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Supplemental Information

The Ubiquitin-Proteasome System Is Indispensable for the Maintenance of Muscle Stem Cells

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Figure S1

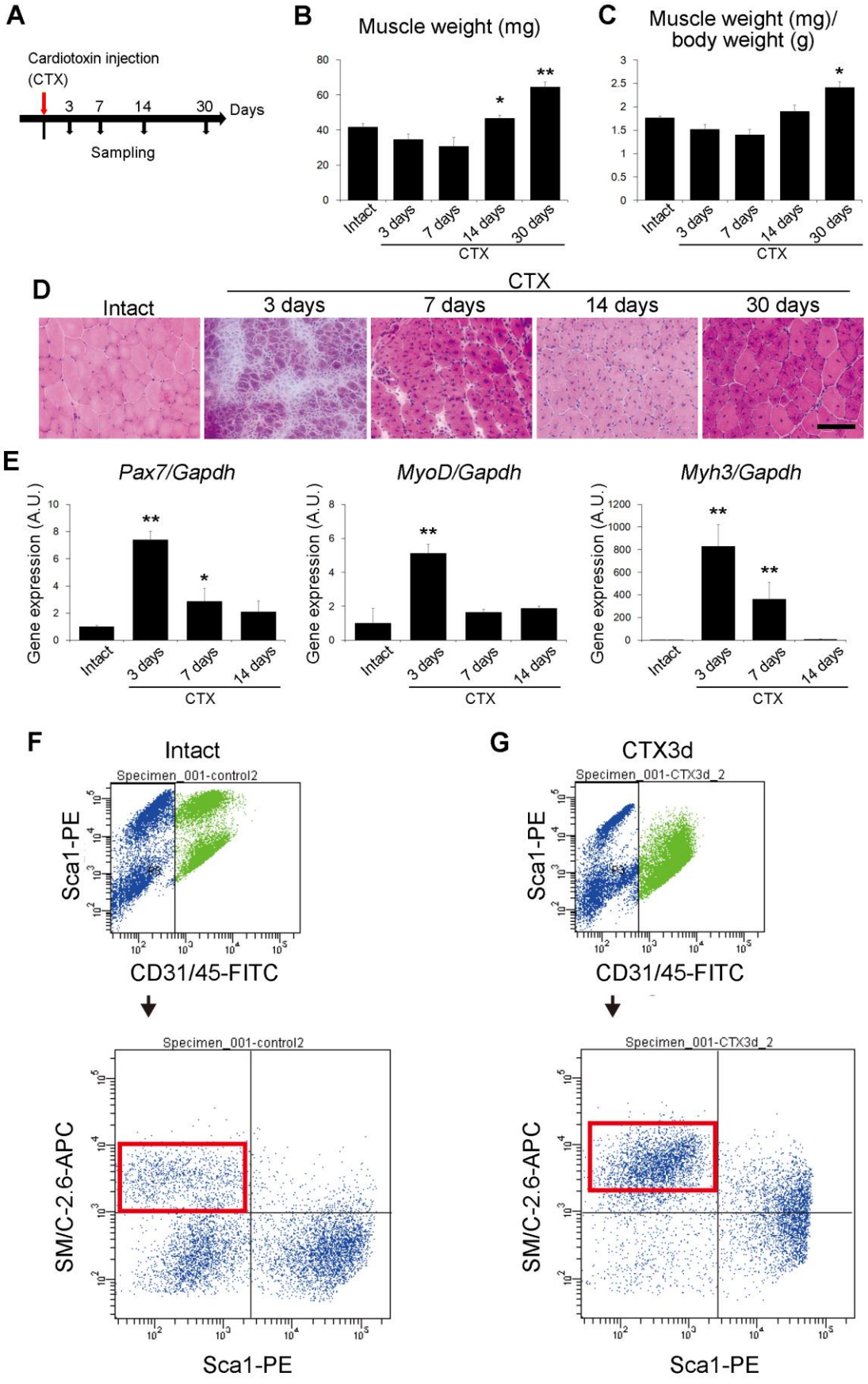
A
Cardiotoxin injection
(CTX)
3
7
14
30
Days
Sampling
B
Muscle weight (mg)
Intact
3 days
7 days
14 days
30 days
CTX
C
Muscle weight (mg)/
body weight (g)
D
Intact
CTX
3 days
7 days
14 days
30 days
E
Pax7/Gapdh
MyoD/Gapdh
Myh3/Gapdh
Gene expression (A.U.)
F
Intact
Specimen_001-control2
Sca1-PE
CD31/45-FITC
SM/C-2.6-APC
G
CTX3d
Specimen_001-CTX3d_2

Figure S1. Histological and gene expression analysis during muscle regeneration. Related to Figure 1. (A) Time course for cardiotoxin (CTX) treatment and tissue harvesting. (B and C) Change in muscle weight (mg) and muscle weight (mg)/body weight (g). Data represent mean ± s.e.m. (t-test: *P < 0.05, **P < 0.01 vs intact (untreated) tissue, n = 5 per group). (D) HE staining of intact muscles and those injured by CTX injection analyzed at 3, 7, 14, and 30 days after injury. Scale Bar: 100 μm. Also shown in Figure 2F. (E) Real-time RT-PCR was used to measure the mRNA expression of myogenic regulatory factor genes (*Pax7* and *MyoD*) and *Myh3* in the tibialis anterior (TA) muscles at 3, 7, and 14 days after injury. Data represent means ± s.e.m. (t-test: *P < 0.05, **P < 0.01 vs intact; n = 3–5 per group). AU, arbitrary units. (F and G) FACS profiles of mononuclear cells derived from intact or injured muscles 3 days after cardiotoxin (CTX) injection. The red gated profiles show satellite cell fractions (SM/C-2.6+ CD31− CD45− Sca1−).

Figure S2

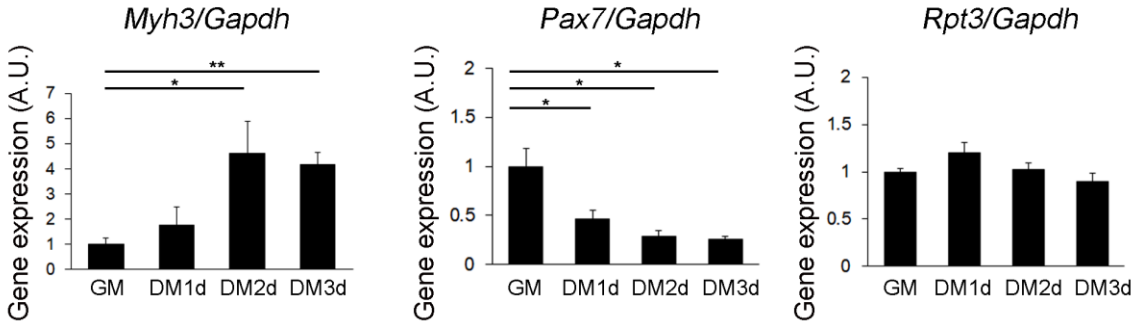

Figure S2. Relative expression of *Rpt3* mRNA in primary myoblasts during proliferation and differentiation process. Related to Figure 2. Relative expression of *Myh3*, *Pax7*, and *Rpt3* mRNA in primary myoblasts during proliferation and differentiation process. Data represent means ± s.d. (t-test: *P < 0.05, **P < 0.01; n = 3 per group). GM, growth medium. DM, differentiation medium. AU, arbitrary units.

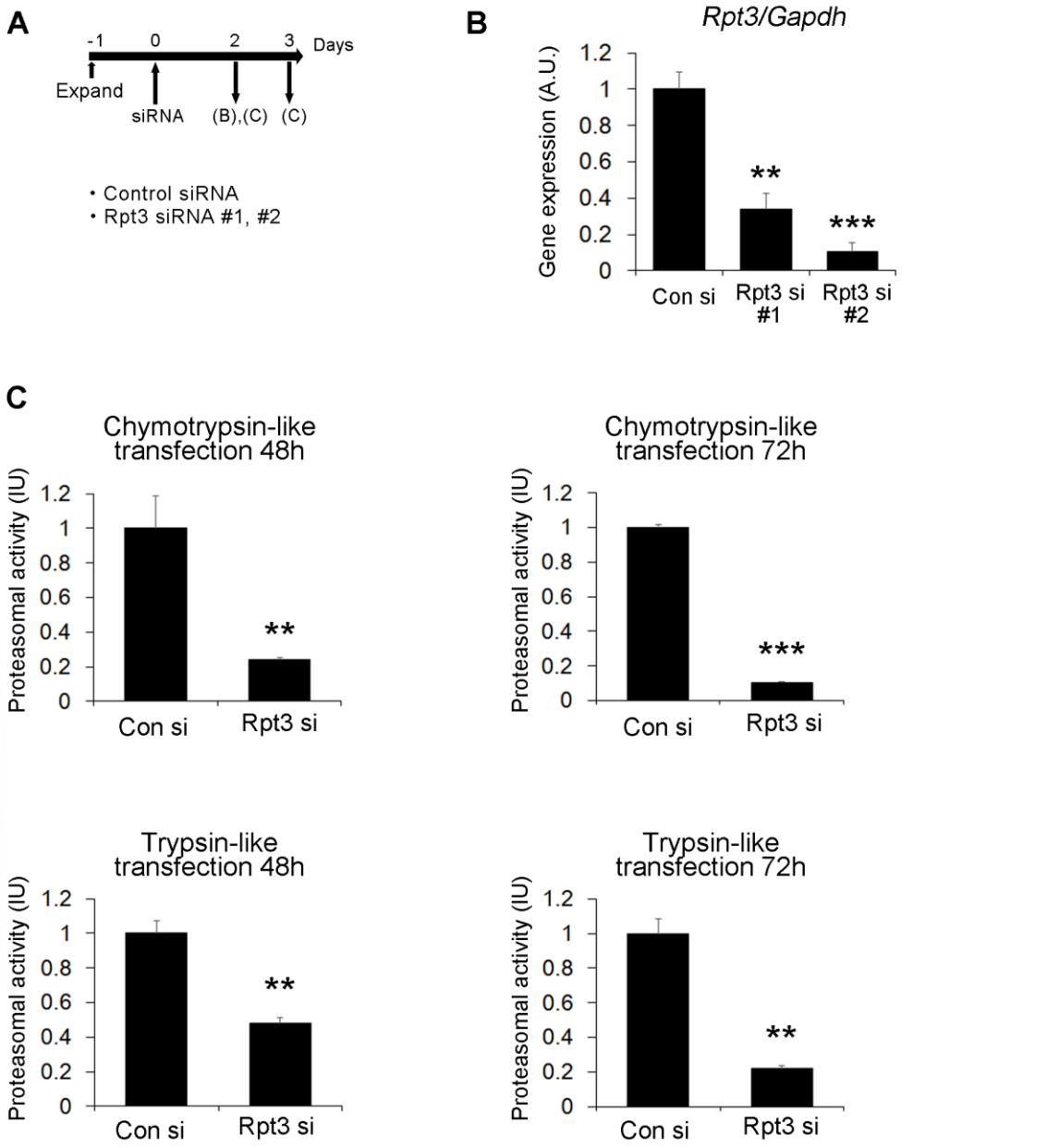

Figure S3. Inactivation of proteasome activity by *Rpt3* knockdown in C2C12 cell line. Related to Figure 2. (A) Time course of *Rpt3* siRNA knockdown in C2C12 cell line. (B) Relative expression of *Rpt3* and *Gapdh* mRNA in C2C12 cells after *Rpt3* siRNA transfection. Data represent means ± s.e.m. (t-test: **P < 0.01, ***P < 0.001; n = 3 per group). AU, arbitrary units. (C) Chymotrypsin-like and trypsin-like proteasome activities (relative to control siRNA) in C2C12 cell line. Data represent means ± s.e.m. (t-test: **P < 0.01, ***P < 0.001; n = 3 per group). IU, international units.

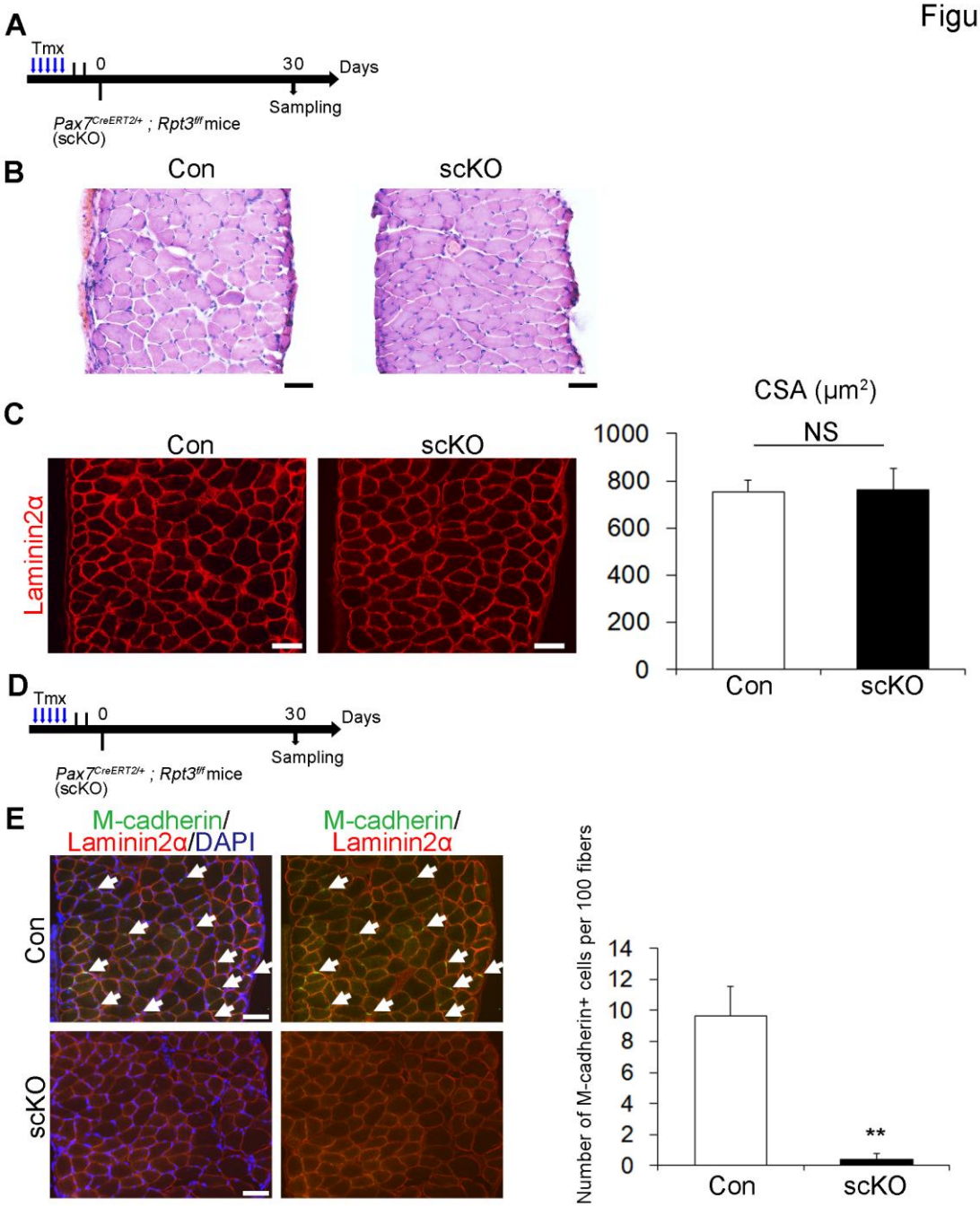

Figure S4. Histological analysis in diaphragm muscle 1 month after tmx treatment. Related to Figure 2 and Figure 4.

(A) Time course for tamoxifen (Tmx) treatment and tissue harvesting. Con indicates *Rpt3*$^{f/f}$ mice and scKO indicates satellite cell-specific *Rpt3* knockout mice (*Pax7*$^{CreERT2/+}$; *Rpt3*$^{f/f}$). (B) HE staining of intact diaphragm muscles. Scale Bar: 50 μm. (C) Immunostaining for Laminin2α in intact diaphragm muscle cryosections from scKO mice. Scale Bar: 50 μm. The y-axis shows the mean cross-sectional area (CSA). Data represent means ± s.d. (t-test: NS, no significance; n = 4 per group). (D) Time course for Tmx treatment and tissue harvesting. (E) Immunostaining for M-cadherin (green), Laminin2α, and DAPI (blue) in intact diaphragm muscle cryosections from scKO mice. Arrows indicate satellite cells. Scale Bar: 50 μm. The y-axis shows average number of M-cadherin+ cells per 100 fibers. Data represent means ± s.d. (t-test: **P < 0.01; n = 3 per group).

Figure S5

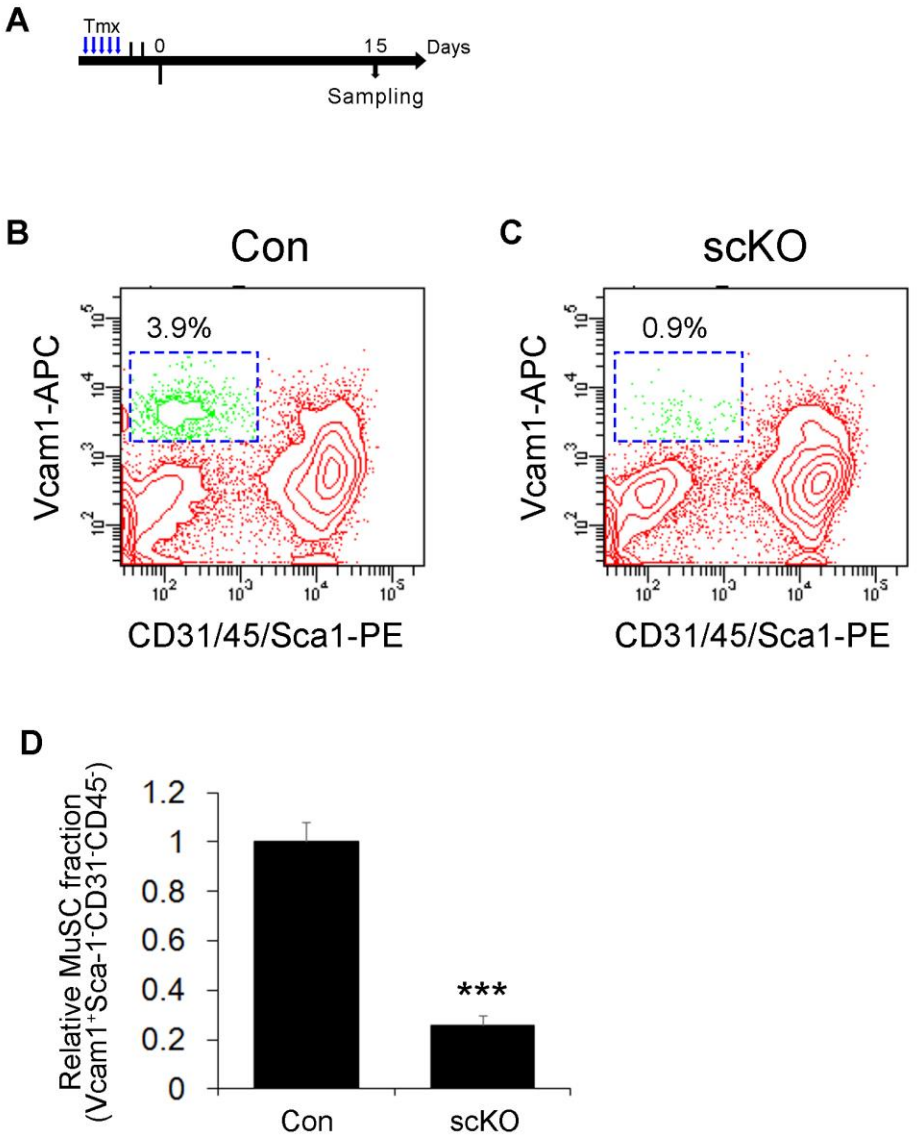

Figure S5. FACS profiles of mononuclear cells derived from scKO mice 15 days after Tmx treatment. Related to Figure 4. (A) Time analysis of tamoxifen (Tmx) treatment and tissue harvesting. Con indicates *Rpt3*$^{f/f}$ mice and scKO indicates satellite cell-specific *Rpt3* knockout mice (*Pax7*$^{CreERT2/+}$; *Rpt3*$^{f/f}$). (B and C) FACS profiles of mononuclear cells derived from Con and scKO mice 15 days after Tmx treatment. The blue gated profiles show satellite cell fractions (Vcam1+ CD31− CD45− Sca1−) from Con and scKO mice. (D) Relative satellite cell fractions from Con and scKO mice. Data represent mean ± s.e.m. (t-test: *** $P < 0.001$; n = 3 for each group).

Figure S6

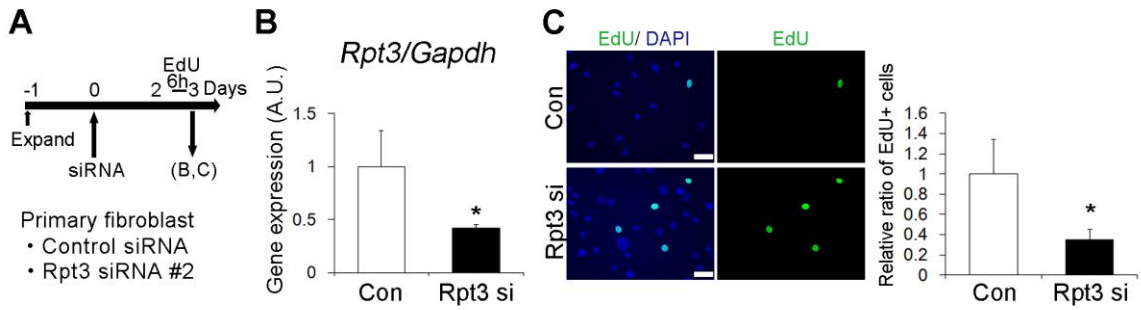

Figure S6. Proliferation defects in *Rpt3* knockdown of primary fibroblast. Related to Figure 6.

(A) Time course of *Rpt3* siRNA knockdown in primary fibroblast. (B) Relative expression of *Rpt3* and *Gapdh* mRNA in primary fibroblast after *Rpt3* siRNA transfection. Data represent means ± s.e.m. (t-test: *P < 0.05; n = 3 per group). AU, arbitrary units. (C) Immunostaining for EdU (green) and DAPI (blue) in primary fibroblast treated after *Rpt3* siRNA transfection. Scale bar: 50 μm. The y-axis shows the relative ratio of EdU-positive cells. Data represent means ± s.e.m. (t-test: *P < 0.05; n = 3 per group).

Figure S7

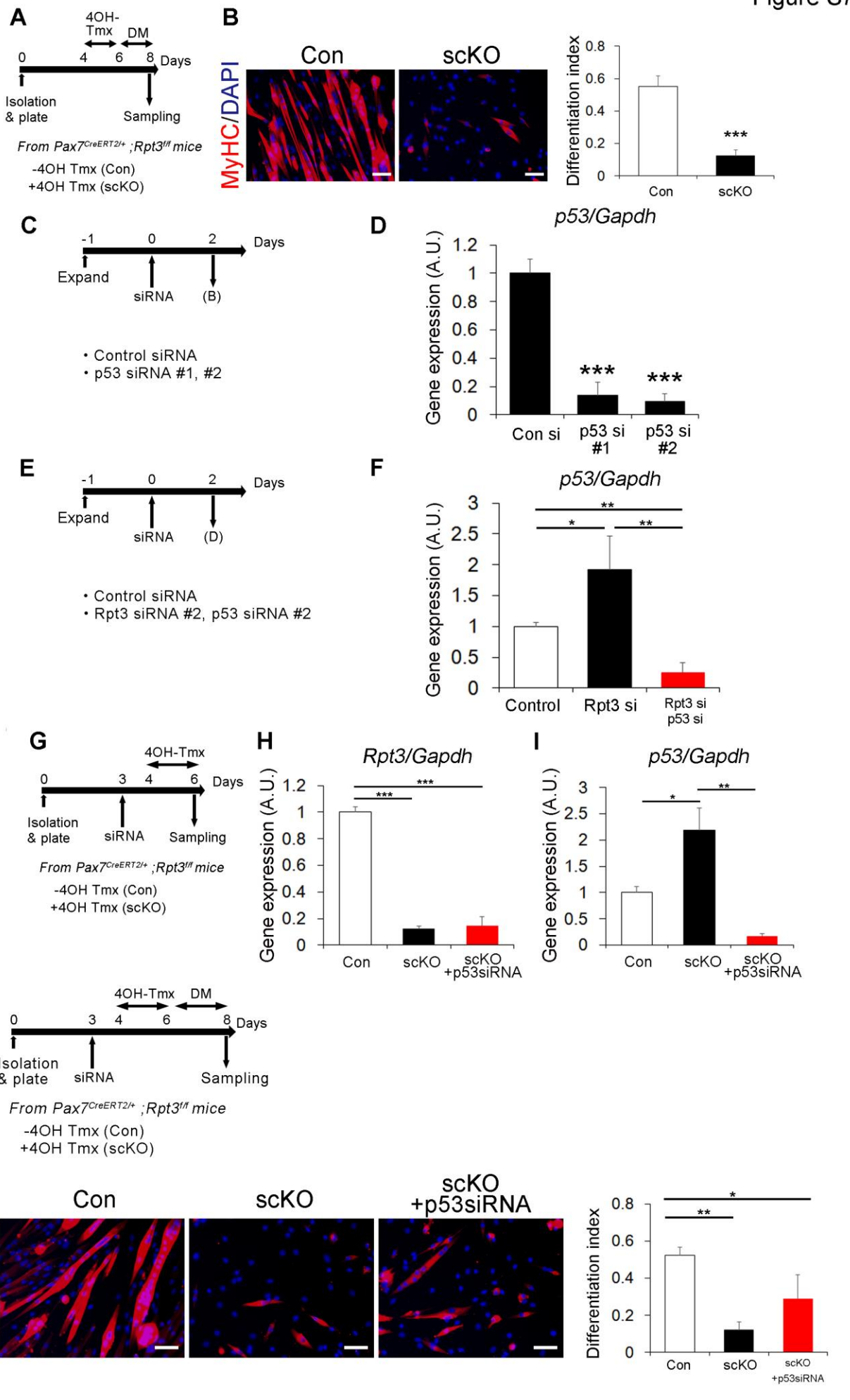
A
4OH-Tmx
DM
0
4
6
8
Days
Isolation & plate
Sampling
From Pax7CreERT2/+ ;Rpt3f/f mice
-4OH Tmx (Con)
+4OH Tmx (scKO)
B
Con
scKO
MyHC/DAPI
Differentiation index
0.8
0.6
0.4
0.2
0
***
Con
scKO
C
-1
0
2
Days
Expand
siRNA
(B)
• Control siRNA
• p53 siRNA #1, #2
D
p53/Gapdh
Gene expression (A.U.)
1.2
1
0.8
0.6
0.4
0.2
0
***
***
Con si
p53 si #1
p53 si #2
E
-1
0
2
Days
Expand
siRNA
(D)
• Control siRNA
• Rpt3 siRNA #2, p53 siRNA #2
F
p53/Gapdh
Gene expression (A.U.)
3
2.5
2
1.5
1
0.5
0
**
*
**
Control
Rpt3 si
Rpt3 si p53 si
G
4OH-Tmx
0
3
4
6
Days
Isolation & plate
siRNA
Sampling
From Pax7CreERT2/+ ;Rpt3f/f mice
-4OH Tmx (Con)
+4OH Tmx (scKO)
H
Rpt3/Gapdh
Gene expression (A.U.)
1.2
1
0.8
0.6
0.4
0.2
0
***
***
Con
scKO
scKO +p53siRNA
I
p53/Gapdh
Gene expression (A.U.)
3
2.5
2
1.5
1
0.5
0
*
**
Con
scKO
scKO +p53siRNA
J
4OH-Tmx
DM
0
3
4
6
8
Days
Isolation & plate
siRNA
Sampling
From Pax7CreERT2/+ ;Rpt3f/f mice
-4OH Tmx (Con)
+4OH Tmx (scKO)
K
Con
scKO
scKO +p53siRNA
MyHC/DAPI
Differentiation index
0.8
0.6
0.4
0.2
0
*
**
Con
scKO
scKO +p53siRNA

Figure S7. Deletion of Rpt3 in satellite cells induces differentiation defects. Related to Figure 6 and Figure 7.

(A) Time course analysis of tamoxifen (Tmx) treatment. (B) Myoblasts were induced to differentiate for 2 days and stained for MyHC (red); nuclei were counterstained with DAPI (blue). Scale bar: 50 μm. The differentiation index was calculated by dividing the number of nuclei in myotubes (MyHC-positive elongated cells) by the total number of nuclei. Data are shown as mean ± s.d. (t-test: ***P < 0.001; n = 4 per group). (C) Time course analysis of p53 siRNA knockdown in C2C12 cell line. (D) Relative expression of *p53* and *Gapdh* mRNA in C2C12 cells after p53 siRNA transfection. Data represent means ± s.e.m. (t-test: ***P < 0.001; n = 3 per group). AU, arbitrary units. (E) Time course analysis of *Rpt3* and *p53* siRNA knockdown in C2C12 cell line. (F) Relative expression of *p53* and *Gapdh* mRNA in C2C12 cells after *Rpt3* and *p53* siRNA transfection. Data represent means ± s.e.m. (one-way ANOVA followed by the Bonferroni post hoc test: * P < 0.05, ** P < 0.01; n = 4 per group). AU, arbitrary units. (G) Time course for analysis of *p53* siRNA knockdown and Tmx treatment. (H) Real-time RT-PCR was used to measure the mRNA expression of *Rpt3*. Data represent means ± s.e.m. (one-way ANOVA followed by the Bonferroni post hoc test: *** P < 0.001; n = 5-6 per group). AU, arbitrary units. (I) Real-time RT-PCR was used to measure the mRNA expression of *p53*. Data represent means ± s.e.m. (one-way ANOVA followed by the Bonferroni post hoc test: * P < 0.05, ** P < 0.01; n = 5-6 per group). AU, arbitrary units. (J) Time course analysis of *p53* siRNA knockdown and Tmx treatment. (K) Myoblasts were induced to differentiate for 2 days and stained for MyHC (red); nuclei were counterstained with DAPI (blue). Scale bar: 50 μm. The differentiation index was calculated by dividing the number of nuclei in myotubes (MyHC-positive elongated cells) by the total number of nuclei. Data are shown as mean ± s.d. (one-way ANOVA followed by the Bonferroni post hoc test: *P < 0.05, **P < 0.01; n = 3–5 per group).

Supplementary Table 1. Primers for quantitative RT-PCR. Related to Figure 2, Figure 6, Figure 7, and Figure S1, S2, S3, S6, S7.

Pax7-F	GTGCCCTCAGTGAGTTCGATTAGC	*Pax7-R*	CCACATCTGAGCCCTCATCCA
MyoD-F	AGCACTACAGTGGCGACTCA	*MyoD-R*	GCTCCACTATGCTGGACAGG
Rpt3-F	CTACAGGCCTTGCTCAGCTC	*Rpt3-R*	AGATTGTGGGCGTCTGTAGG
Gapdh-F	AACTTTGGCATTGTGGAAGG	*Gapdh-R*	CACATTGGGGGTAGGAACAC
Tbp-F	CAGATGTGCGTCAGGCGTTC	*Tbp-R*	TAGTGATGCTGGGCACTGCG
Myh3-F	GCCAGGATGGGAAAGTCACTGTGG	*Myh3-R*	GGGCTCGTTCAGGTGGGTCAGC
p53-F	ACGCTTCTCCGAAGACTGG	*p53-R*	AGGGAGCTCGAGGCTGATA
p21(Cdkn1a)-F	CGGTGTCAGAGTCTAGGGGA	*p21(Cdkn1a)-R*	AGGATTGGACATGGTGCCTG
Cyclind1(Ccnd1)-F	AGTGCGTGCAGAAGGAGATT	*Cyclind1(Ccnd1)-R*	CTCTTCGCACTTCTGCTCCT
Cdk4-F	GGCCCTCAAGAGTGTGAGAG	*Cdk4-R*	CATCAGCCGTACAACATTGG

Supplementary Table 2. Microarray analysis identified 1254 genes that were up- or down-regulated with p-values < 0.05 (satellite cells from satellite cell-specific *Rpt3* mice vs satellite cells from control mice). Related to Figure 7.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse strains

The Experimental Animal Care and Use Committee of Nagasaki University approved animal experimentation (Ref. No. 1203190970). Rpt3-floxed mice (Kitajima et al., 2014; Tashiro et al., 2012) were crossed with *Pax7*^{CreERT2} mice (Lepper and Fan, 2010) to generate *Pax7*$^{CreERT2/+}$; *Rpt3*$^{f/f}$ (satellite cell-specific *Rpt3* knockout) mice. All mice used for these experiments were between 2 and 3 months of age.

Mouse tissue preparation

The body and wet muscle were weighed. The TA muscles were collected individually using standard dissection methods and cleaned of excess fat, connective tissue, and tendons. The TA and diaphragm muscles were frozen in isopentane cooled with liquid nitrogen for histological and immunocytochemical analysis, and directly in liquid nitrogen for RNA isolation, and stored at -80 °C.

Mouse treatments

Tamoxifen (Tmx; Sigma) was dissolved in corn oil at a concentration of 20 mg/ml, and experimental and control mice were injected intraperitoneally with 150 μl (3mg) once per day for 5 days to induce Cre mediated excision.

Immunostaining

Immunocytochemistry of satellite cells and isolated single fibers was performed as described previously (Kitajima et al., 2016). Samples were incubated with primary antibodies at 4°C overnight following blocking/permeabilization with phosphate-buffered saline containing 0.3% triton X-100 and 5% goat serum for 20 min at RT. For immunohistochemistry, cryosections of muscle tissue were fixed with 4% PFA, blocked with 5 % goat serum or the M.O.M kit (Vector Laboratories) for 30 min at room temperature, and incubated with primary antibodies at 4 °C overnight. All immunostaining samples were visualized using appropriate species-specific Alexa Fluor 488 and/or 546 fluorescence-

conjugated secondary antibodies (Life Technologies). Samples were then observed using an Olympus fluorescence microscope IX83 (Olympus).

For EdU detection, the Click chemical reaction was performed after primary and secondary staining according to the manufacturer's instructions using a Click-iT EdU Imaging Kit (Life Technologies).

In vivo EdU-uptake assay

EdU was dissolved in PBS at 0.5 mg/ml and injected intraperitoneally at 0.1 mg per 20 g body weight at the time points indicated.

Immunoblotting

Total protein lysates were extracted from the mouse TA muscle and cultured satellite cells for immunoblotting analysis. We used the BCA method to determine protein concentrations. Then, the protein fractions were extracted with a reducing sample buffer containing 5% β-mercaptoethanol and complete protease inhibitor cocktail (Roche). The protein (20 μg per lane) samples were separated on a 10-20 % gradient SDS-polyacrylamide gels and subsequently transferred to polyvinylidene difluoride membranes (Millipore) at 250 mA for 1 h. The membrane was then incubated with primary antibodies. Specific signals were detected using the enhanced chemiluminescence method (GE Healthcare), as described previously (Kitajima et al., 2014). Densitometry was measured using ImageJ software (National Institute of Health).

Muscle injury

Cardiotoxin (CTX; Sigma-Aldrich) was prepared by dissolving a freshly opened tube in 0.9%NaCl at 10μM. Next, 50μl of CTX (10 μM) was injected percutaneously into the left TA muscles of anesthetized mice. Mice were allowed to recover for 3,7,14, or 30 days post-injury. Injected and contralateral TA muscles were harvested for sectioning and staining.

Preparation and FACS analyses of satellite cells

Mononuclear cells from uninjured limb muscles were prepared using 0.2% collagenase type II (Worthington Biochemical) as previously described (Kitajima et al., 2016). Mononuclear cells derived from skeletal muscle were stained with FITC-conjugated anti-CD31 (Biolegend, Cat. No. 102405), anti-CD45 (Biolegend, Cat. No. 103107), PE-conjugated anti-Sca-1 (Biolegend, Cat. No. 122507), and biotinylated anti-SM/C-2.6 (Fukada et al., 2004) antibodies. Cells were then incubated with streptavidin-APC (BD Biosciences, Cat. No. 554067) on ice for 30 min, and resuspended in PBS containing 2% FBS. Cell sorting was performed using a FACS Aria II flow cytometer (BD Immunocytometry Systems). Debris and dead cells were excluded by forward scatter, side scatter, and PI gating. Data were collected using FACS Diva software (BD Biosciences).

Proteasome activity

Proteasome activity was assessed using a Proteasome-Glo™ Assay kit (Promega), as we previously described (Gomes et al., 2001; Kitajima et al., 2014). The chymotrypsin-like and trypsin-like proteasome activities assays were conducted in a total volume of 100 μl in opaque 96-well plates. For the assays, protein contents from TA muscles or cells were added to assay buffer containing 20 mM Tris HCl (pH 7.2), 0.1 mM EDTA, 5 mM ATP, 1 mM β-mercaptoethanol, 20% glycerol and 0.04% Nonidet P40. The individual proteasome reagents were added separately, and the luminescence was recorded as relative light units on a Varioskan luminometer 30 min later (Thermo Scientific). Each sample was measured in duplicate or triplicate.

Real time PCR

Total RNA was isolated using RNeasy (Qiagen). For real-time PCR, first-strand cDNA was synthesized using oligo-dT primers. The expression levels of selected genes were analyzed using the Bio-Rad CFX96 system according to the manufacturer's instructions and quantitative PCR analysis was performed in triplicate using specific primers (Supplementary Table 1).

Gene expression microarrays

The cRNA was amplified, labeled, and hybridized to a 60K Agilent 60-mer oligomicroarray according to the manufacturer's instructions. All hybridized microarray slides were scanned by an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1). Raw microarray data are available from the GEO public depository under the accession number: GEO: GSE114354.

Detection of apoptotic cells

For TUNEL staining, slides were fixed in 4% PFA for 10 min and then subjected to the TUNEL reaction using MEBSTAIN Apoptosis TUNEL Kit Direct (MBL), strictly following instructions provided by the manufacturer. Samples treated with DNaseI for 30 min before TUNEL staining was used as the positive control.

Antibodies

The following antibodies from Cell Signaling Technology were used: anti-GAPDH (Cat. No. 2118), phospho-p53 (Cat. No. 9281), p53 (Cat. No. 2524), Cyclin D3 (Cat. No. 2936), phospho-Rb (Cat. No. 9308), cleaved-caspase 3 (Cat. No. 9664), and M-cadherin (Cat. No. 40491). Antibodies against the following proteins were obtained from Biolegend: FITC-conjugated anti-CD31 (Cat. No. 102405), anti-CD45 (Cat. No. 102405), PE-conjugated anti-Sca-1 (Cat. No. 122507), and APC-conjugated anti-Vcam1 (Cat. No. 105718). Rpt3 (Cat. No. HPA002044) and laminin (Cat. No. L9393) were also from Sigma. The Pax7 (Cat. No. MAB1675) and MyHC (Cat. No. MAB4470) antibodies were purchased from R&D. We also used antibodies against MyoD (Cat. No. sc760) and eMyHC (Cat. No. sc53091) from Santa Cruz Biotechnology, ubiquitin (LifeSensors, Cat. No. VU101), and Collagen type I (Southern Biotech, Cat. No. 1310-01). Biotinylated anti-SM/C-2.6 antibody was a gift from Dr. Fukada (Fukada et al., 2004).

Running capacity assessment

The protocol of the maximal exercise test was performed as our described previously (Nunomiya et al., 2017). Briefly, before the test, the mice ran for 5 min at 10 m min $^{-1}$ as a warm-up. During the first 30 min, the speed was set at 10 m min^{-1} and was increased by 2 m min $^{-1}$ every 15 min. Throughout the warm-up and test running, the treadmill was set without slopes. Exhaustion was determined to be point at which the animal would not resume running for 15 s despite gentle brushing on the tail and a mild electrical foot shock.

Statistical analysis

Statistical analyses were performed with SPSS software (IBM) to determine significant differences based on a two-tailed distribution using a Student's t-test. For comparisons of more than two groups, a one-way analysis of variance (ANOVA) for repeated measures followed by the Bonferroni post hoc test was used. P values are indicated on each figure as < 0.05 (*), < 0.01 (**) and < 0.001 (***). All error bars represent means ± s.d. or s.e.m. NS represents statistically non-significant.

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