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Supplemental Information

Recapitulation of Extracellular LAMININ Environment Maintains Stemness of Satellite Cells *In Vitro*

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Figure S1

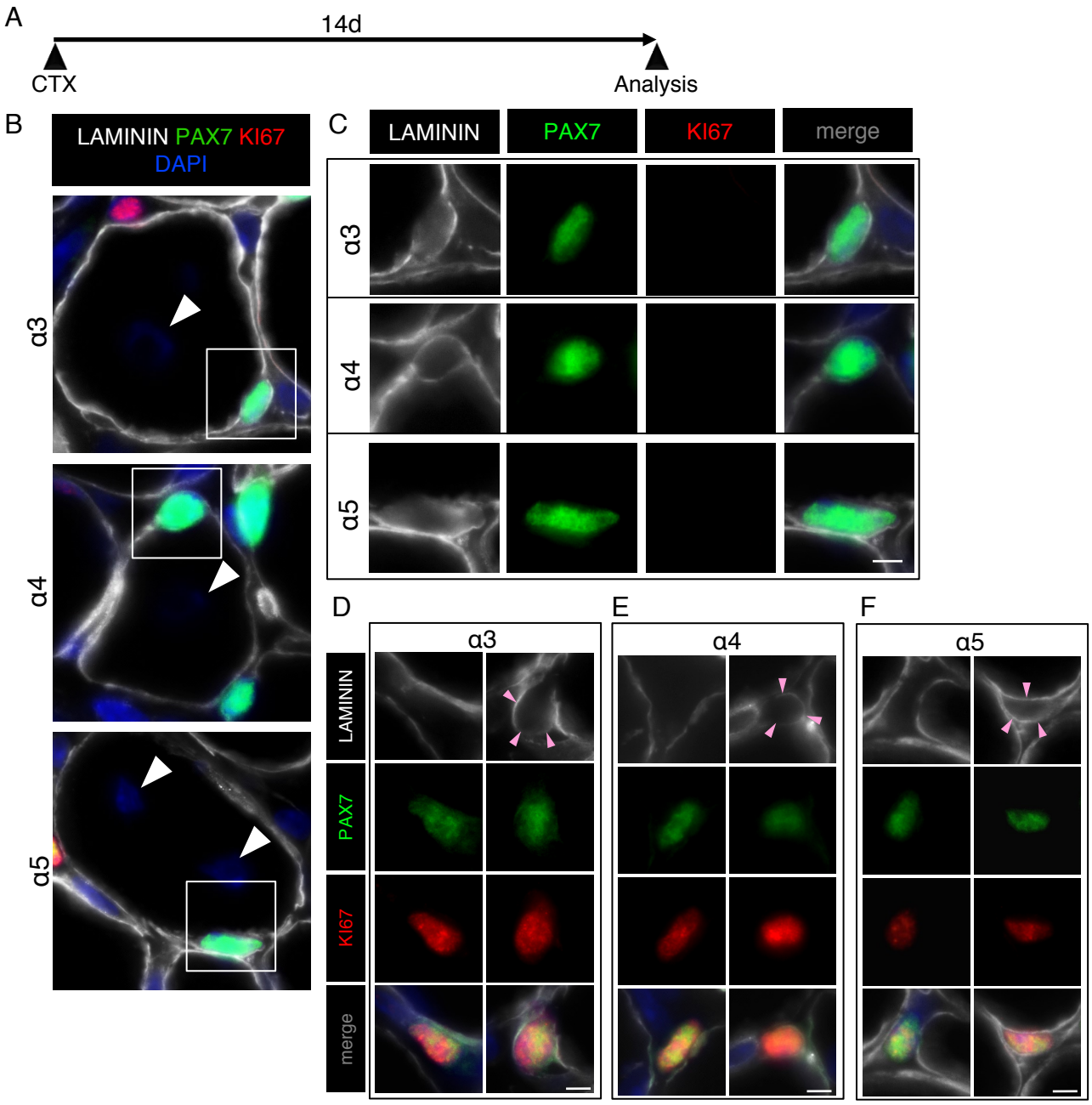

Figure S1. Expression patterns of LMa3, a4, and a5 around self-renewed and activated satellite cells. (A) Sections were obtained from TA muscle 14 days after CTX injection. (B-C) Expression patterns of LMa3, a4, and a5 around PAX7+KI67– self-renewed satellite cells after CTX injection. Muscle tissue stained with anti-PAX7 antibody (green), anti-KI67 antibody (red), and anti-LMa3-5 antibody (white). Arrowheads indicate the centrally located nuclei in regenerated fibers. Scale bar represents 10 μm. (D–F) Expression patterns of LMa3, a4, and a5 around $PAX7^{+}KI67^{+}$ activated satellite cells after CTX injection. Muscle tissue stained with anti-PAX7 antibody (green), anti-KI67 antibody (red) and anti-LMa3–5 antibody (white). Arrowheads indicate expression of LMα3, α4 and α5 around activated satellite cells. Scale bar represents 10 μm.

Figure S2

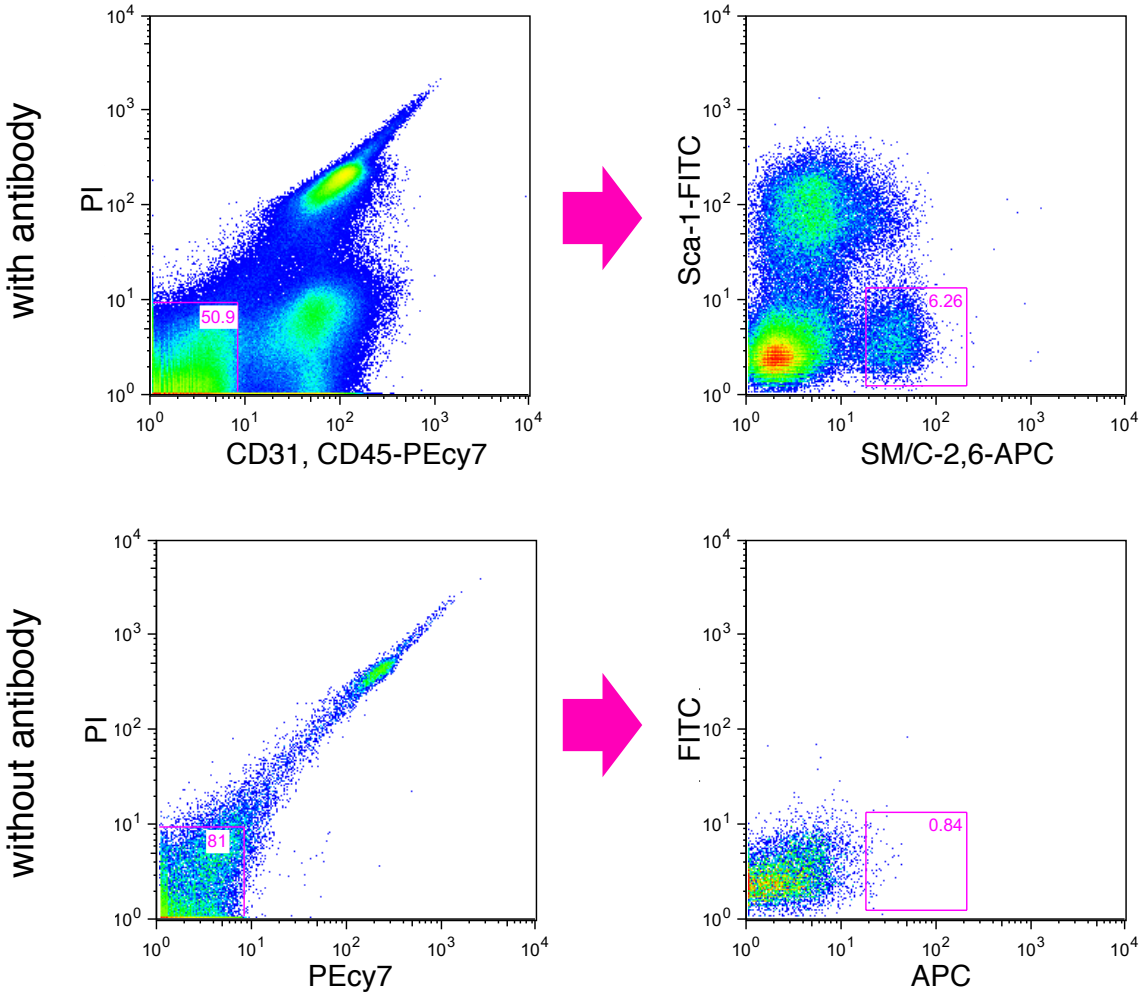

Figure S2. FACS profiles of satellite cells from WT mice. CD31−CD45−Sca-1−SM/C-2.6+ cells were analyzed as muscle satellite cells using MoFlo.

Figure S3

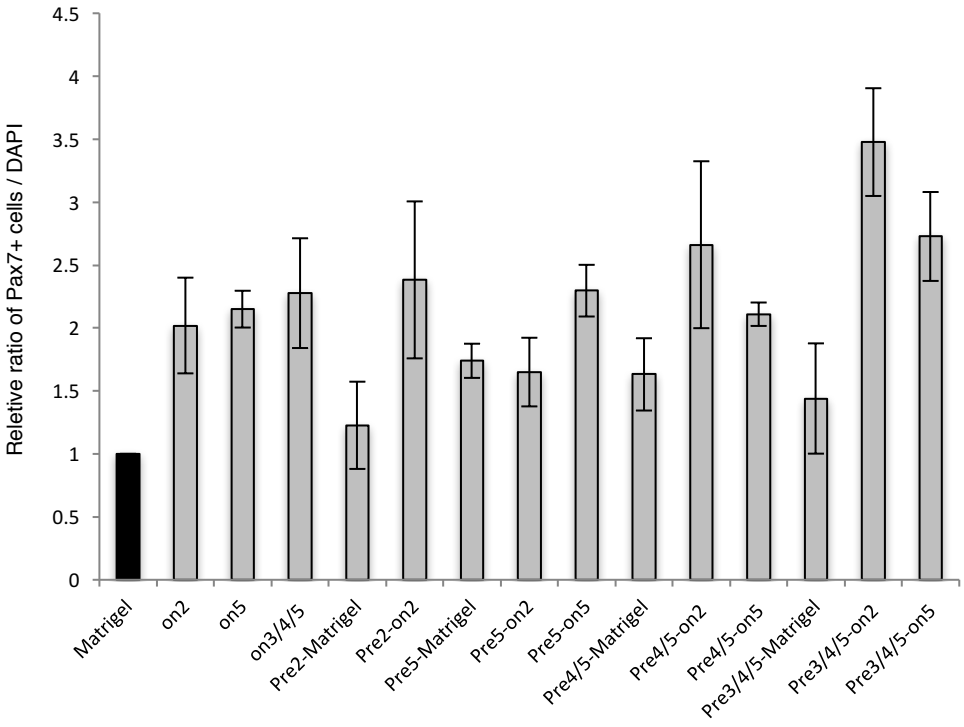

Figure S3. The ratio of PAX7 positive cells in several different culture conditions using the LME8 fragments. Matrigel; cultured on Matrigel coating dish (control), on2; cultured on LM211-E8 coated dish, on5; cultured on LM511-E8 coated dish, on3/4/5; cultured on LM332-, 411- and 511-E8 coated dish, Pre2-Matrigel; cultured on Matrigel coated dish after pre-treated with LM211-E8, Pre2-on2; cultured on LM211-E8 coated dish after pre-treated with LM211-E8, Pre5-Matrigel; cultured on Matrigel coated dish after pre-treated with LM511-E8, Pre5-on2; cultured on LM211-E8 coated dish after pre-treated with LM511-E8, Pre5-on5; cultured on LM511-E8 coated dish after pre-treated with LM511-E8, Pre4/5-Matrigel; cultured on Matrigel coated dish after pre-treated with LM411- and 511-E8, Prelm4/5-lm2; cultured on LM211-E8 coated dish after pre-treated with LM411- and 511- E8, Prelm4/5-lm5; cultured on LM511-E8 coated dish after pre-treated with LM411- and 511-E8, Prelm3/4/5-mt; cultured on Matrigel coated dish after pre-treated with LM332-, 411- and 511-E8, Prelm3/4/5-lm2; cultured on LM211-E8 coated dish after pre-treated with LM332-, 411- and 511- E8, Prelm3/4/5-lm5; cultured on LM511-E8 coated dish after pre-treated with LM332-, 411- and 511-E8. The ratio of the Matrigel was set as 1.0. Error bars, s.e.m.

Figure S4

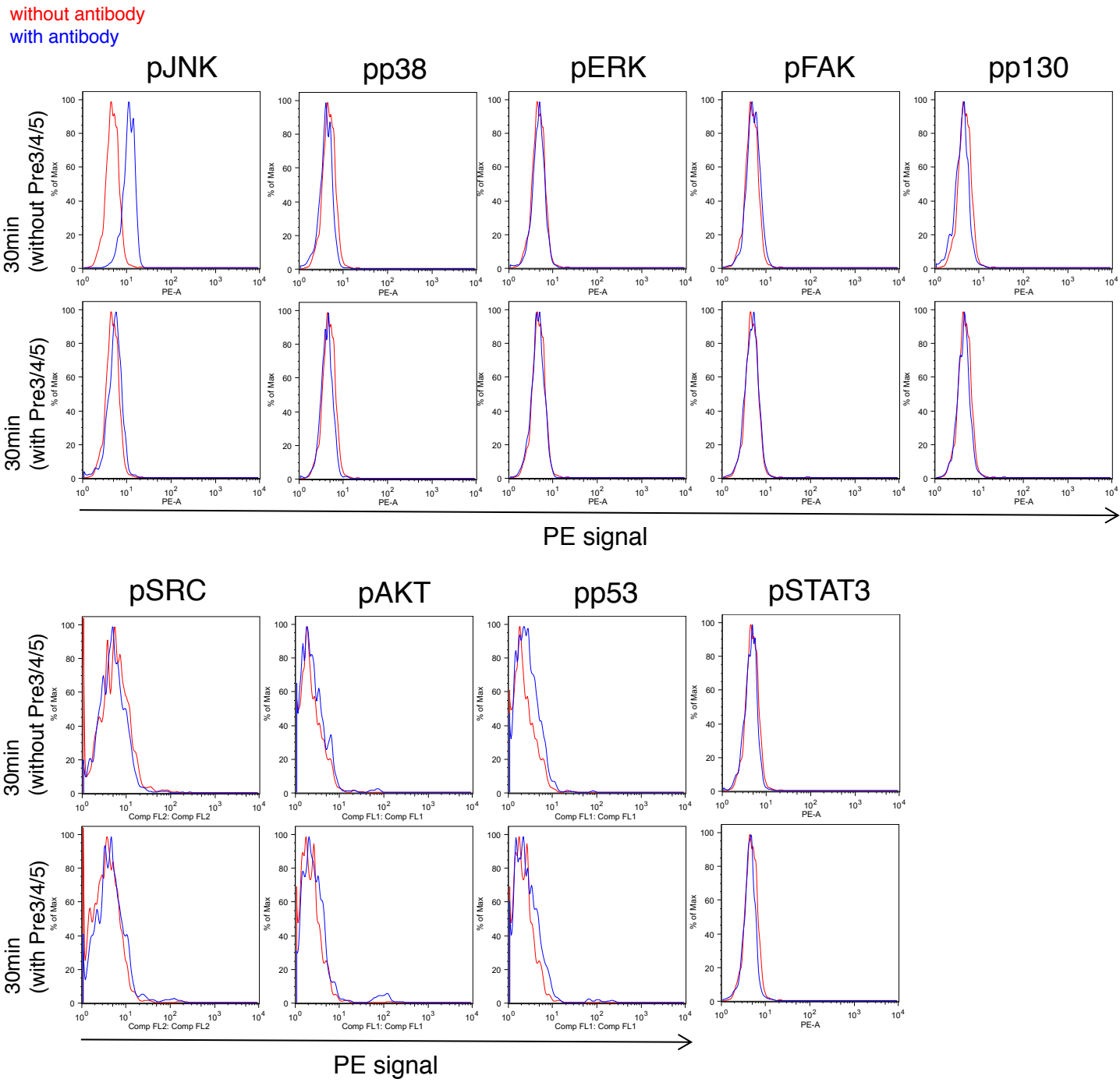

Figure S4. The phosphorylation levels of JNK, ERK, FAK, p38, p130, STAT3, SRC, AKT and p53 in satellite cells. Expression of phosphorylated molecules JNK, ERK, FAK, p38, p130, STAT3, SRC, AKT and p53 were measured by FACS on isolated satellite cells incubated with/without LM332-E8, 411-E8, and 511-E8 for 30 minutes. Histograms represent levels of protein expression. Blue line histogram; controls (without antibodies) and red line histogram; samples (incubated with/without LM-E8).

Figure S5

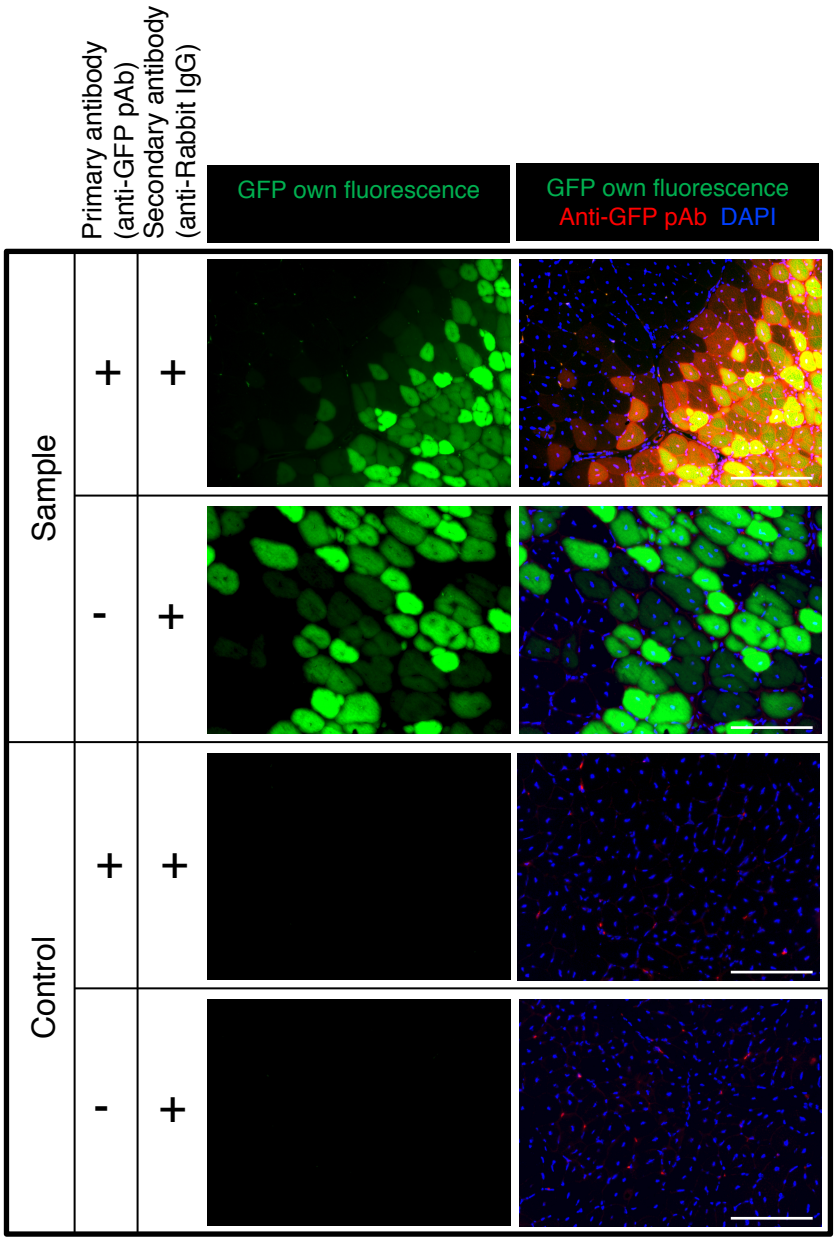

Figure S5. Detection of GFP-positive myofibres from TA muscles transplanted with GFP positive satellite cells. The specificity of GFP-positive myofibres was confirmed by immunostaining with an anti-GFP antibody (red). Scale bar represents 200 μm.

Figure S6

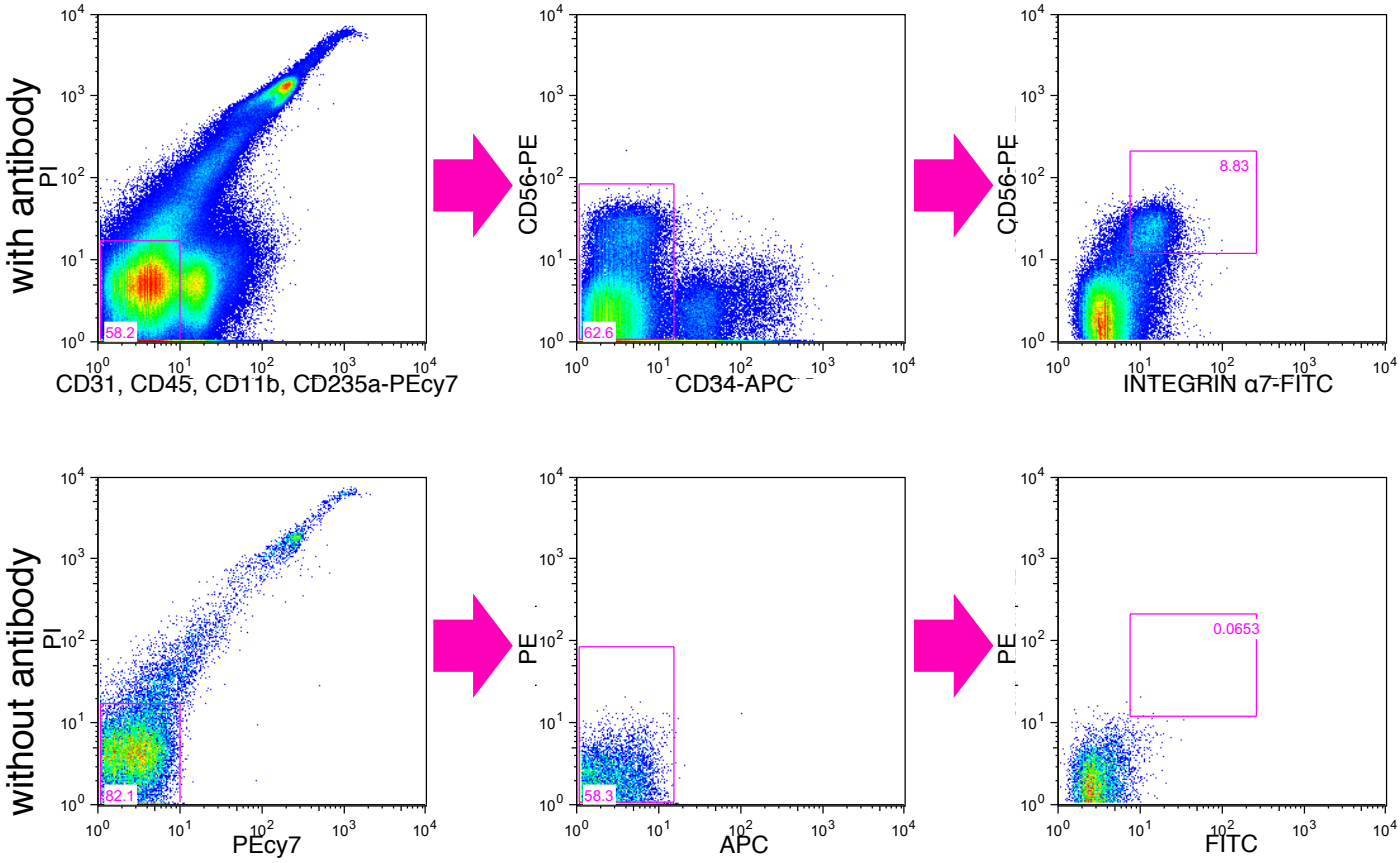

Figure S6. FACS profiles of satellite cells from human.
The CD31−CD45−CD11b−CD235a−CD34−CD56+INTEGRIN α7+ cells were analyzed as human muscle satellite cells using the MoFlo.

Figure S7

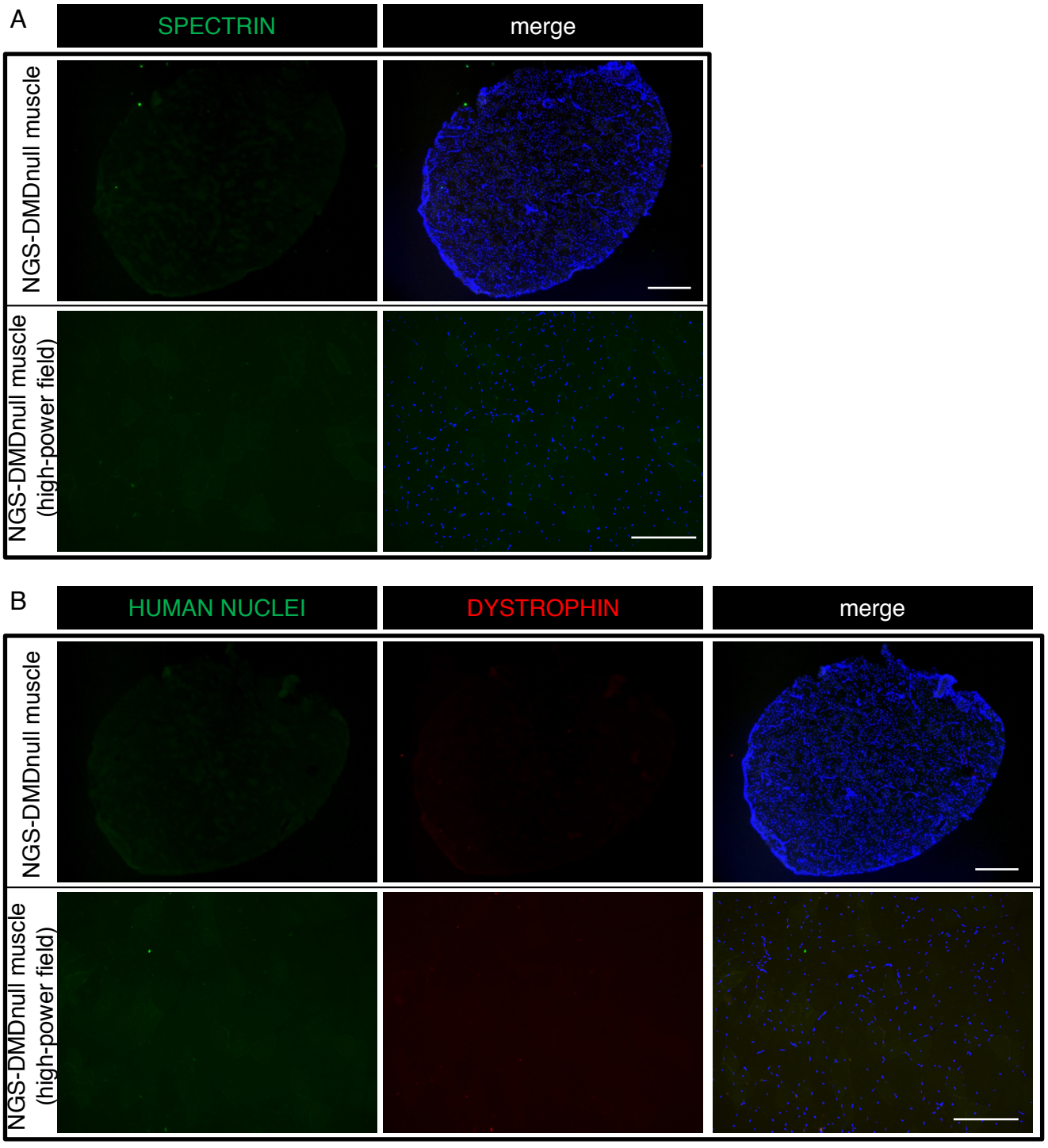

Figure S7. Immunostaining of TA muscle of NSG-DMDnull mice. (A) Immunostaining of cross-section of the contra lateral TA muscle of NSG-DMDnull mice for HUMAN SPECTRIN antibody (green) and DAPI (blue). (B) Immunostaining of cross-section of the contra lateral TA muscle of NSG-DMDnull mice for DYSTROPHYN antibody (red) and HUMAN NUCLEI antibody (green) and DAPI (blue). Scale bar represents 500 μm (upper figures) and 200 μm (lower figures).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse and human samples

C57BL/6 wild-type mice and C57BL/6-Tg (CAG-EGFP) mice were purchased from CLEA Japan, Inc. and Japan SLC, Inc., respectively. Eight to twelve-week-old male mice were analyzed. All procedures for animal experiments were approved by the Tokyo Medical and Dental University Animal Care and Use Committee (Protocol number: 0170282C). Human samples were obtained from semitendinosus muscle from patients undergoing anterior cruciate ligament reconstruction at Tokyo Medical and Dental University Hospital. All experiments were approved by the local Institutional Review Board of Tokyo Medical and Dental University (No. 2121) and all study participants provided written informed consent. NSG mice were purchase from Charles River Japan, Inc. DMD-null/NSG mice were generated by mating DMD-null/NODscid mice (Tanaka et al., 2013) and NSG mice in the Animal Facility of Center for iPS Cell Research and Application (CiRA), Kyoto University. Some animal experiments were carried out in the Animal Facility of CiRA following protocols approved by the Animal Research Committee of Kyoto University (No.10-1-11) and by the Ethics Committee of the Graduate School of Medicine, Kyoto University and the Kyoto University Hospital (No. 57).

Muscle injury model

Mice were anesthetized with isoflurane, and hairs in their hind limbs were shaved. One hundred microliters of CTX (10 μM in 0.9% NaCl; Sigma-Aldrich) was injected into the tibialis anterior (TA) muscle using a 29-gauge needle. Seven or 14 days after injections, mice were euthanized and the frozen tissue sections were prepared for analysis as described above.

Cryosectioning

Mouse TA muscle and human semitendinosus muscle were dissected out and frozen in liquid nitrogen-cooled isopentane (Wako). Using a cryostat (Leica), the frozen TA muscles were sectioned transversely at a thickness of 8 μm, and sections from the widest part of the muscle were attached to MAS-coated slide glasses (MATSUNAMI). Cryosections were kept at −80°C until used for immunostaining.

Immunofluorescence

The cryosections described above were used for immunohistochemistry. Primary satellite cells were cultured on 8-well chamber slides (MATSUNAMI) coated with Matrigel (BD Biosciences) or laminin-E8 fragment. Tissue sections or cells were fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature, and then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in phosphate-buffered saline

(PBS) for 15 minutes at room temperature. After blocking with Power Block Universal Blocking Reagent (BioGenex) or M.O.M. kit (Vector Laboratories), the fixed cells were incubated with primary antibodies overnight at 4°C. After washing, bound primary antibodies were labeled with fluorescence-conjugated secondary antibodies for one hour at room temperature. The immunostained samples were mounted with mounting medium for fluorescence with DAPI (Vector Laboratories). Primary and secondary antibodies were as follows: anti-LAMININ α2 (Sigma-Aldrich, L0663), anti-PAX7 (SantaCruz, sc-81648), anti-KI67 (Leica, NCL-Ki67p), anti-MYOD (SantaCruz, sc-760), anti-pJNK (Cell Signaling, 9251S), anti-p-p38 (Cell Signaling, 9211S), anti-HUMAN NUCLEI (Millipore, MAB1281), anti-SPECTRIN (Leica, NCL-SPEC1), anti-DYSTROPHIN (Abcam, ab15277) and mouse/rabbit/rat IgG-Alexa488, -Alexa594, or Alexa647 (Life Technologies). Fluorescent images were obtained using a BZ-X710 (Keyence) and a LSM700 laser scanning confocal microscope (ZEISS). Fluorescence intensity was quantified by automated image analysis using the BZ-X analysis software (Keyence).

Flow cytometry

Skeletal muscle samples from mouse fore- and hind-limbs and human semitendinosus muscle were dissected out and digested with 0.14% collagenase type II (Worthington Biochemical) for one hour at 37°C. Then, the digested tissue was filtered through 100 μm- and 40 μm-cell strainers (BD Biosciences). The

filtered mononuclear cells were stained with phycoerythrin (PE)-conjugated anti-CD31 (BD Biosciences, 553373), PE-conjugated anti-CD45 (BD Biosciences, 552848), PE-conjugated anti-SCA1 (BD Biosciences, 553335), and biotinylated anti-SM/C-2.6 antibodies and streptavidin-allophycocyanin (APC) (Becton, Dickinson and Company, 555516). In human: PEcy7-conjugated anti-CD31 (BD Biosciences, 563651), PEcy7-conjugated anti-CD45 (BD Biosciences, 557748), PEcy7-conjugated anti-CD11b (BD Biosciences, 557743), PEcy7-conjugated anti-CD235a (BD Biosciences, 563666), APC-conjugated anti-CD34 (BD Biosciences, 555824), PE-conjugated anti-CD56 (BD Biosciences, 555516) and INTEGRIN α7 Antibody (3C12) [FITC] (Novus Biologicals, NBP1-54412) on ice for 30 minutes. Cell sorting was performed using a MoFlo flow cytometer (Beckman), and $CD31^{-}$, $CD45^{-}$, $SCA\text{-}1^{-}$, and $SM/C\text{-}2.6^{+}$ cells were collected as mouse satellite cells, and $CD31^{-}$, $CD45^{-}$, $CD11b^{-}$, $CD235a^{-}$, $CD34^{-}$, $CD56^{+}$, INTEGRIN $\alpha7^{+}$ cells were collected as human satellite cells.

Phosphorylated signal analysis

For intracellular staining of phosphorylated JNK, ERK, FAK, p38 MAPK, p130CAS, STAT3, SRC, AKT and p53, stimulated cells were fixed with fixation buffer (BD bioscience) for 15 min at 37°C, and then permeabilized with perm wash buffer (BD bioscience) for 25 min at 37°C. Cells were incubated with PE

Mouse anti-JNK (pT183/pY185) (BD Bioscience, 562480), PE Mouse anti-ERK1/2 (pT202/pY204) (BD Bioscience, 612566), PE Mouse anti FAK (pS910) (BD Bioscience, 558450), PE Mouse anti-p38 MAPK (pT180/pY182) (BD Bioscience, 612565), PE Mouse anti-p130CAS (pY249) (BD Bioscience, 558538), PE Mouse anti-STAT3 (pY705) (BD Bioscience, 612569), PE Mouse anti-SRC (BD Bioscience, 560094), Alexa Fluor 488 Mouse anti-Akt (pS473) (BD Bioscience, 560404) and Alexa Fluor 488 Mouse anti-p53 (pS37) (BD Bioscience, 560282). Phospho-flow analysis was performed using an Aria flow cytometer (BD Bioscience).

RT-PCR

Total RNA was isolated from sorted cells and primary myogenic culture using TRY reagent (Sigma-Aldrich). cDNA was generated from 0.5 µg total RNA using SuperScript III First- Strand Synthesis SuperMix for qRT-PCR (Invitrogen), previously treated with DNAse. RT-PCR was performed using Applied Biosystems Step One Real Time PCR System. PCR was performed in duplicate with reaction volumes of 10 µl, containing Fast SYBER Green Master Mix (Applied Biosystems), forward and reverse primers and cDNA template. Data were analyzed using a comparative critical threshold (Ct) method where the amount of target normalized to the amount of endogenous control reactive to control value is given by

$2^{-\Delta\Delta Ct}$. We used following primers: *HPRT*: 5′-tcagtcaacggggacataaa-3′ (forward), 5′-ggggctgtactgcyyaaccag-3′ (reverse), *Pax7*: 5′-ctcagtgagttcgattagccg-3′ (forward), 5′-agacggttccctttgt-3′ (reverse), *Myogenin*: 5′-gaaatgaatgaggccttcg-3′ (forward), 5′-caaatgatctcctgggttgg-3′ (reverse).

Statistical analysis

Sample sizes, replicate numbers, and p-values are stated in the figure legends. Three or more animals were used per experiment. Biological replicates were tested with individual mouse and human samples. Sample sizes were not based on power calculations. No animals used for the experiments were excluded from analyses. Results were shown as means ± standard deviations or standard errors of the mean, depending on the size of the samples. *P* values are indicated either with the number on the graphs or with asterisks: **P*<0.05, ***P*<0.01, and ****P*<0.001. Differences between groups were assessed by using the Student's two-tailed t test for independent samples. Differences among more than two groups were analyzed using one-way ANOVA followed by Tukey-Kramer post-hoc tests. Values of *P*<0.05 were considered statistically significant. All data are means ± SEM.