****	ns	****	***,****
P15 *vs* P56	****	****	****	****
P21 *vs* P28	*	ns	***	ns
P21 *vs* P56	****	ns	****	****
P28 *vs* P49	****	ns	ns	ns
P28 *vs* P56	****	**	*	**
P49 *vs* P56	ns	ns	ns	ns

Supplemental Experimental Procedures

Validation of the coupled antibodies used for flow cytometry analysis. Related to Figure S2B-D. $CD45^-TER\text{-}119^-CD31^-SCA\text{-}1^-CD34^+ITGA7^+$ myogenic cells were purified from P7 mouse muscles, plated on gelatin-coated dish and expanded for 4 days in GM. After trypsinization, cells were fixed and permeabilized. Fixed cells were then divided into 3 tubes and respectively immunostained with the coupled antibodies anti-PAX7-AF647 or anti-MYOD-PE or anti-MYOG-AF488 (see **Table S3**) and FACS sorted. The positive and negative fractions were cytospun and immunostained with antibodies commonly used in the field for immunofluorescence from different species except for PAX7: namely mouse IgG1 anti-PAX7 (sc-81648), rabbit anti-MYOD M-318 (sc-760) and rabbit anti-MYOG M-225 (sc-576) (see **Table S4**). Cells were incubated overnight at 4°C with the relative primary antibody of interest. Then, cells were washed with PBS and incubated with the appropriate secondary antibodies (**Table S4**) for 1 hour at room temperature. Nuclei were stained with DAPI. Cells were mounted on microscopy slides with Fluoromount-G. Images were acquired with Zeiss LSM 800 confocal microscope at 20x magnification. For each myogenic marker, the number of double-positive cells was quantified and expressed as percentage. At least 50 cells were quantified per condition.

Co-immunostaining of PAX7 and MYOG on myogenic cells cultured for 5 days in GM. **Related to Figure S3H.** Cells were fixed, permeabilized and then incubated for one hour with BSA 5%. Cells were then incubated with primary anti-PAX7 antibody (sc-81648) for 1 hour, washed 3 times with PBS and incubated with secondary GaM1-Cy3 antibody for 1 hour. Cells were then washed 3 times with PBS, and incubated 1 hour with coupled anti-MYOG-AF488 antibody (sc-52903, Santa Cruz). Nuclei were stained with DAPI.