Supplemental Data File

TRAF6 regulates satellite stem cell self-renewal and function during regenerative myogenesis

By

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1). Table S1
2). Figure S1-12 and their Legends
3). Supplemental Methods

SUPPLEMENTAL FIGURES

FIGURE S1

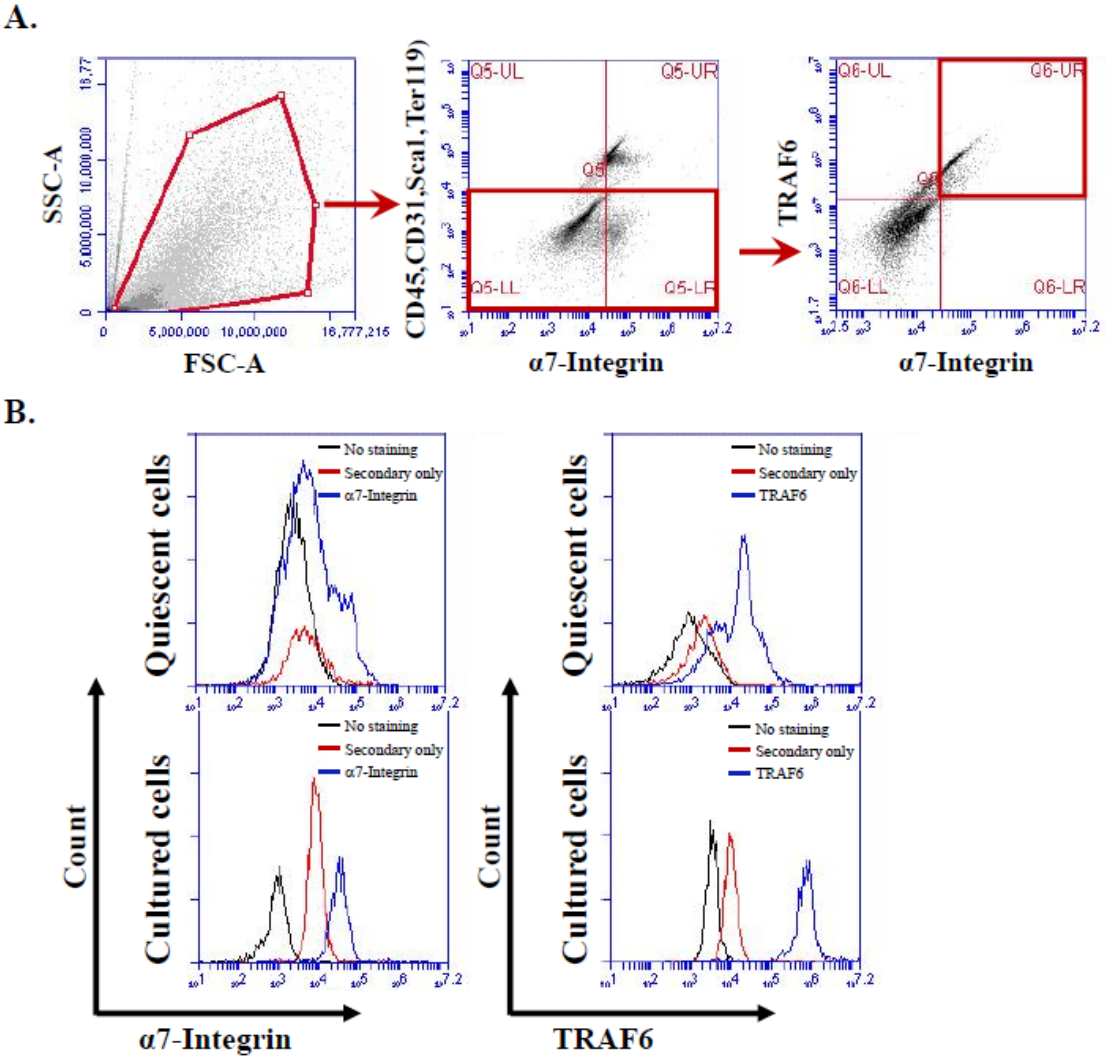

FIGURE S1. FACS gating schematic for identification of satellite cells. (A) Satellite cells were gated for α7-Integrin after eliminating all CD45, CD31, Sca-1, and Ter119 positive cells from all mononuclear cells. After which TRAF6 expression was analyzed in the a7-Integrin^{+} population. **(B)** FACS histograms showing specificity of antibody labeling in both quiescent and activated satellite cells**.**

FIGURE S2

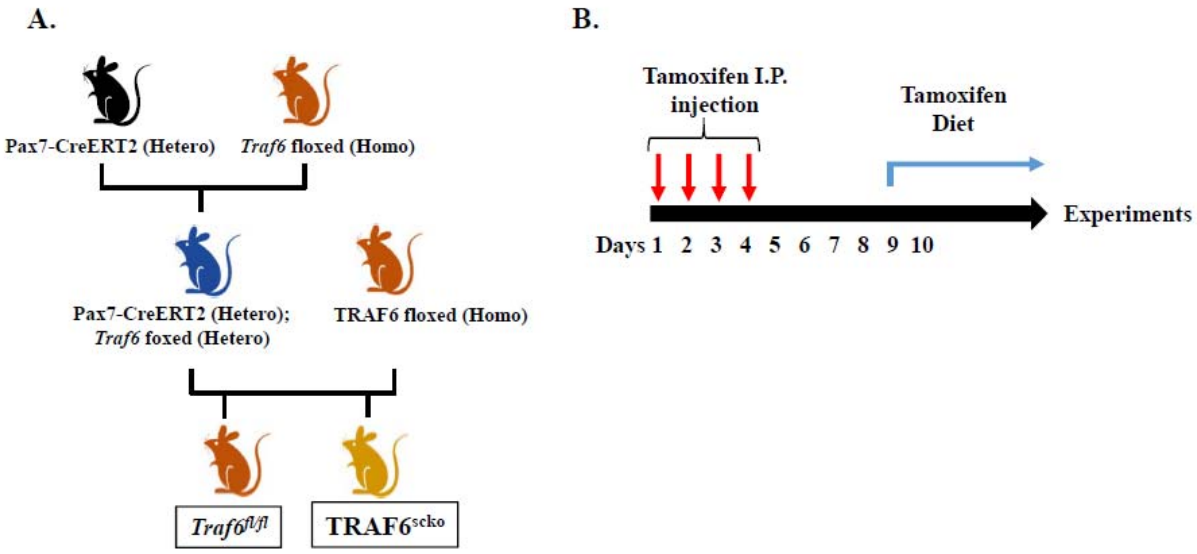

FIGURE S2. Generation of conditional satellite cell-specific TRAF6-knockout (TRAF6^{scko}) mice. (A) Schematic illustration of the breeding strategy to obtain TRAF6^{scko} and littermate *Traf6*$^{fl/fl}$ mice. **(B)** Representation of the time line of tamoxifen-mediated Cre recombination**.**

FIGURE S3

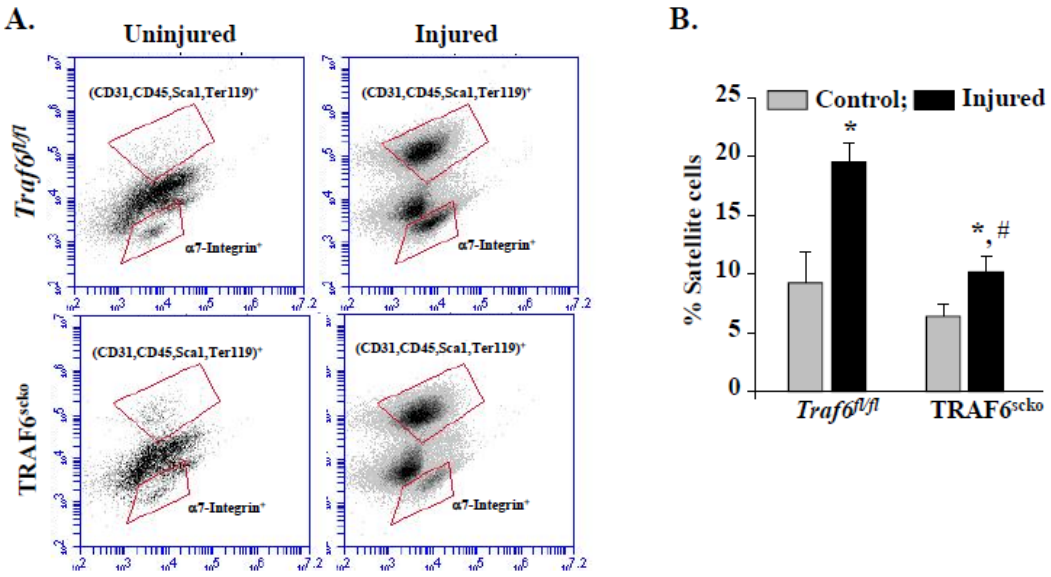

FIGURE S3. Role of TRAF6 in regulating satellite cells in adult skeletal muscle. (A) Representative FACS dot plots demonstrating the percentage of α7-integrin^{+} cells in uninjured and 5d-injured TA muscle of *Traf6*$^{fl/fl}$ and TRAF6^{scko} mice. **(B)** Quantification of α7-integrin^{+} satellite cells in uninjured and 5d-injured TA muscle of *Traf6*$^{fl/fl}$ and TRAF6^{scko} mice assayed by FACS. N=4 in each group. $^{*}p< 0.05$, values significantly different from corresponding uninjured muscle of *Traf6*$^{fl/fl}$ or TRAF6^{scko} mice by paired t test. $^{\#}p<0.05$, values significantly different from injured muscle of *Traf6*$^{fl/fl}$ mice by paired t test.

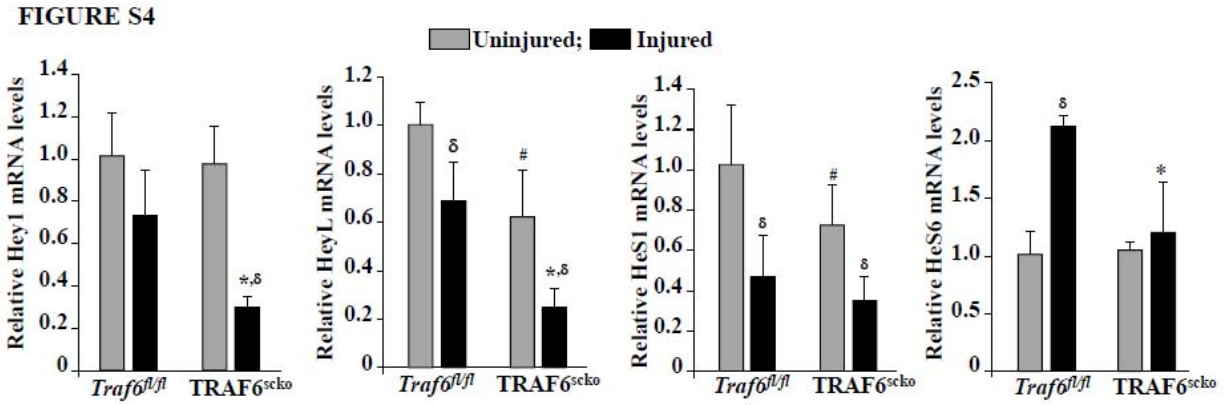

FIGURE S4. Notch signaling is compromised in skeletal muscle of TRAF6^{scko} mice. TA muscle of *Traf6*$^{fl/fl}$ and TRAF6^{scko} mice were injured by $BaCl_2$ injection. After 3d, the muscle was collected and processed for QRT-PCR analysis. Representative bar diagrams illustrating relative mRNA levels of Notch pathway target genes in uninjured and injured TA muscles of *Traf6*$^{fl/fl}$ and TRAF6^{scko} mice. Error bars represent SD. N=3 in each group. $^{\delta}p<0.05$, values significantly different from corresponding uninjured TA muscle of *Traf6*$^{fl/fl}$ or TRAF6^{scko} mice by paired t test. $^{*}p<0.05$, values significantly different from injured TA muscle of *Traf6*$^{fl/fl}$ mice by paired t test. $^{\#}p<0.05$, values significantly different from uninjured TA muscle of *Traf6*$^{fl/fl}$ mice by paired t test.

FIGURE S5

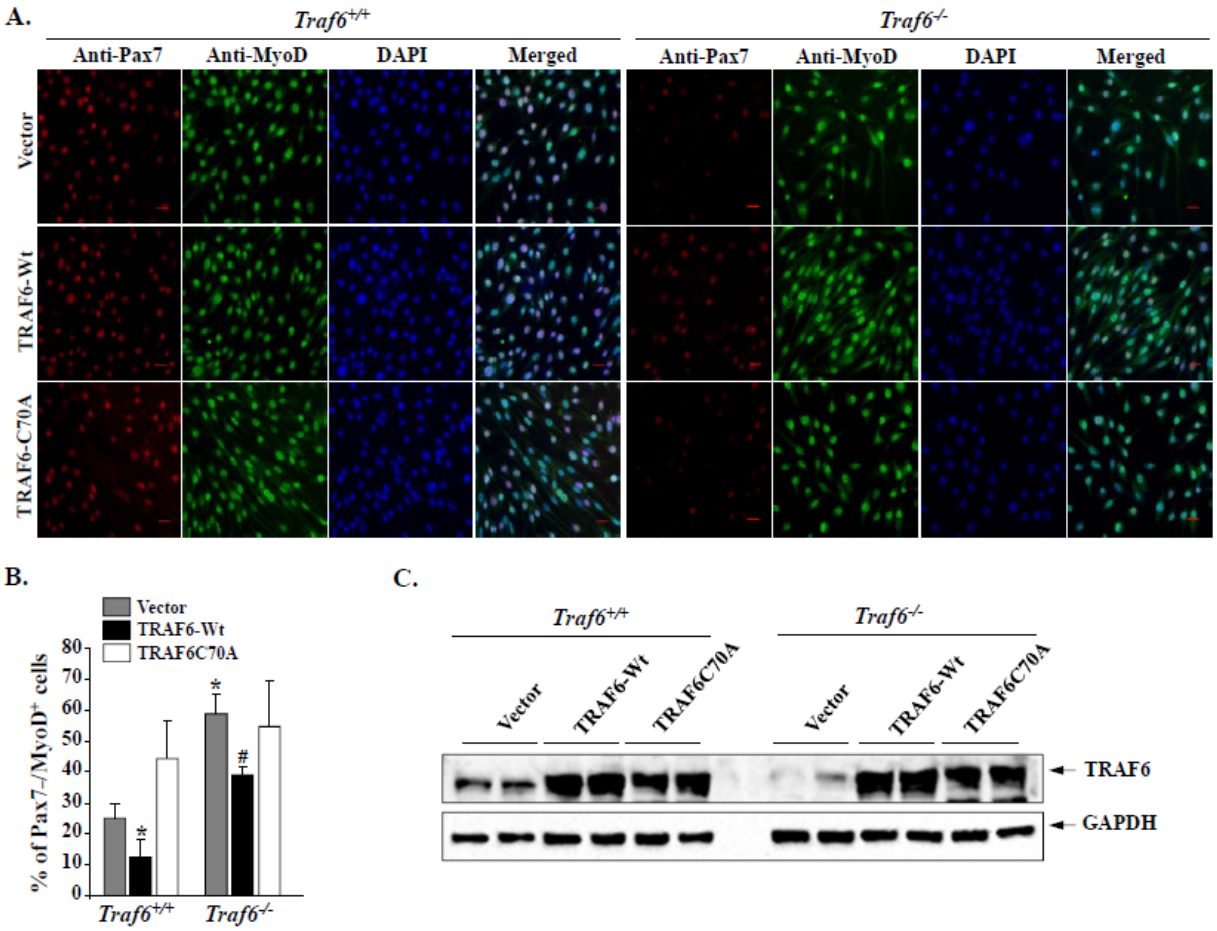

FIGURE S5. E3 ubiquitin ligase activity of TRAF6 is required for maintaining Pax7 expression in satellite cells. *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ primary myoblasts were transfected with vector alone (pcDNA3), TRAF6-Wt, or TRAF6C70A cDNA. **(A)** Representative individual staining for Pax7, MyoD, and DAPI and merged images are presented here. Scale bar: 20µm. **(B)** Quantitative estimation of percentage of Pax7^{-}/MyoD^{+} cells (normalized with DAPI) in *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ cultures. **(C)** Representative immunoblots presented here demonstrate increased levels of TRAF6-Wt or TRAF6C70A in transfected cells. Error bars represent SD. N=4 in each group. $^{*}p< 0.05$, values significantly different from *Traf6*$^{+/+}$ cells transfected with vector alone by paired t test. #, $p<0.05$, values significantly different from *Traf6*$^{-/-}$ cells transfected with vector alone by paired t test.

FIGURE S6

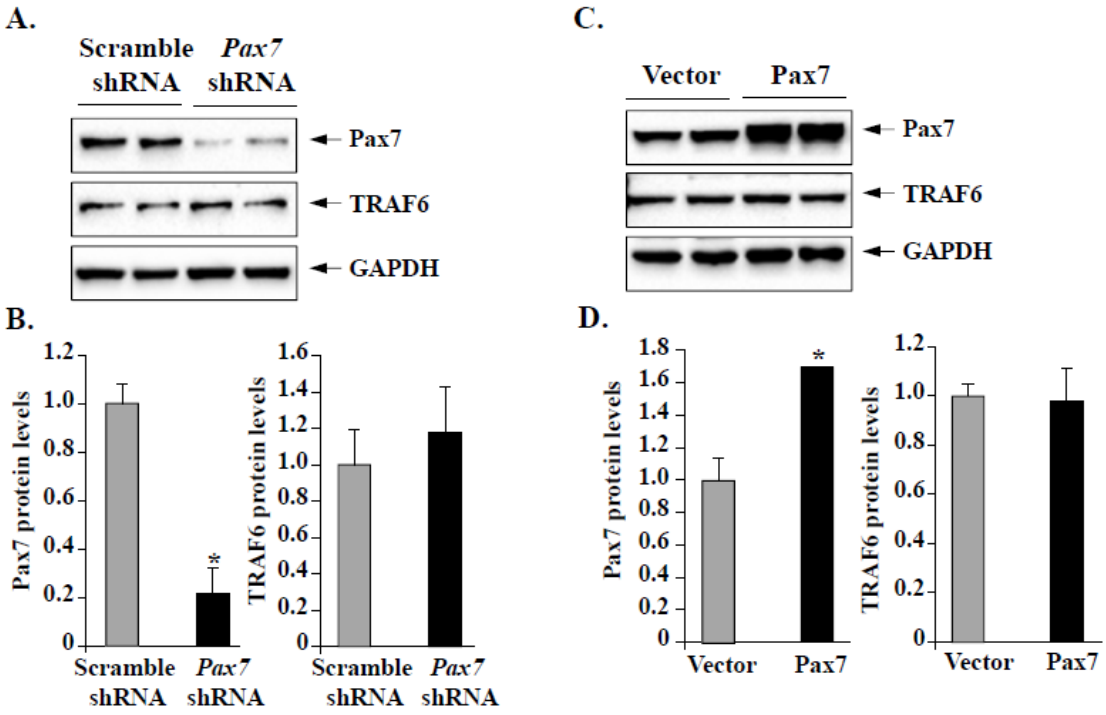

FIGURE S6. Role of Pax7 in regulation of TRAF6 levels. (A) Primary myoblasts prepared from wild-type mice were transfected with scrambled shRNA or *Pax7* targeting shRNA and the protein levels of Pax7, TRAF6 and an unrelated protein GAPDH were measured by performing Western blot. **(B)** Densitometry quantification of Pax7 and TRAF6 protein in scrambled shRNA or *Pax7* shRNA transfected cultures. **(C)** Representative immunoblots demonstrating the levels of Pax7 and TRAF6 and an unrelated protein GAPDH in myoblast cultures transfected with vector alone or Pax7 cDNA. **(D)** Densitometry quantification of Pax7 and TRAF6 protein in cultures transfected with Pax7 cDNA. *p<0.01, values significantly different from corresponding scrambled shRNA or vector alone transfected cultures by unpaired t test.

FIGURE S7

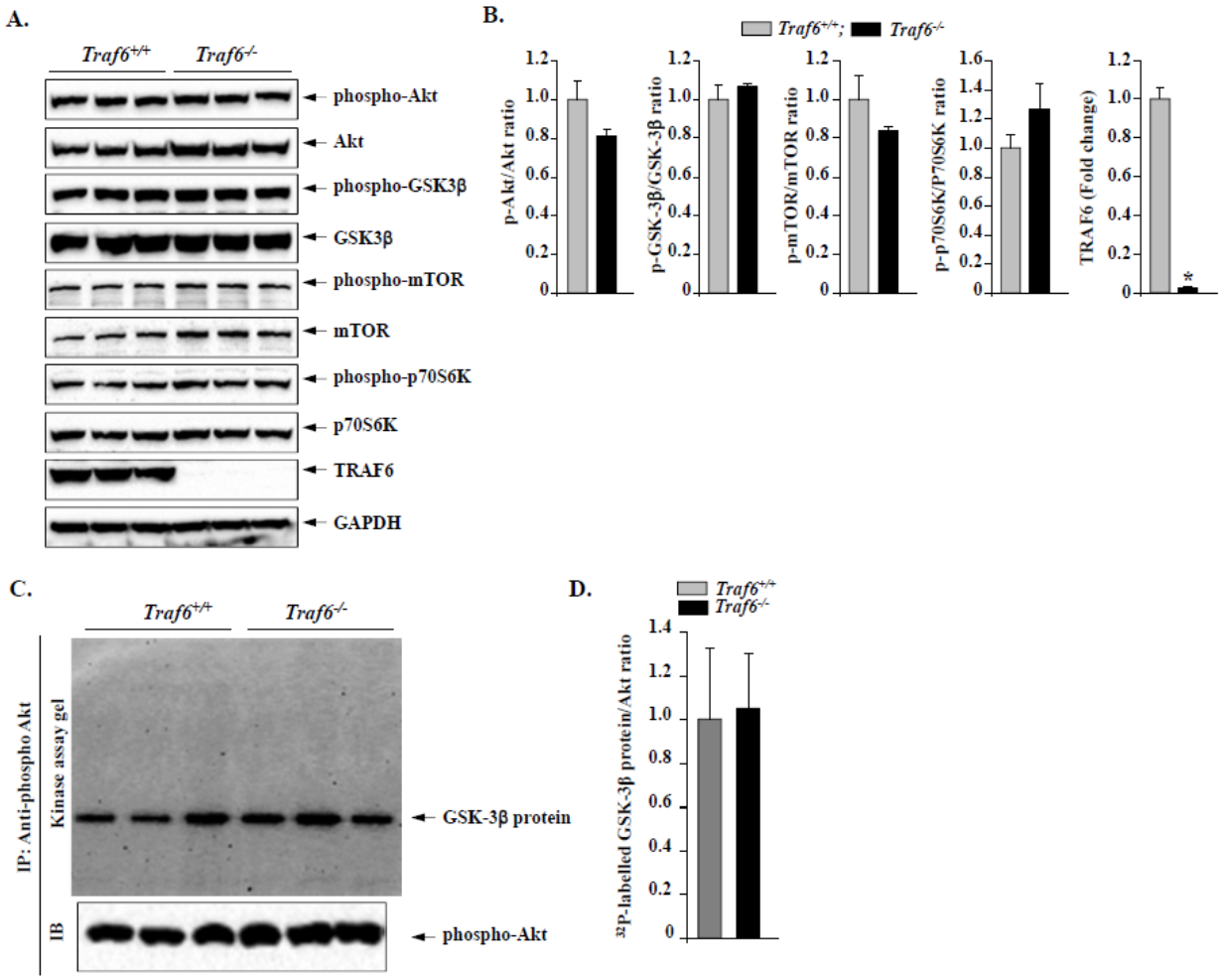

FIGURE S7. Role of TRAF6 in regulating Akt/mTOR pathway in satellite cells. (A) Representative immunoblots demonstrating phosphorylated and total protein levels of AKT, GSK-3β, mTOR, p70S6K, TRAF6, and unrelated protein GAPDH in *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ cultures. **(B)** Densitometric quantification of ratio of phosphorylated and total Akt, GSK-3β, mTOR, p70S6K, and total TRAF6 and GAPDH protein bands in *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ cultures. N=3 in each group. **(C)** Enzymatically active Akt protein was immunoprecipitated (IP) from cell lysates made from cultured *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ myogenic cells using phospho-Akt (Ser473) antibody followed by in vitro kinase assays using GST-GSK-3β protein as substrate. A representative image of kinase assay gel form two independent experiments is presented here. Immunoblot (IB) demonstrates the levels of phospho-Akt post IP. **(D)** Densitometric quantification of ratio of phosphorylated GSK-3β and phosphorylated Akt. N=3 in each group. Error bars represent SD. $^{*}p<0.05$, values significantly different from *Traf6*$^{+/+}$ cells by unpaired t test.

FIGURE S8

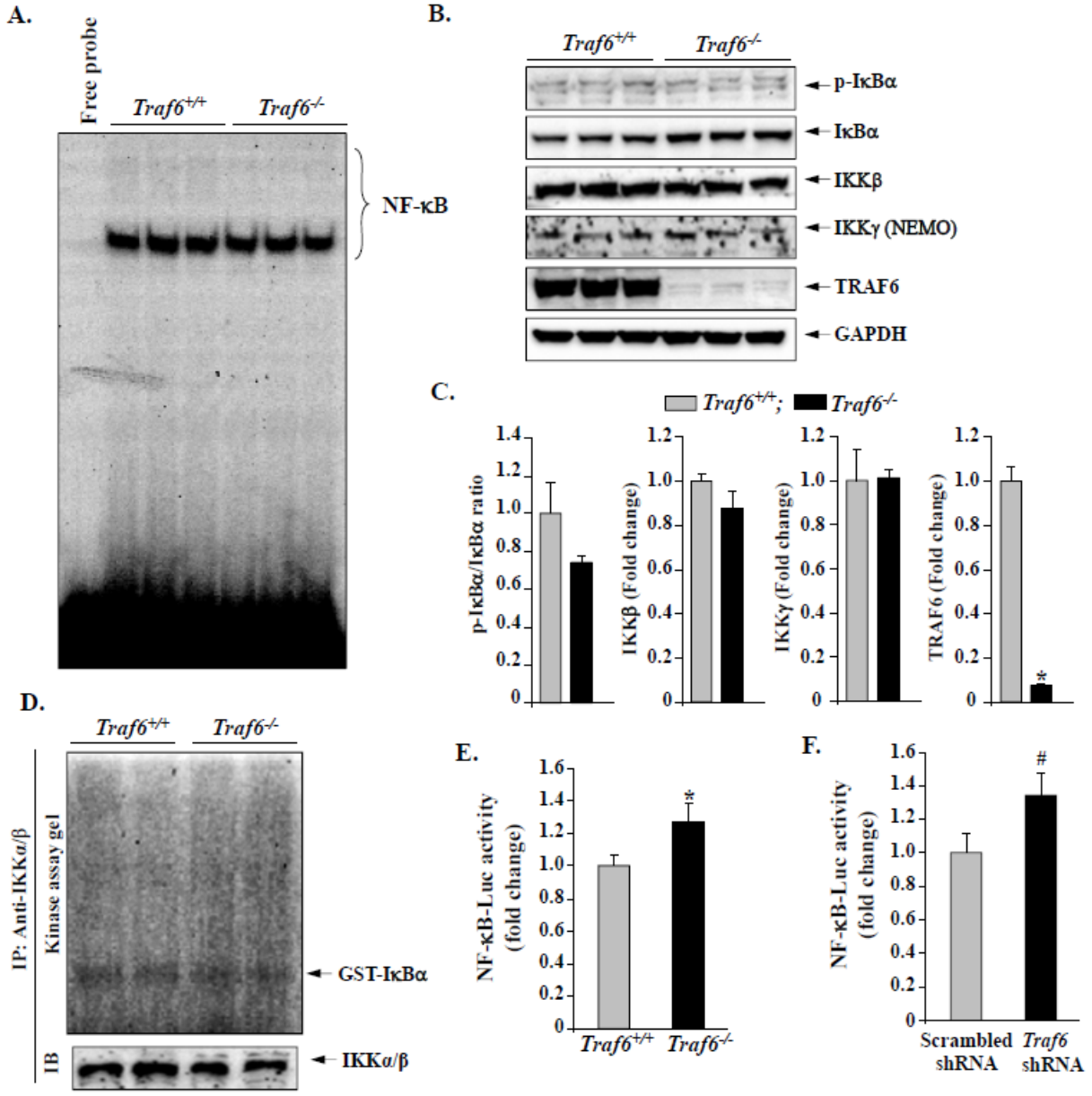

FIGURE S8. Role of TRAF6 in activation of NF-κB in satellite cells. (A) Nuclear extracts prepared from *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ cells were analyzed for DNA-binding activity of NF-κB by performing electrophoretic mobility shift assay (EMSA). Representative EMSA gel is presented here. **(B)** Representative immunoblot demonstrating phosphorylated and total protein levels of IκBα, IKKβ, IKKγ (i.e. NEMO), TRAF6, and unrelated protein GAPDH in cultured *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ myogenic cells. **(C)** Densitometric quantification of ratio of phosphorylated and total IκBα and levels of IKKβ, IKKγ, TRAF6 bands in immunoblots of *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ cultures. N=3 in each group. **(D)** Protein lysates prepared from *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ cells were immunoprecipitated using IKKβ antibody followed by *in vitro* kinase assay using GST-IκBα protein as substrate. A representative image of kinase assay gel and immunoblot (IB) for IKKα/β levels in immunoprecipitation is presented here. **(E)** *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ cells were transfected

with pNF-κB-Luc plasmid along with pRL-TK plasmid for 48h following by measuring the amounts of luciferase and renilla using Dual luciferase assay kit (Promega). Fold change in luciferase/renilla ratio is presented here. **(F)** Primary myoblasts prepared from wild-type mice were transfected with scrambled shRNA or that targeting *Traf6* along with pNF-κB-Luc and pRL-TK plasmids. After 48h of transfection, luciferase and renilla activity in cell lysates was measured. Fold change in luciferase/renilla ratio is presented here**.** Error bars represent SD. $^{*}p<0.05$, values significantly different from *Traf6*$^{+/+}$ cells by unpaired t test. $^{\#}p<0.05$, values significantly different from wild-type cells transfected with scrambled shRNA by unpaired t test.

FIGURE S9

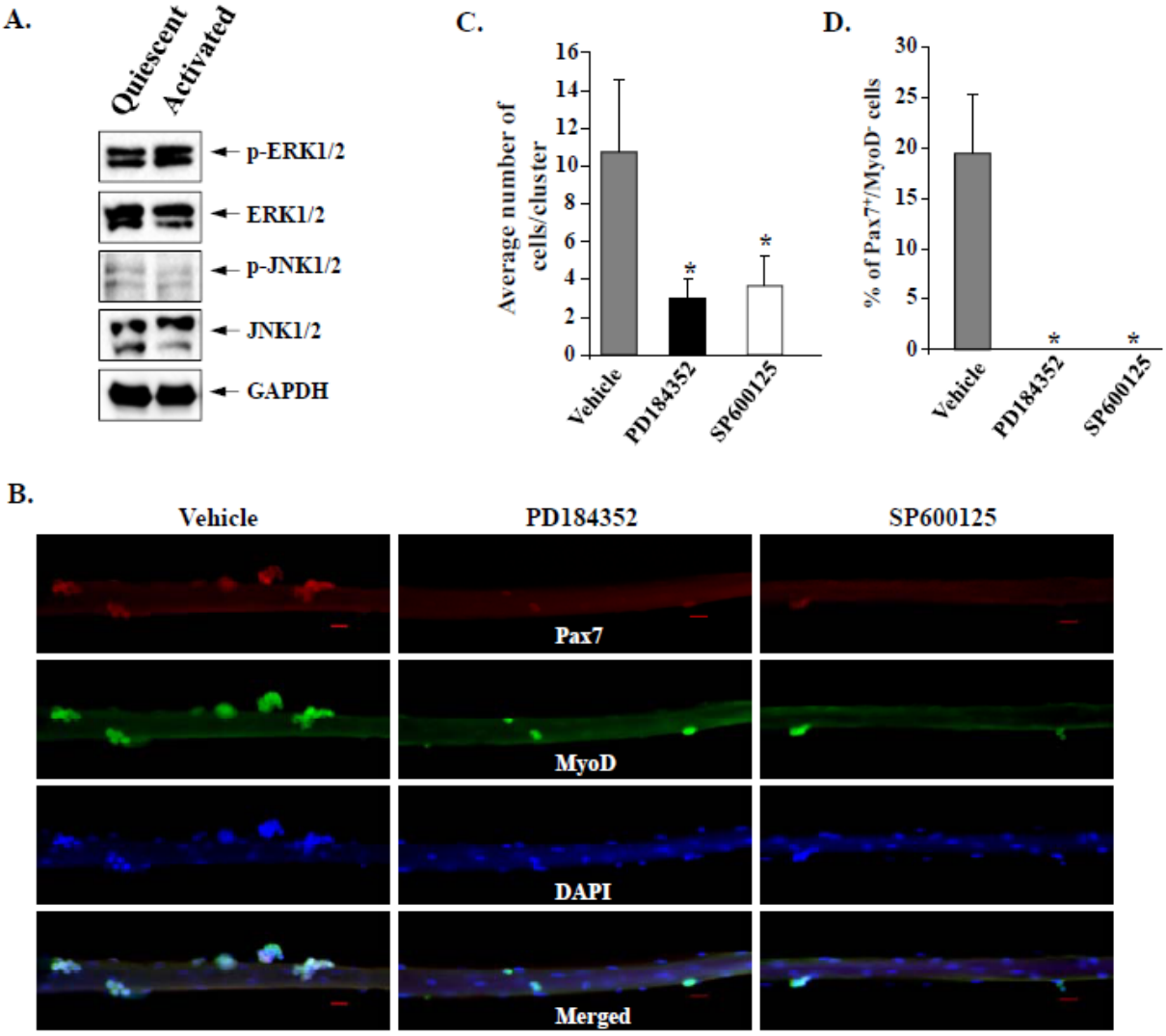

FIGURE S9. Role of ERK1/2 and JNK1/2 in satellite cell proliferation and self-renewal. (A) Freshly sorted satellite cells (quiescent) or cultured primary myoblasts (activated) were analyzed for phosphorylated and total levels of ERK1/2 and JNK1/2 proteins. Representative immunoblots presented here demonstrate levels of phosphorylated and total protein levels of ERK1/2 and JNK1/2. **(B)** Single myofiber cultures were established from EDL muscle of wild-type mice. Fibers were immediately treated with vehicle alone, 2 μM PD184352, (ERK1/2 inhibitor) or 20μM SP6100125 (JNK1/2 inhibitor) and cultured for 72h. Representative individual and merged photomicrographs of Pax7, MyoD, and DAPI staining are presented here. Quantification of **(C)** average number of cells/cluster and **(D)** percentage of self-renewing ($Pax7^+/MyoD^-$) cells. N=20-25 myofibers in each group. Error bars represent SD. *$p<0.01$, values significantly different from myofiber cultures treated with vehicle alone by paired t test.

FIGURE S10

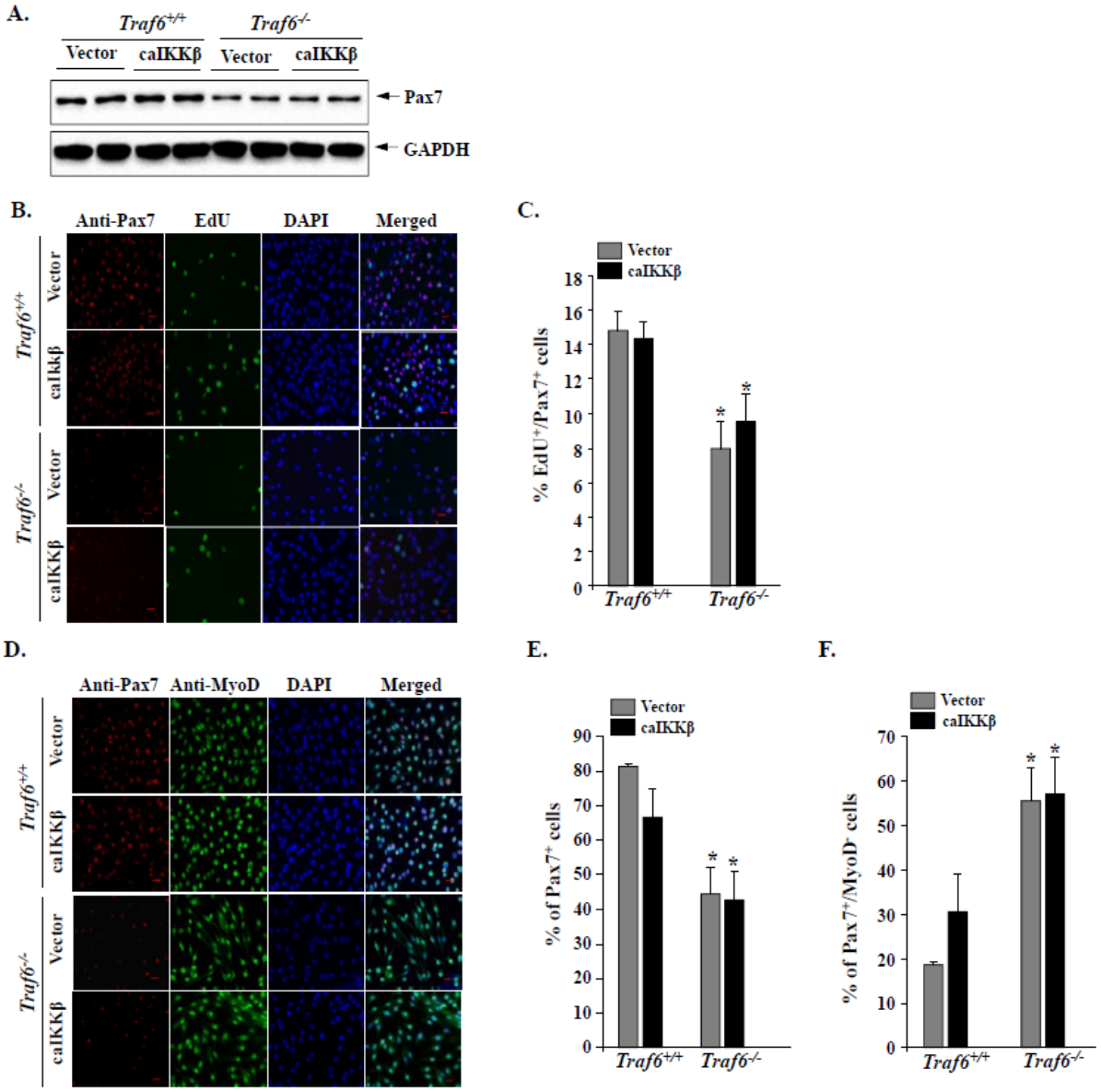

FIGURE S10. Role NF-κB in regulating Pax7 levels in cultured myogenic cells. (A) *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ primary myogenic cells were transfected with vector alone (pcDNA3) or constitutively active mutant of IKKβ (caIKKβ) plasmid. After 72h, the cells were analyzed for Pax7 and an unrelated protein GAPDH by performing western blot. Representative immunoblots are presented here. **(B)** *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ cells were pulse-labeled with EdU for 90 min and analyzed for the expression of Pax7 protein and EdU incorporation. Nuclei were identified by staining with DAPI. Representative photomicrographs are presented here. Scale bar: 20μm. **(C)** Quantification of the percentage of Pax7^{+}/EdU^{+} cells in *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ cultures transfected with vector alone or caIKKβ cDNA. **(D)** Representative individual and merged photomicrographs labeled with Anti-Pax7, Anti-MyoD, and DAPI. Quantification of percentage of **(E)** Pax7^{+} and **(F)** Pax7^{-}/MyoD^{+} cells in *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ cultures transfected with vector

alone or caIKKβ. Error bars represent SD. *p< 0.05, values significantly different from corresponding *Traf6*$^{+/+}$ cultures transfected with vector alone or caIKKβ by paired t test.

FIGURE S11

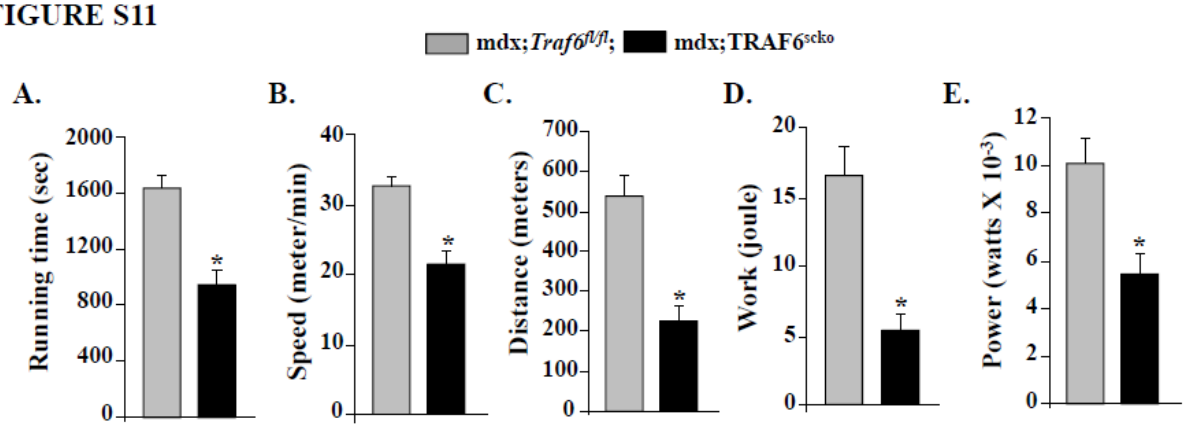

FIGURE S11. Deletion of TRAF6 in satellite cells reduces exercise capacity of mdx mice. After acclimatization, male mdx;*Traf6*$^{fl/fl}$ and mdx;TRAF6^{scko} mice were run on a treadmill with a 10% slope and increasing speed to exhaustion. Maximum speed and running time were monitored and distance and work were calculated based on the individual performance. Data presented here show differences in **(A)** running time, **(B)** maximum running speed, **(C)** distance, **(D)** work, and **(E)** power between 8-week old mdx;*Traf6*$^{fl/fl}$ and mdx;TRAF6^{scko} mice. N = 4 in each group. Error bars represent SD. *p<0.05, values significantly different from littermate mdx;*Traf6*$^{fl/fl}$ mice by unpaired t test.

FIGURE S12

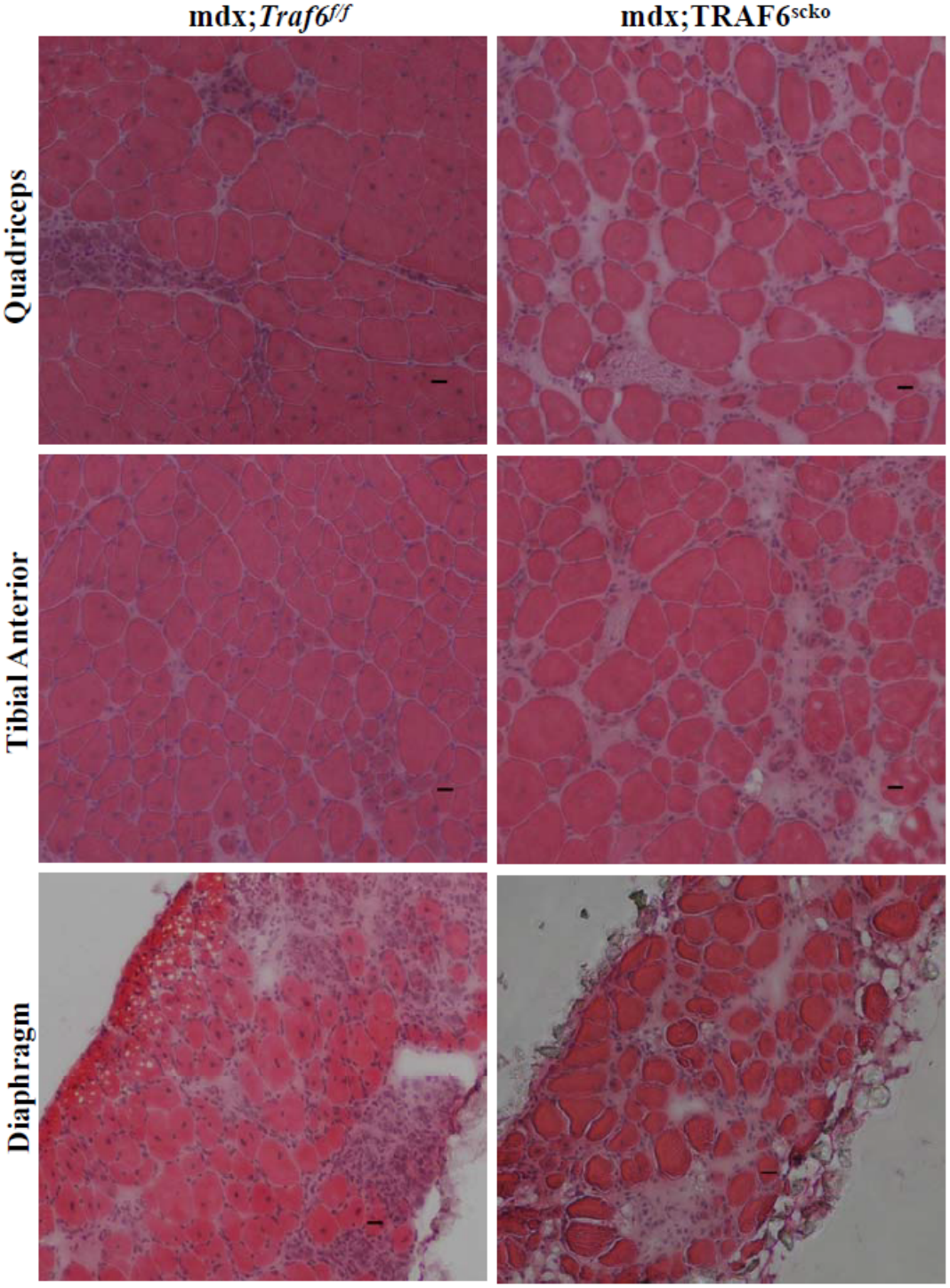

FIGURE S12. Deletion of TRAF6 in satellite cells exacerbates myopathy in mdx mice. Representative photomicrograph of H&E-stained transverse sections of quadriceps, TA, and diaphragm from 8-week old mdx;*Traf6*$^{fl/fl}$ and mdx;TRAF6^{scko} mice. Scale bar: 20µm.

Table S1: Sequence of the primers.

Name	Assay	Sequence
TRAF6	QRT-PCR	GCAGTGAAAGATGACAGCGTGA TCCCGTAAAGCCATCAAGCA
Pax-7	QRT-PCR	CAGTGTGCCATCTACCCATGCTTA GGTGCTTGGTTCAAATTGAGCC
Myf5	QRT-PCR	TGAAGGATGGACATGACGGACG TTGTGTGCTCCGAAGGCTGCTA
MyoD	QRT-PCR	TGGGATATGGAGCTTCTATCGC GGTGAGTCGAAACACGGATCAT
Myogenin	QRT-PCR	CATCCAGTACATTGAGCGCCTA GAGCAAATGATCTCCTGGGTTG
Myh3	QRT-PCR	ACATCTCTATGCCACCTTCGCTAC GGGTCTTGGTTTCGTTGGGTAT
Hey1	QRT-PCR	TGAATCCAGATGACCAGCTACTGT TACTTTCAGACTCCGATCGCTTAC
HeyL	QRT-PCR	CAGATGCAAGCCCGGAAGAA ACCAGAGGCATGGAGCATCT
Hes1	QRT-PCR	GCACAGAAAGTCATCAAAGCC TTGATCTGGGTCATGCAGTTG
Hes6	QRT-PCR	GCCGGATTTGGTGTCTACAT TCCTGAGCTGTCTCCACCTT
c-Jun	QRT-PCR	AAGAACGTGACCGACGAGCA TGAAGTTGCTGAGGTTGGCG
β-actin	QRT-PCR	CAGGCATTGCTGACAGGATG-3' TGCTGATCCACATCTGCTGG
RT	Reverse transcription for miRs	GCATACGAGCTCTTCCGATCTTTTTTTTTTTTTTTTTTTTTTTTTTT
miR-R (common reverse primer for all miRs)	QRT-PCR	GCATACGAGCTCTTCCGATC
miR1 F	QRT-PCR	TGGAATGTAAAGTATGTAT
miR-206 F	QRT-PCR	TGGAATGTAAGGAAGTGTGTGG
miR-133a F	QRT-PCR	TTTGGTCCCCTTCAACCAGCTG
Let7 F	QRT-PCR	TGAGGTAGTAGGTTGTATAGTT
U6 F	QRT-PCR	GAACGATACAGAGAAGATTAGC
Site: -308	ChIP	TCTGGGCTCAGGAACTTCGG AGTGGAAGGTGGCGACAAGG
Site: -466	ChIP	ACTTACCCAGCTGATCGCTCG CGGAGTGGCTTTCTTTGGGT
Site:-1207	ChIP	AAAGCCCTGACAGGTATCAGCG TTGGGCTGGGATCTTCTCTGTC
Site: -2767	ChIP	TGAAATTGCTCTCCAACAGGCG ATCCTTGGTGACCACAGCATCCTT

Supplemental Methods

Histology and Morphometric Analysis. For the assessment of muscle morphology, 10μm thick transverse sections of TA, GA, quadriceps, and diaphragm muscle were stained with Hematoxylin and Eosin (H&E). For quantitative analysis, cross-sectional area (CSA) of myofibers was analyzed in H&E-stained TA or GA muscle sections using Nikon NIS Elements BR 3.00 software (Nikon). For each muscle, the distribution of fiber CSA was calculated by analyzing approximately 250 myofibers. Masson's Trichrome staining was performed to analyze fibrosis using a commercially available kit and following a protocol suggested by the manufacturer (Richard-Allan Scientific).

Creatine kinase (CK) assay. Muscle injury in mdx;*Traf6*$^{fl/fl}$ and mdx;TRAF6^{scko} mice was also evaluated by quantifying the levels of muscle CK in serum collected by cardiac puncture. CK assays were performed using a commercially available kit and following the protocol suggested by the manufacturer (Stanbio Laboratory, TX, USA).

Short hairpin RNA (shRNA) and plasmid constructs. The pLKO.1-mCherry-Puro plasmid was kindly provided by Dr. Renzhi Han of the Ohio State University. The target siRNA sequence were identified using BLOCK-iT™ RNAi Designer online software (Life Technologies). At least 2-3 siRNA sequence for each gene were tested for efficient knockdown of target mRNA. The shRNA oligonucleotides were synthesized to contain the sense strand of target sequences for mouse TRAF6 (i.e. GCAAGTATGAGTGTCCCATCT), mouse c-Jun (i.e. GGAACAGGTGGCACAGCTTAA), mouse Pax7 (i.e. GCCGAGTGCTCAGAATCAAGT), mouse Mekk1 (i.e. GCTGCTTATTCTCTAGAAACT), mouse Fbw7 (i.e. GCAGCAAGCGAGACATCAAGA), mouse Itch (i.e. GCAGCTTGTAGGTGACAAAGA), mouse Det1 (i.e. GCACATTCAAGTGTGACAAAG), or mouse MuRF1 (i.e.

GCCTGGAGATGTTTACCAAGC), short spacer (CTCGAG), and the reverse complement sequences followed by five thymidines as an RNA polymerase III transcriptional stop signal. Oligonucleotides were annealed and cloned into pLKO.1-mCherry-Puro with AgeI/EcoRI sites. pBRIT Pax7 TAP plasmid containing mouse Pax7 cDNA was obtained from Addgene (plasmid # 17528). Pax7 cDNA was excised from pBRIT Pax7 TAP plasmid using BamHI and XhoI restriction enzymes and cloned at the same sites in pcDNA3 vector (Invitrogen, Carlsbad, CA). The insertion of shRNA and cDNA sequence in the plasmids was confirmed by DNA sequencing.

Electrophoretic Mobility Shift Assay (EMSA). DNA-binding activity of NF-κB was measured by performing EMSA using same method as previously described (60, 61). In brief, 2×10^6 *Traf6*$^{+/+}$ or *Traf6*$^{-/-}$ cells were washed with ice-cold PBS and suspended in 0.1 ml of lysis buffer (20 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitor cocktail). The cells were allowed to swell on ice for 10 min, after which 3.25 µl of 10% IGEPAL (Sigma) was added. The tube was then vigorously mixed on a vortex machine for 10s, and the homogenate was centrifuged for 1 min at 14,000 rpm. The nuclear pellet was resuspended in 25µl of ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitor cocktail), and the tube was incubated on ice for 45 min with intermittent mixing. The tube was then centrifuged for 5 min at 14,000 rpm at 4°C, and the supernatant (nuclear extract) was collected and assayed for protein concentration.

EMSA was performed by incubating 15µg of nuclear extract with 16 fmol of ^{32}P end-labelled NF-kB consensus oligonucleotide 5'-AGTTGAGGGGACTTTCCCAGGC-3' (Promega) at 37^{o}C for 20 min. The incubation mixture included 2-3 µg of poly(dI-dC) in a

binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM DTT, 1% IGEPAL, 5% glycerol, and 50 mM NaCl). The DNA-protein complex formed was separated from free oligonucleotide on 7.5% native polyacrylamide gel using buffer containing 50 mM Tris, 200 mM glycine, pH 8.5, and 1 mM EDTA and then the gel was dried. The radioactive bands from the dried gel were visualized and quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuaNT software.

In vitro Kinase Assay: The activity of IKKβ and Akt was determined by immunoprecipitation followed by an *in vitro* kinase assay. In brief, cell extracts from *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ cells were made in lysis buffer (50mM Tris-Cl (pH 8.0), 200mM NaCl, 50mM NaF, 1mM dithiotheritol, 1mM sodium orthovanadate, 0.3% IGEPAL and protease inhibitors), and the concentration of protein was measured. About 500 μg of protein was immunoprecipitated with anti-IKKα/β (Catalog sc-7607, Santa Cruz Biotechnology) or phospho-Akt antibody (Catalog 4060, Cell Signaling Technology) and the immune complex was collected using protein A-Sepharose beads. After washing two times with lysis buffer and two times with kinase buffer (50 mM HEPES [pH 7.4], 10 mM MgCl2, and 1 mM DTT), the beads were suspended in 20μl of kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 2 mM DTT, 10 μCi of [γ-^{32}P]ATP, 1 μM unlabeled ATP, and 2 μg of either GST-IκBα (aa 1-54) (for IKK) or GSK-3β fusion protein (for Akt, Cell Signaling Technology) as substrate. After incubation at 37°C for 30 min, the reaction was terminated by boiling with 20 μl of 2× Laemmli sample buffer for 3 min. Finally, the protein was resolved on a 10% polyacrylamide gel, the gel was dried, and the radioactive bands were visualized by exposing to a PhosphorImager screen and quantified using ImageQuant TL (GE Healthcare) software.

Grip Strength Measurements. A digital grip-strength meter (Columbus Instruments) was used to measure forelimb or four- limb grip strength in mice similar to as previously described (21). Mice were acclimatized for 10 min before starting test. The mouse was allowed to grab the metal pull bar with the forepaws and in a separate experiment with all four paws. The mouse tail was then gently pulled backward in the horizontal plane until it could no long grasp the bar. The force at the time of release was recorded as the peak tension. Each mouse was tested five times with a 20-40s break between tests. The average peak tension from three best attempts was defined as forelimb or total grip strength.

Exercise Tolerance Test: We measured the exercise capacity of mdx;*Traf6*$^{fl/fl}$ and mdx;TRAF6^{scko} mice. In brief, all animals were run on a treadmill (Eco3/6 treadmill; Columbus Instruments, Columbus, OH, USA) at 10 m/min for 5 min at 0% degree incline for acclimation for 3 days. On the exercise testing day, animals ran on the treadmill with a fixed slope of 10%. Mice first ran at 10 m/min for 5 minutes and the speed was increased by 2 m/min every two minutes until they were exhausted or a maximal speed of 46 m/min was achieved. The criterion of exhaustion was defined as the inability of the animal to run on the treadmill for 10 seconds despite mechanical prodding. Running time and maximum speed achieved was measured whereas running distance, work and power were calculated similar to as described (48, 49).