Large Polyglutamine Repeats Cause Muscle Degeneration in SCA17 Mice

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Supplemental Information

Experiment Procedures

Antibodies and plasmids

Primary antibodies from commercial sources used in this study include: 1C2 (Millipore/Chemicon, MAB1574), 1TBP18 (QED bioscience, 70102), MyoD (Santa Cruz, 5.8A; s-17; m-318), Myf5 (Santa Cruz, C-20), Myogenin (Thermo, Ab-1), γ-tubulin (Sigma, T-6557), GAPDH (Chemicon, mAB374), Sydecan4 (a gift from Dr. Grace Pavlath's lab), and EMyHC (F1.652, Developmental Studies Hybridoma Bank). Rabbit polyclonal antibody EM395 was generated previously by our laboratory using a GST fusion protein containing a N-terminal fragment of TBP (aa1-20). All secondary antibodies were purchased from Jackson ImmunoResearch.

pRK-MyoD-HA plasmid was generated by PCR cloning of MyoD cDNA from mouse muscle tissue using primers (forward: 5'-AAG GAT CCG CTA TGG AGC TTC TAT CGC-3'; reverse: 5'-ATG AAT TCA AGC ACC TGA TAA ATC GC-3'). The PCR product was digested with BamH1 and EcoR1 restriction enzymes and was then inserted into the pRK vector in which the MyoD is in-frame fused to the influenza hemagglutinin (HA) epitope. TBP13Q and 105Q plasmid were generated as described previously (Friedman et al., 2007).

Mouse behavior tests

Mouse body weight and growth were measured monthly. The motor function of mice was assessed using a rotarod test (AccuScan Instruments, Inc). Rotarod performance was evaluated as described previously (Friedman et al., 2007). Five-week-old mice were trained for 5 min on 2 separate days on a rod that rotated at 5 RPM. Testing commenced the following week on a rod that rotated at 15 RPM. Mice were subjected to 3 trials, each with a maximum duration of 180 s, and latency to fall was recorded. Mice were tested every 3 months at the same time of day. Mouse stride length test was evaluated as described previously (Detlef et al., 2008). Mouse hind-paws were dipped in nontoxic water-based

paints. Mice were then allowed to walk down an enclosed runway lined with white paper. Three trials were performed on 3 separate days within 1 week. Two to 4 steps from the middle portion of each run were measured for hind-stride length. At least 9 steps were measured for each mouse. Mean values were used for statistical analysis. Grip strength was evaluated as described previously (Janet et al. 2011). Mice were allowed to grip the metal grids of a grip meter (Ametek Chatillon) with all their limbs, and they were gently pulled backwards by the tail until they could no longer hold the grids. The peak grip strength observed in 5 trials was recorded.

Cell culture

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100μg/ml streptomycin, and 0.25 μg/ml amphotericin B.

C2C12 mouse myoblasts were maintained in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution at 37°C and 8% CO2. C2C12 cell differentiation was initiated by incubating the cells in differentiation media (DM) consisting of DMEM supplemented with 2% horse serum (Mediatech) and 1% penicillin-streptomycin. For MyoD cycloheximide (CHX) chase assay, C2C12 cells were infected with Ad-GFP-TBP13Q/105Q virus for 24 h. Cells were then incubated in DM for 3 days. At the fourth day, cells were treated with CHX (100 μg/ml) for the indicated hours, and western blotting was performed. For the MG132 assay, C2C12 cells were infected with Ad-GFP-TBP13Q/105Q virus for 24 h.

Cells were then incubated in DM for 3 days and treated with (+) or without (-) MG132 (10 μM) overnight, and western blotting was performed.

Western blot, immunohistochemistry, electron microscopy, and immunoprecipitation

Methods for western blot and immunohistochemistry of mouse brain tissue were described previously (Huang et al., 2011). For muscle immunohistochemistry, fresh muscle samples were fixed in 10% neutral-buffered formalin and paraffin-embedded. Four-mm sections were cut, baked at 60°C for 30 min, and cooled. Sections were deparaffinized and rehydrated through graded alcohols to water. Antigen was retrieved by incubating slides in Antigen Unmasking Solution (Vector lab) at 100°C 1 h before staining with 1TBP18 antibody. Light micrographs and fluorescent images were acquired using a Zeiss microscope (Axiovert 200 MOT) with a digital camera (Hamamatsu Orca-100) and OpenLAB software (Improvision Inc). Both 20x and 63x objectives were used for image acquisition. Method for neuromuscular junction staining was adopted from a previous publication (Wu and Mei, 2013), and α-bungarotoxin was purchased from Invitrogen (B-13423). For electron microscopy, after mice were sacrificed, tibialis anterior (TA) muscle and diaphragm were collected. The samples were transferred to 0.1 M sodium phosphate buffer (PB, PH=7.3) containing 4% paraformaldehyde and 0.2% glutaraldehyde overnight. The ultra-thin sections were used for electron microscopic examination as described in our previous publication (Li et al., 2001). For immunoprecipitation, muscle tissue or transfected HEK293 cells were homogenized in NP-40 buffer (150 mM NaCl, 50 mM Tris-HCl pH8.0, 1% NP-40). Protein concentration was determined by BCA protein assay kit (Thermo Scientific, 23227), and 500 μg of protein was used for one experiment. The protein was first pre-cleared with protein A-beads for 1 h, and then incubated with primary antibody overnight. Protein A-beads were then added into the

mixture and kept for 1 h. The precipitated antibody-protein complexes with protein-A beads were used for western blot.

RNA isolation, reverse transcriptase PCR, and real-time PCR

RNA from mouse TA muscle was isolated by RNeasy Lipid Tissue Mini Kit (QIAGEN, 74804). RNA concentration was determined by Synergy H4 reader, and an equal amount of RNA was used for cDNA synthesis with SuperScript III First-Strand synthesis system (Invitrogen, 18080-051). For reverse transcriptase PCR, rTaq, reaction buffer, and dNTP used are all from TaKaRa. For real-time PCR, RealMaster Mix 2.5× from 5 Prime was used. The PCR reaction was performed in Mastercycler realplex (Eppendorf). Primers used are for mouse myogenin (mMyog) (forward: 5′-TCG TAC ATC GTG GAC AGC ATC AC -3′; reverse: 5′-ACA TCT CAG TTG GGC ATG GTT TC -3′); mouse myostatin (mMstn) (forward 5′- TGT GCA AAT CCT GAG ACT CAT CAA ACC-3′; reverse: 5′-TGG GCT CAT GTC AAG TTT CAG AGA TCG-3′); mouse tripartite motif-containing 72 (mTrim72) (forward: 5'-ACT ACA CGC GGT GCC CTC A-3'; reverse: 5'-ACT CCA CGT GCG CCT CCA GG-3'); mouse myogenic differentiation 1 (mMyoD1) (forward: 5'-AGA AGA GTG CGG CTG TGT CGA GC-3'; reverse: 5'-ACG GGG GCT GTC TGT GGA GAT GC-3'); mouse myosin binding protein C, fast type (mMybpc2) (forward: 5'-TGA CGG CTC CCA AGT TCC TGA C-3'; reverse: 5'-TGA CCT CTG ACG GCA CAG TTG A-3'); mouse creatine kinase, muscle (mCKM) (forward: 5'-ACA GGT GCA GCT GGT GGT GGA TG-3'; reverse: 5'-TTC TGC GCG GGG ATC ATG TCG TC-3'), and mouse calcium channel, voltage-dependent, L type, alpha 1S subunit (mCacna1s) (5'- GGC TCT GGT TCG AGG AGG ACT A-3'; reverse: 5'-CAC CTC GAC TTC CTC TGG TTC C-3').

Mass spectrometry analysis

The mouse brain and muscle protein samples were prepared as described before (Huang et al., 2011). The proteins were analyzed by RP LC-MS/M at the Emory core facility. All analyses were performed on an LTQ-Orbitrap ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Acquired MS/MS spectra were searched against the mouse reference database of the National Center for Biotechnology Information.

In vivo chromatin immunoprecipitation (ChIP)

ChIP assays with semi-quantitative PCR was performed as described previously (Friedman et al., 2007, Huang et al., 2011). For ChIP with semi-quantitative PCR, fresh TA muscle from 3-4-month-old TBP-105Q mice and age-matched wild-type littermates was used (n = 3 mice per genotype). For each immunoprecipitation (IP), 1000 µg of precleared whole-cell lysate and 5 µg of anti-MyoD or rabbit IgG were used. The experiment was performed using a ChIP Assay Kit (Millipore). Semi-quantitative PCR, using myosin heavy chain 4 (MHC4) and muscle creatine kinase (MCK) primers in separate reactions, was performed on DNA recovered from IP samples and inputs (5% pre-cleared lysate). IP with rabbit IgG DNA served as negative controls. Promoter sequences were acquired using the UCSC Genome Browser. The size of the amplicons for MHC4 and MCK were 127 bp and 104 bp, respectively. Primer sequences and PCR cycling parameters are as follows: mouse MHC4 primers: sense 5'-AAA CAC ATG GAA CAG ATG GGC AG-3' and antisense 5'-TTT CTG TAT TAT AAA CCA CAT TTG AGA GC -3'; mouse MCK primers: sense 5'-ATT TCC CCC CAG GTG CTG TAG GCT AGA G -3' and antisense 5'- AAG GTA TGT GTC ATT TTT TGC AGT TGG AG-3'. PCR program: 1. 96°C for 3 min; 2. 96°C for 45s; 3. 55°C for 45s; 4. 72°C

for 1 min, and repeat steps 2-4; 5.72°C for 5 min.

Microarray assay

Total muscle RNA was extracted with Trizol reagent (Invitrogen) and further purified with RNeasy columns (Qiagen) following manufacturers' instructions. RNA was isolated from 3 3-month-old mice of each genotype (SCA17 KI and WT). Independent hybridizations (3 for SCA17 KI vs. WT) of synthesized cRNAs were performed on the Gene Expression 8 Affymetrix Mouse Gene1.0ST by Emory University Cancer Genomics Shared Resource.

Raw data obtained via the GCOS software (Affymetrix) were subjected to background adjustment and normalization with RMAExpress, using all chips at the same time. RMA-normalized values were averaged across hybridizations. Log2-scaled RMA results were analyzed to calculate ANOVA two-sided P-values. Natural-scaled results from RMAExpress were used to calculate log ratios. Specifically, the mutant TBP group RMA average was divided by the normal TBP group RMA average, and the base-2 logarithm (Log2) of this value constituted the log ratio. To combine the P-value and log ratio calculations and weight each with the same importance, we used the following method for selecting significantly changed probes from RMA and ANOVA values. First, we ascertained the number of probe sets that met the threshold P-value (< 0.01). Second, the absolute value of log ratios was taken, and a cutoff set so that the same number of probe sets was obtained based on ranked log ratios as was obtained based on ranked P-values. Finally, the probe sets that fulfilled thresholds for both P-value and log ratio were selected as significantly changed.

Quality control metrics were evaluated for each chip. Chips were not used in the final results if they showed signs of RNA degradation, contamination, poor amplification, or abnormal hybridization. Expression changes were confirmed by semi-quantitative RT-PCR for a subset of the identified genes.

Virus generation and expression

The pAAV-MyoD-F2A-CFP plasmid was generated by releasing MyoD from pRK-MyoD-HA using BamHI and EcoRI restriction sites and F2A-ECFP from pCAG-HTT-2A-ECF (Yang et al., 2010) using EcoRI and XbaI restriction sites, then inserting MyoD and F2A-ECFP into pAAV-MCS vector. This vector coexpresses MyoD and a fluorescent protein CFP that is released from MyoD-F2A-CFP via F2A cleavage. Plasmids encoding mouse TBP13Q, 44Q, 68Q and 98Q were generated as described before (Friedman et al., 2007). The coding sequences of TBP13Q, 44Q, 68Q and 98Q were released using PstI and ClaI restriction sites and inserted into pAAV-MCS vector. The viral vectors were packaged by the Viral Vector Core at Emory University for virus production and purification. MyoD shRNA (mouse) lentiviral (Lv) Particles (sc-35991-v) and Lv-GFP virus were purchased from Santa Cruz. Adenoviruses expressing GFP or TBP13Q/105Q were generated by Welgen (Worcester, MA, USA) as described previously (Shah et al., 2009).

For intramuscular injections (IM), mice were anesthetized with a 2.5% solution of Avertin (Sigma, T48402) at 0.15 ml per 10 g body weight intraperitoneally, and the viruses were injected into the tibialis anterior (TA) with 2 x 10^{10} vector genomes diluted in 25 μl of phosphate-buffered saline. The mice were sacrificed 45 days after the virus injection and the TA muscle were used for hematoxylin and eosin (H&E) and immunofluorescence staining.

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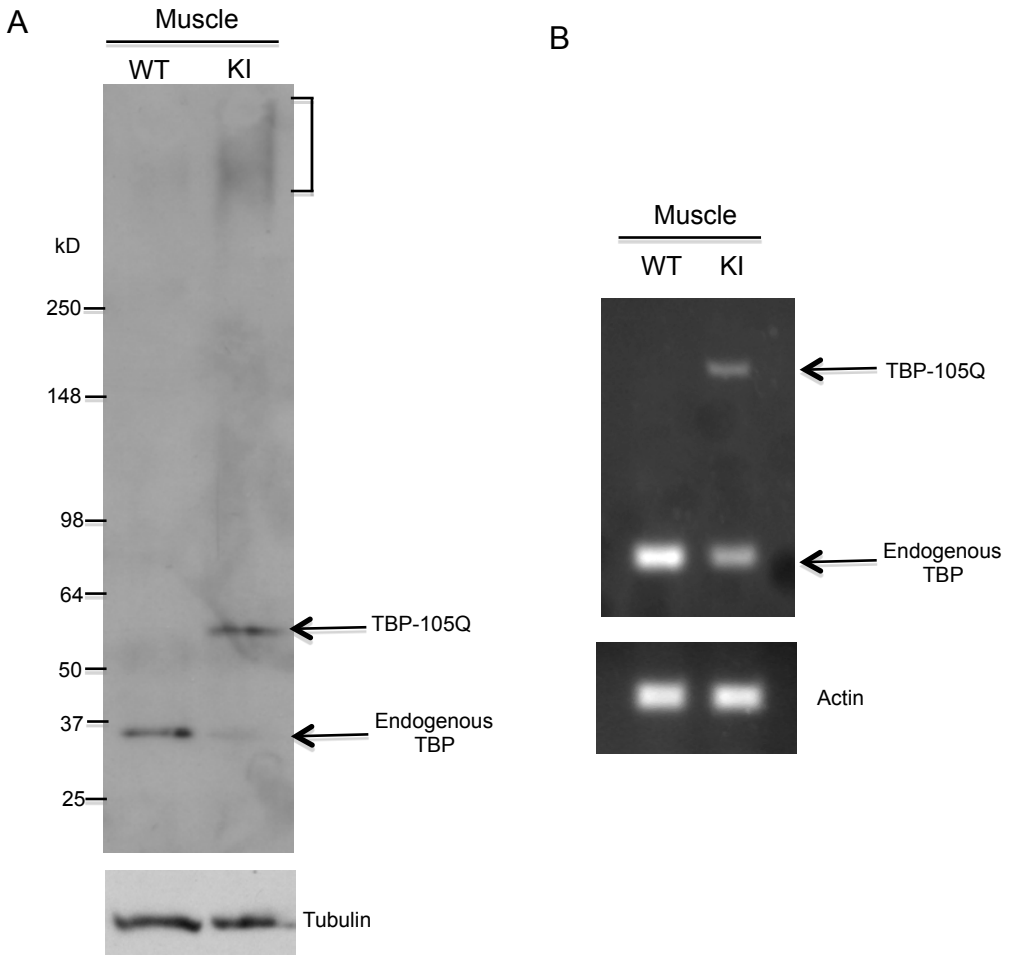

Figure S1 (Related to Figure 1). Reduced level of endogenous mouse TBP in SCA17 mouse muscle tissues. (**A**) Mutant TBP and normal TBP from WT or SCA17 KI skeletal muscle tissues were immunoprecipitated with anti-TBP, EM395. Western blot analysis showing that endogenous mouse TBP is reduced when mutant TBP is expressed. Bracket indicates aggregated TBP, and arrows indicate soluble TBP. (**B**) RT-PCR showing reduced mRNA level of endogenous TBP in the presence of mutant TBP (TBP-105Q) in SCA17 KI skeletal muscle tissues as compared with WT muscle tissues.

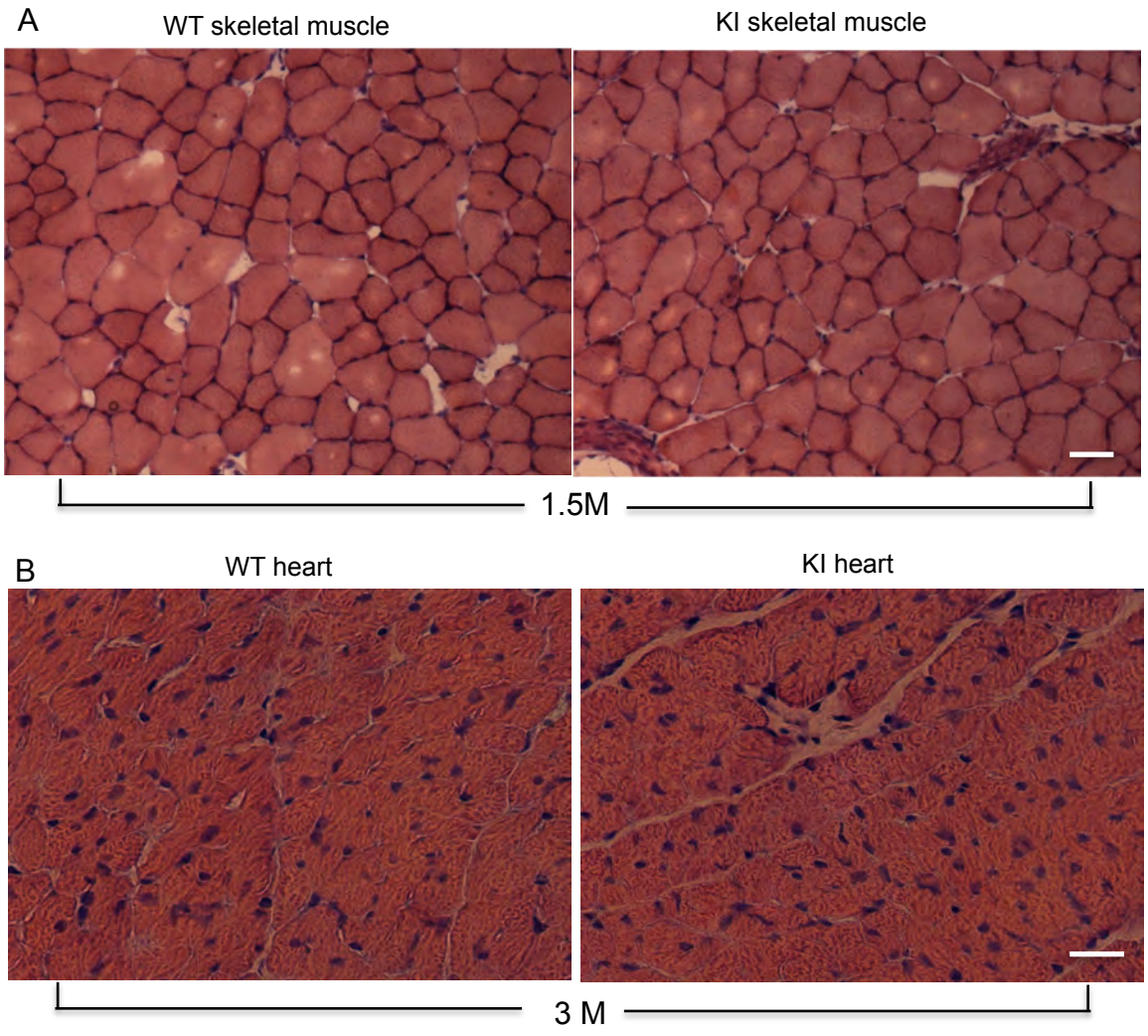

Figure S2 (Related to Figure 2). Mutant TBP dose not cause muscle atrophy in young mice at 1.5 months of age or in the heart. (A) Haematoxylin and eosin (H&E) cross-section staining of tibialis anterior (TA) muscles from 1.5-month-old WT and SCA17 KI mice**. (B**) Haematoxylin and eosin (H&E) staining of heart muscles from 3-month-old WT and KI mice showing no morphological changes in muscle-KI heart muscle. Scale bars: 40 μm.

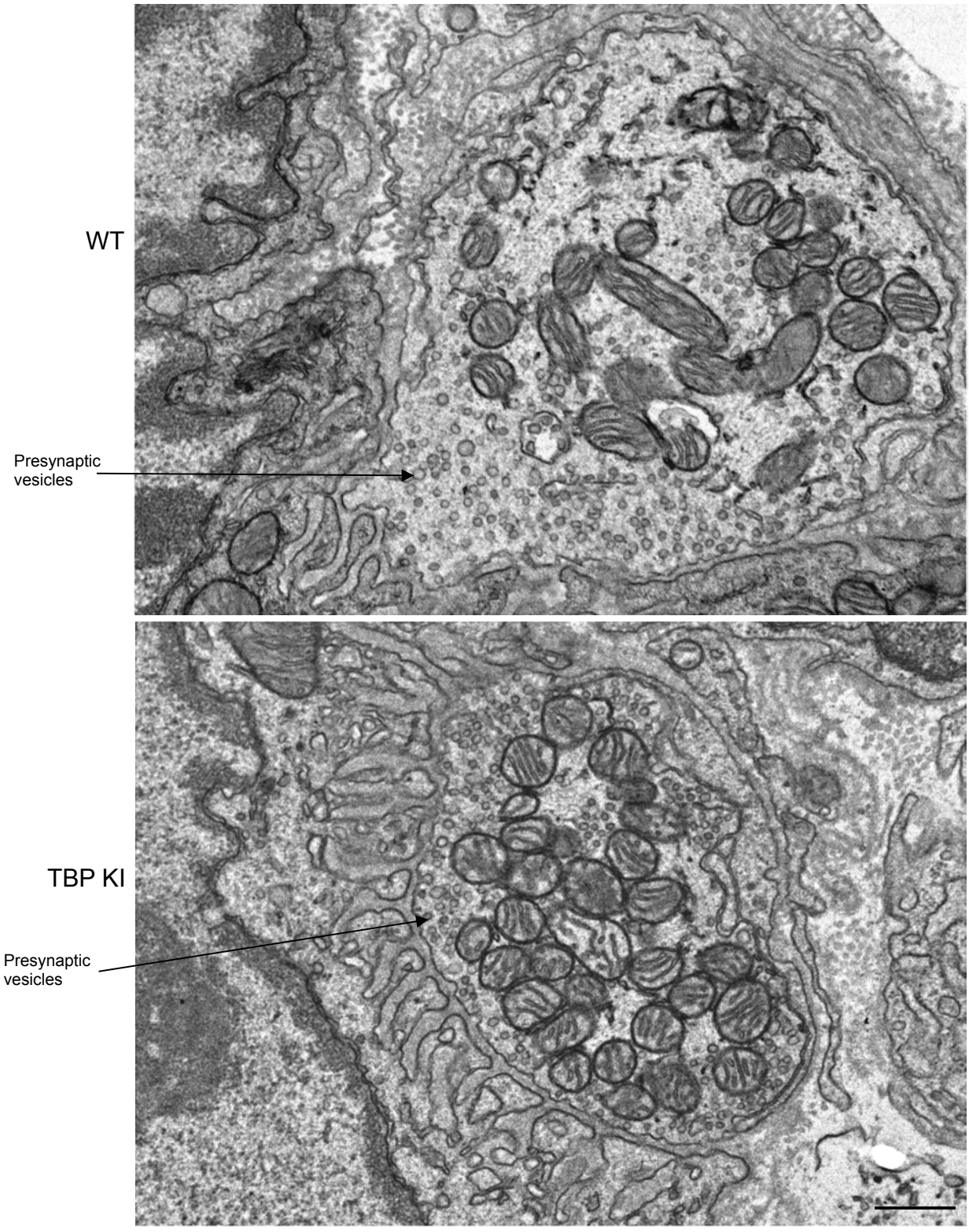

Figure S3 (Related to Figure 2). Mutant TBP caused no neuron muscular junction morphological changes. Electron microscopy showing the presynaptic structure of the neuron muscular junction (NMJ) of WT and SCA17 KI diaphragm muscles at 3 months of age. Arrows indicate presynaptic vesicles. The shape, number, and density of presynaptic vesicles in the NMJ are no different in WT versus SAC17 KI diaphragm muscles. Scale bar: 0.5 μm.

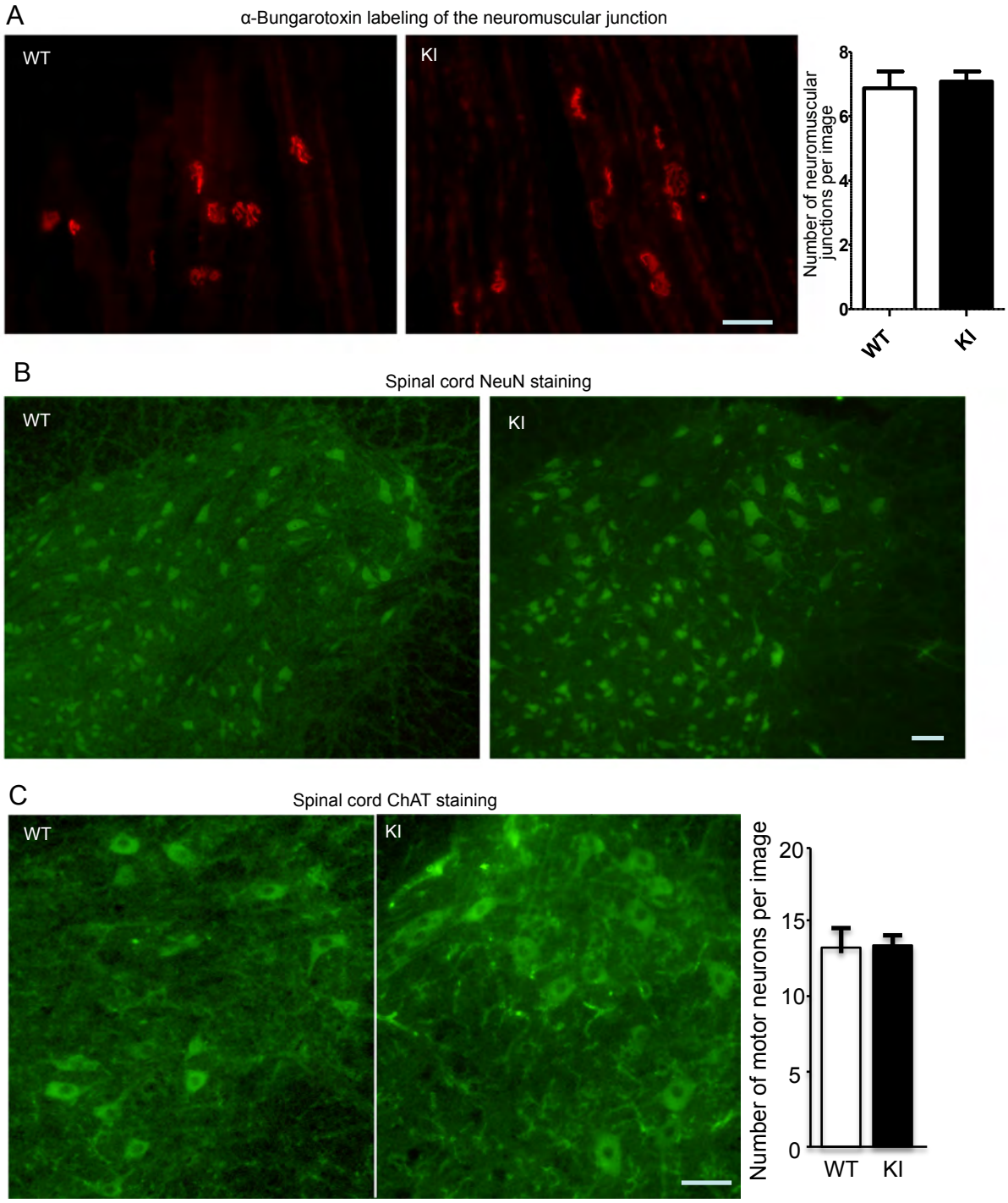

Figure S4 (Related to Figure 2). The absence of neuronal loss in the spinal cord in SCA17 mice. (A) α-Bungarotoxin labeling of the neuromuscular junction revealing no difference between WT and SCA17 KI mice at 5 months of age. (**B**) Immunofluroescent staining of the spinal cord with anti-NeuN antibody in the spinal cord of wild type (WT) and SCA17 KI mice at 5 months of age**. (C**) Immunofluroescent staining of the spinal cord with an antibody to choline acetyltransferase (ChAT) was also used to identify the spinal cord neurons**.** The data are mean+/-SE (n=9 per group). Scale bars: 20 μm.

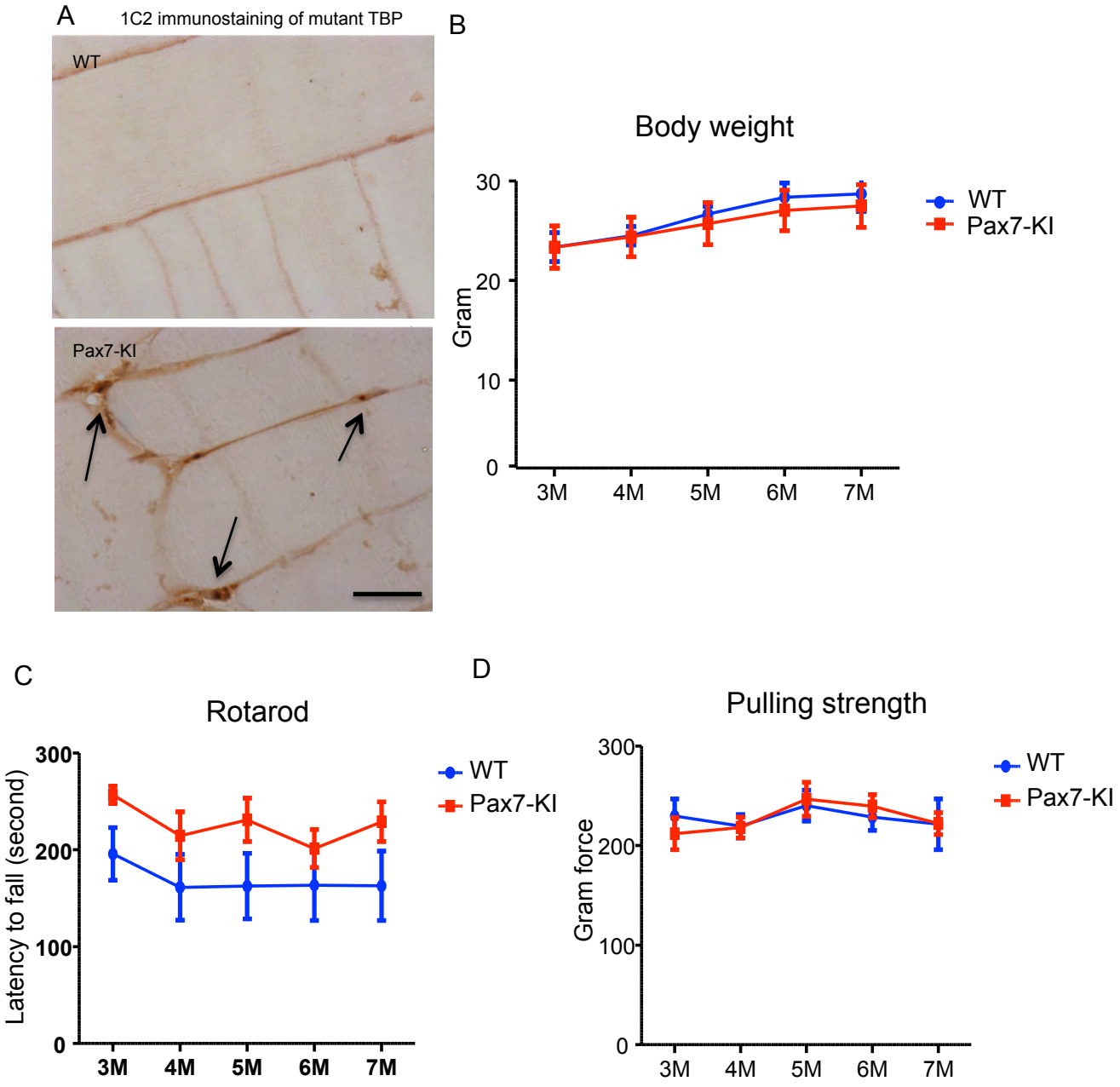

Figure S5 (Related to Figure 4). Expression of mutant TBP in muscle progenitor cells under the promoter of Pax7 does not lead to abnormal growth and muscle dysfunction. (A) Immunostaining of mouse muscle tissues with 1C2 shows the selective expression of mutant TBP in muscle satellite cells (arrows) that are located between the basal lamina and sarcolemma of muscle fibers in Pax7-KI mouse. (**B-D**) Body weight (B), rotarod performance (C), and grip strength test (D) show no defects in Pax7- KI mice compared with WT mice**.** The data are mean+/-SE (n= 5 mice per group). Scale bar: 20 μm.

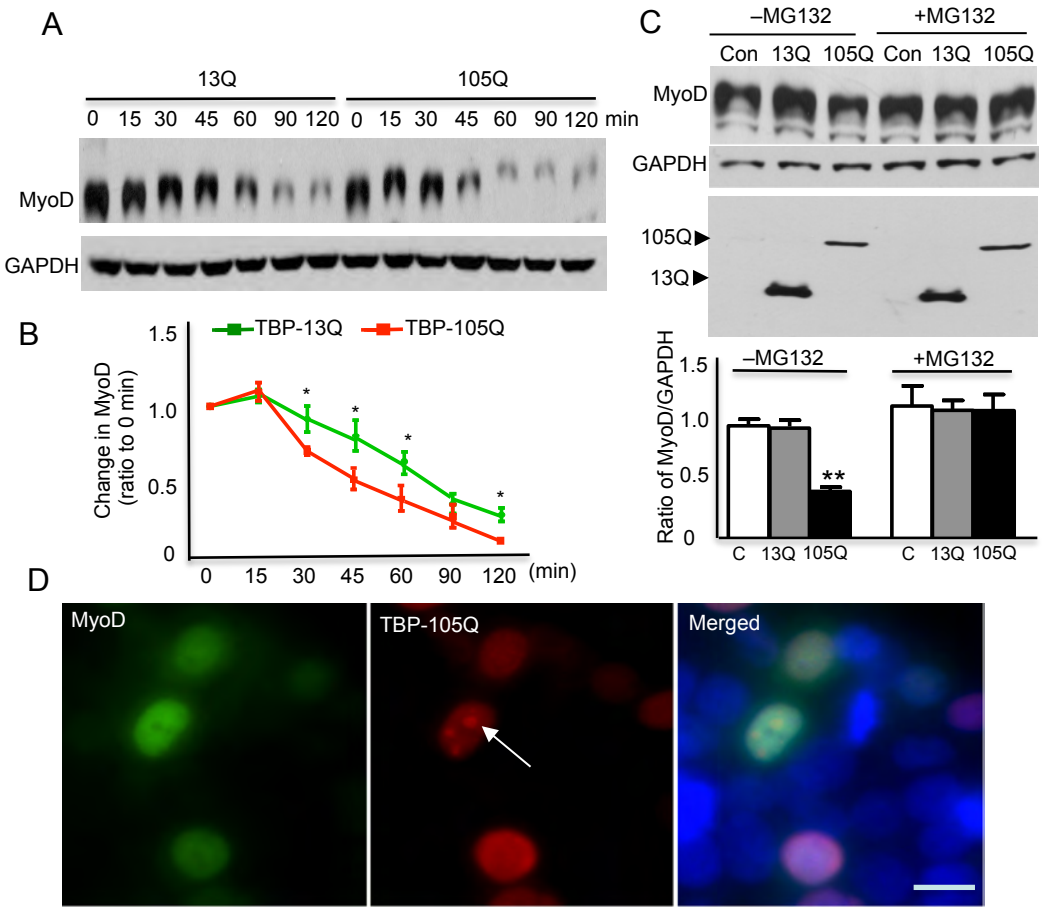

Figure S6 (Related to Figure 6). Increased degradation of MyoD by mutant TBP. (**A**) Western blot analysis of MyoD level in adenoviral TBP-13Q- or TBP-105Q-infected C2C12 cells with cycloheximide (100 μg/ml) treatment at indicated times after differentiation. (**B**) The densitometric ratios of MyoD expression at indicated times to 0 min showing increased MyoD degradation in TBP-105Q transfected cells. Data are mean ±SE, * p< 0.05. (**C**) Western blot analysis of MyoD expression in control, TBP-13Q, or TBP-105Q transfected C2C12 cells with (+MG132) or without (-MG132) MG132 (10 μM) overnight treatment after differentiation. The densitometric ratios of MyoD to GAPDH are presented (n=3, ** p< 0.01). (**D**) Immunofluorescent images showing transfected MyoD (green) is not colocalized with transfected TBP-105Q (red) aggregates in the nuclei (blue) of HEK293 cells. Arrow indicates aggregated mutant TBP. Scale bar: 5 μm.

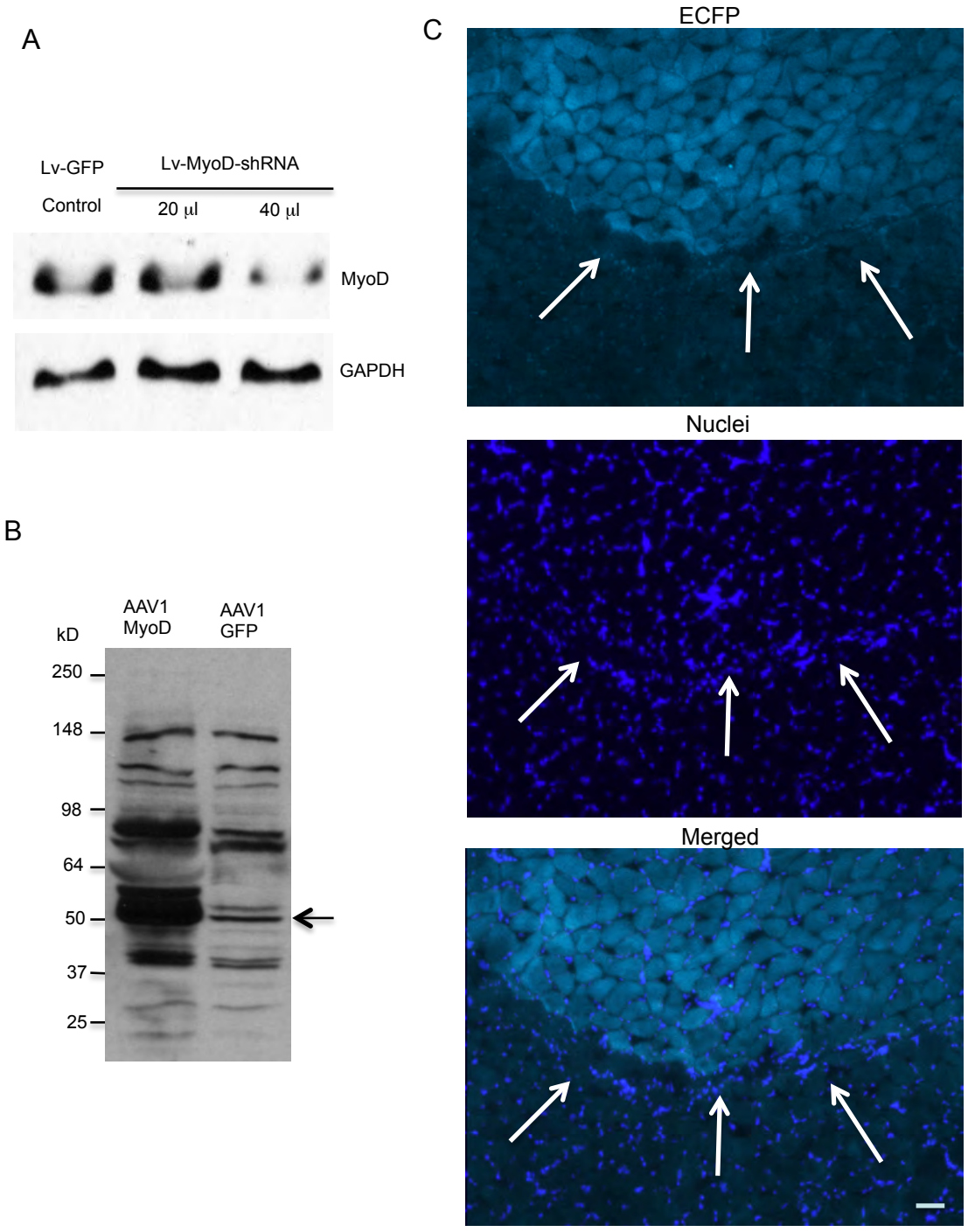

Figure S7 (Related to Figure 7). Expression of AAV1-MyoD-F2A-ECFP in TBPKI mouse TA muscle. (**A**) Western blot analysis of MyoD expression in lentiviral (Lv)-GFP (Control, $1x10^5$ infectious units of virus) or Lv-MyoD-shRNA ($1x10^5$, 20 μl or $2x10^5$, 40μl infectious units of virus) infected C2C12 cells. (**B**) Western blot analysis of MyoD expression in AAV1-MyoD-F2A-ECFP (AAV1-MyoD) or AAV1-GFP (AAV1-GFP) infected HEK293 cells. Arrow indicates MyoD. (**C**) SCA17 KI mouse TA muscle had been injected with $2x10^{10}$ v.g. AAV1-MyoD-F2A-ECFP virus for 45 days. Cross sections were collected, and the representative sections show the ECFP fluorescent signals, indicating the virus-infected area (arrows). Hoechst staining was used for nuclear visualization. Scale bar: 50 μm.

Table S1. Comparison of phenotype between Germline KI, Muscle KI and Nestin KI mice, related to Figure 5.

Genotype	Germline KI	Muscle KI	Nestin KI
Cre recombinase	EIIa-Cre	CKmm-Cre	Nestin-Cre
Tissue expressing mTBP gene	Whole body	Skeletal muscle, cardiac muscle	Brain
Tissue expressing accumulated mTBP	Brain, skeletal muscle	Skeletal muscle	Brain
Life span	5 to 10 month	6 to 12 month	No early death
Earliest appearence of rotarod deficit	3 month	3 month	9 month
Earliest appearence of body weight loss	3 month	4 month	11 month
Earliest appearance of muscle atrophy	3 month	3 month	12 month

Table S2. Down-regulated muscle related genes in KI mice (WT vs KI, Log2WT/KI)*, related to Figure 5.

Gene Symbol	Gene assignment	p-value	Log_2WT/KI
Meg3	maternally expressed 3	0.00566388	1.50163
Actn3	actinin alpha 3	0.00847351	1.5163
Homer1	homer homolog 1 (Drosophila)	0.00190498	1.53935
Jph2	junctophilin 2	0.00196035	1.55806
Casq1	calsequestrin 1	0.0030737	1.6045
Ppapdc3	phosphatidic acid phosphatase type 2 domain containing 3	0.00297483	1.62159
Mfsd7b	major facilitator superfamily domain containing 7B	0.00931034	1.57986
Tcap	titin-cap	0.00606555	1.68684
Pgam2	phosphoglycerate mutase 2	0.00583816	1.83166
Ankrd23	ankyrin repeat domain 23	0.00425885	1.91354
Ky	kyphoscoliosis peptidase	0.000290911	1.97531
Myl4	myosin, light polypeptide 4	0.00293607	2.3349
Map2k6	mitogen-activated protein kinase kinase 6	0.00026115	1.99152
Mylk2	myosin, light polypeptide kinase 2, skeletal muscle	0.00489059	2.49032
Fhl3	four and a half LIM domains 3	0.000272926	2.50155
Mylk4	myosin light chain kinase family, member 4	0.00170262	2.71121
Itgb1bp3	integrin beta 1 binding protein 3	0.00135303	3.38964
Myl3	myosin, light polypeptide 3	0.00587154	5.11264

*Altered muscle related genes in SCA17 KI mice skeletal muscle. P values were obtained via 1-way ANOVA, and Log2WT/KI >1.5 or 0.7 represents more than 1.5 fold differences between WT and KI samples.

Table S3. Up-regulated muscle related genes in KI mice (WT vs KI, Log2WT/KI)*, related to Figure 5.

Gene Symbol	Gene assignment	p-value	Log_2WT/KI
Ctnna1	catenin (cadherin associated protein), alpha 1	0.00058007	0.659295
Jup	junction plakoglobin	0.000271846	0.649925
Pak1	p21 protein (Cdc42/Rac)-activated kinase 1	0.00982691	0.617583
Lrp4	low density lipoprotein receptor-related protein 4	0.000352323	0.594408
Myom3	myomesin family, member 3	0.00449945	0.51258
Runx1	runt related transcription factor 1	0.000610497	0.516977
Atp1a1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	0.000262885	0.496516
Mustn1	musculoskeletal, embryonic nuclear protein 1	0.00199935	0.468677
Smtnl1	smoothelin-like 1	0.0010137	0.440527
Lix1	limb expression 1 homolog (chicken)	0.00212707	0.329293
Musk	muscle, skeletal, receptor tyrosine kinase	0.000260284	0.292846
Casq2	calsequestrin 2	0.000895619	0.307863

*Altered muscle related genes in SCA17 KI mice skeletal muscle. P values were obtained via 1-way ANOVA, and Log2WT/KI >1.5 or 0.7 represents more than 1.5 fold differences between WT and KI samples.

Table S4-1. Down-regulated neuron related genes in KI cerebellum (WT vs KI, Log2WT/KI)*, related to Figure 5.

Gene Symbol	Gene assignment	p-value	Log_2WT/KI
Inpp5a	inositol polyphosphate-5-phosphatase A	0.00119284	1.62603
Plxdc1	plexin domain containing 1	0.00309919	1.62821
Prmt8	protein arginine N-methyltransferase 8	0.00697059	1.63296
Rgs8	regulator of G-protein signaling 8	0.00963827	1.65422
Nab2	Ngfi-A binding protein 2	0.00017597	1.77836

Table S4-2 Up-regulated neuron related genes in KI cerebellum (WT vs KI, Log2WT/KI) *

Gene Symbol	Gene assignment	p-value	Log_2WT/KI
Kcnb2	potassium voltage gated channel, Shab-related subfamily	0.000900211	0.578133
Naip6	NLR family, apoptosis inhibitory protein 6	0.00458344	0.600952

*Altered neuronal related genes in SCA17 KI mice skeletal muscle. P values were obtained via 1-way ANOVA, and Log2WT/KI >1.5 or 0.7 represents more than 1.5 fold differences between WT and KI samples.