YMTHE, Volume *28*

Supplemental Information

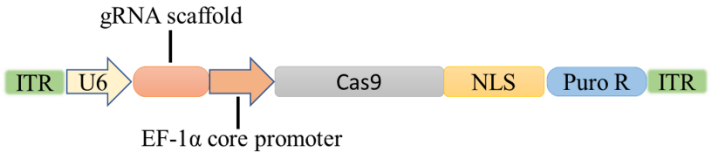
CRISPR/Cas9-Mediated miR-29b Editing as a Treatment of Different Types of Muscle Atrophy in Mice

Jin Li, Lijun Wang, Xuejiao Hua, Haifei Tang, Rui Chen, Tingting Yang, Saumya Das, and Junjie Xiao

Supplemental Figures and Legends:

Figure S1

a



b

	Forward primer(5'-3')	Reverse primer(5'-3')	Locus
1	GGGACTCGAGCACTCTATGTT	AGGGTCATTAGCAGGGTTCC	chr1:+196863288
2	TCATCGGACCTTGACAGCTC	TGTGCCAGGCCAGAGAAAAA	chr9:-101867160
3	AGCCGAGCTATCAATGGGC	TTCATCTGCATTCTGCGCTGT	chr2:-44912669
4	CTGCATTGAGTGCCTTAGCG	GACCATTTGGAAACCGTGTGA	chr11:-26661876
5	GCACTGGGGACATAGGTGAG	AGCCCACCTTGGCAATAGAC	chr5:-68275574
6	CAGTGAGCTTCACAGTTTGCT	GAGTCATACAGTATTTAGGCTGCT	chrX:+93743993
7	AAGGCTGAATGCCGTTCACT	GGCAAGAAGAACCTGGGACA	chr16:+15872481
8	ATGCAGCAGATGCCAGACTT	CTCATGAGCACAGGAGCCAA	chr15:-66654630
9	GACCACCACAATCGGCTGTA	GTTCTTGGCTCCCCTGACTC	chr11:-98951837
10	TGGCTGCCAATACCTATGCT	AGCCATCCCCTCGACTCAAA	chr4:+40629962

c

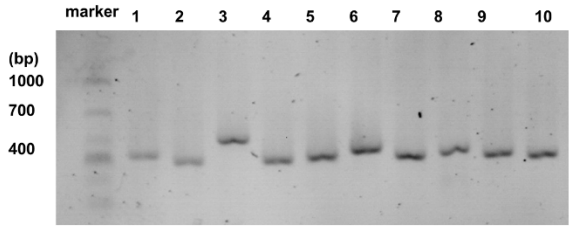


Figure S1. Backbone of lentiCRISPRv2 and T7EI analysis for the top genome-wide off-target sites with genomic DNA from C2C12 myotubes. (a) The backbone of lentiCRISPRv2 (Addgene plasmid # 52961). **(b)** The predicted top 10 potential off-target sites in the mouse genomic (http://crispr.mit.edu/). **(c)** No significant off-target mutagenesis was detected in T7EI cleavage assay.

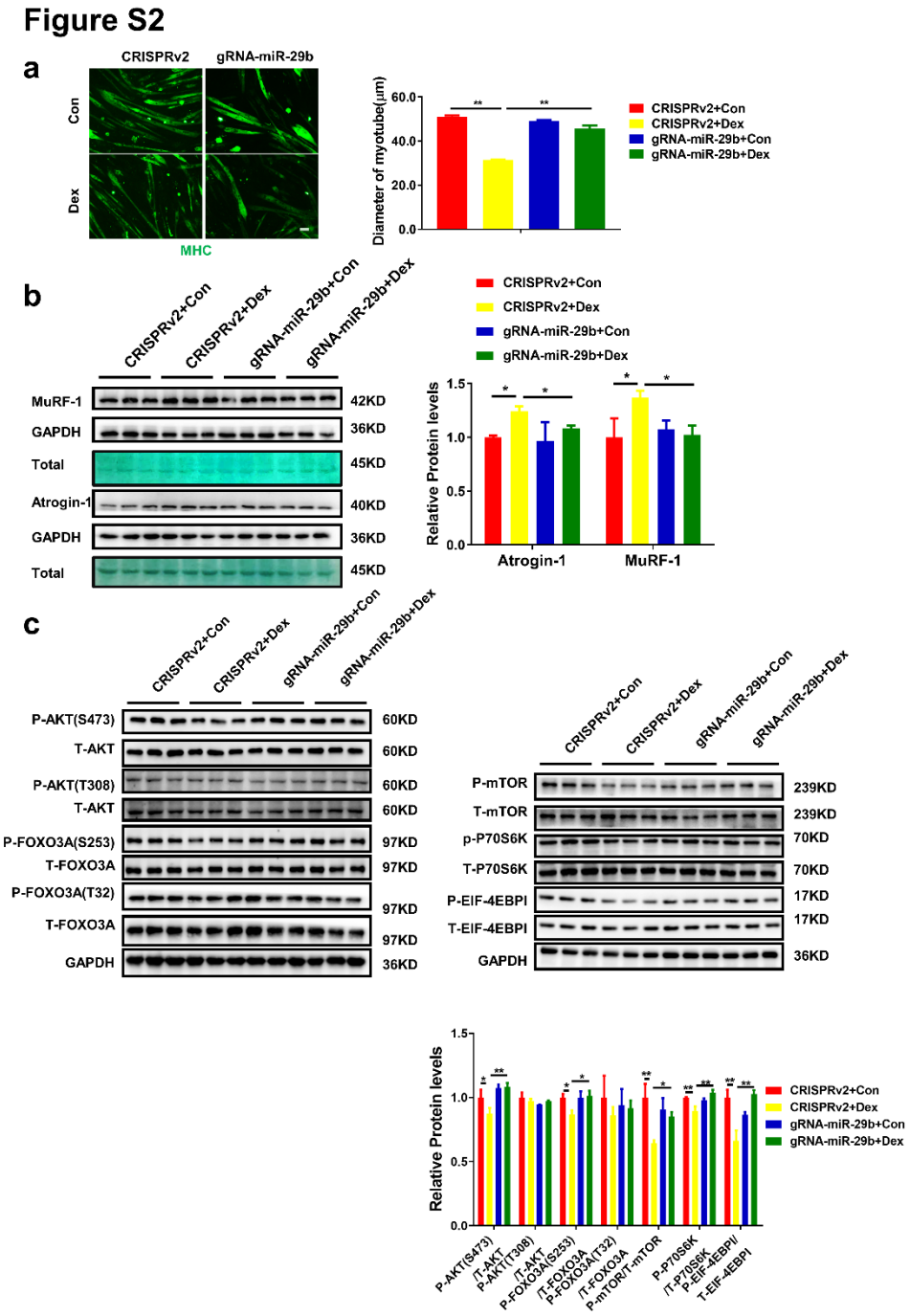


Figure S2. CRISPR/Cas9 mediated miR-29b editing prevents dexamethasone-induced atrophy in cultured C2C12 myotubes. (a) Immunofluorescence staining for diameters of C2C12 myotubes in dexamethasone (Dex)-induced atrophy with or without SpCRISPR-gRNA-miR-29b treatment (n=3, scale bar: 100 μm). **(b)** Western blot and quantitative analysis of MuRF-1 and Atrogin-1 protein levels in dexamethasone-induced atrophy with or without SpCRISPR-gRNA-miR-29b treatment (n=3). GAPDH protein and total proteins are stained for control. **(c)** Western blot and quantitative analysis for the AKT/FOXO3A/mTOR pathway (AKT, FOXO3A, mTOR, P70S6K, EIF-4EBP1) in C2C12 myotubes (n=3). One-way ANOVA test was performed to compare multiple groups followed by Bonferroni or Dunnett T3's post hoc test based on homogeneity of variance test. *p<0.05, **p<0.01 versus respective control.

Figure S3

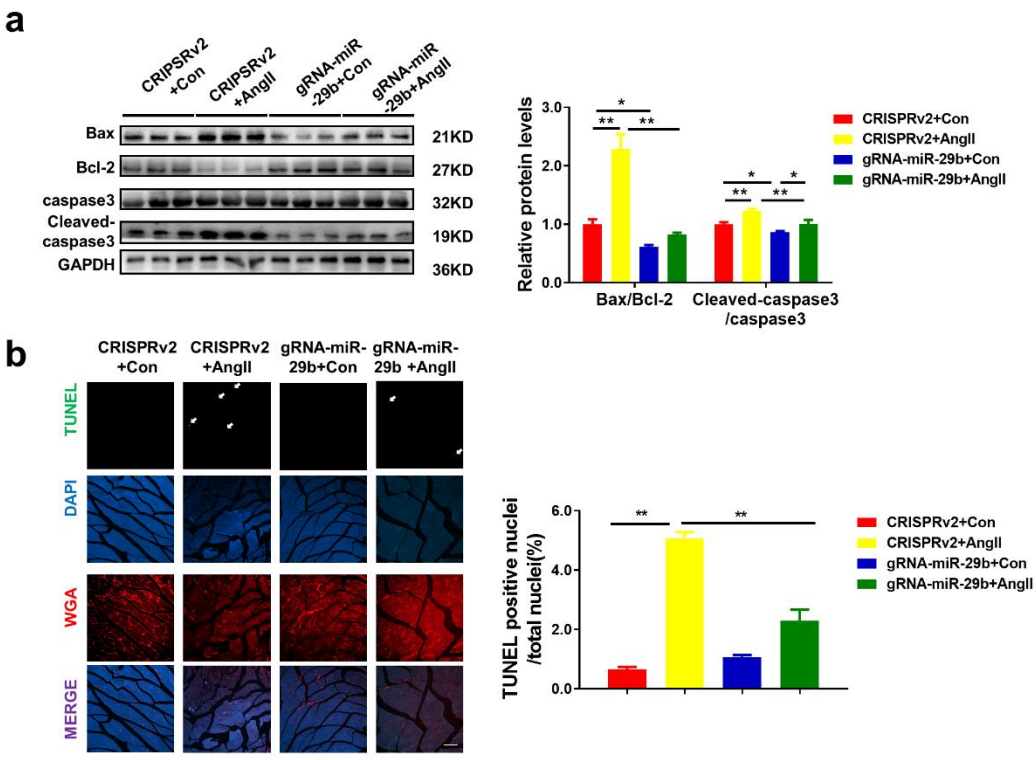


Figure S3. CRISPR/Cas9 mediated miR-29b editing prevents AngII-induced apoptosis. (a) Western blot and quantitative analysis of Bcl-2, Bax, Cleaved-caspase 3, caspase 3 protein levels in AngII-treated mice with or without SpCRISPR-gRNA-miR-29b treatment (n=3). **(b)** TUNEL staining of Gastrocnemius muscle in AngII-treated mice with or without SpCRISPR-gRNA-miR-29b treatment (n=4, scale bar: 20 μm). AngII, Angiotensin II. One-way ANOVA test was performed to compare multiple groups followed by Bonferroni or Dunnett T3's post hoc test based on homogeneity of variance test. *p<0.05, **p<0.01 versus respective control. Data are represented as mean±s.e.m.

Figure S4

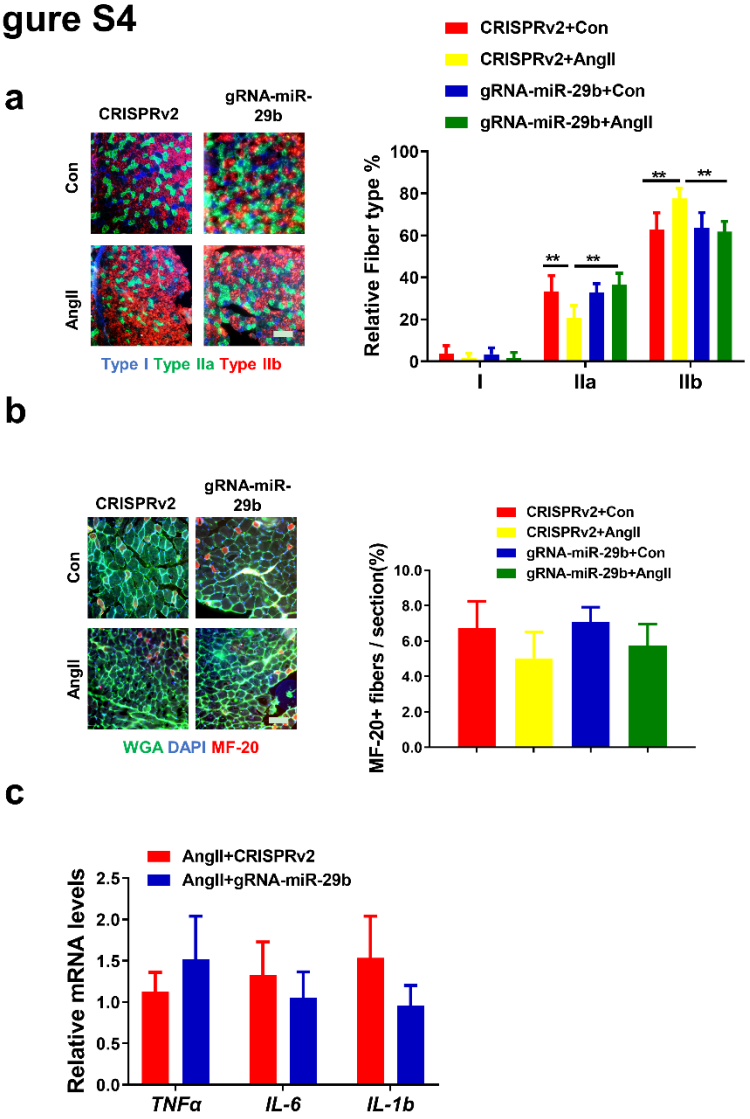


Figure S4. The effects of CRISPR/Cas9 treatment on muscle fiber composition, regeneration, and inflammation in AngII-induced muscle atrophy. (a) Immunofluorescence staining of myosin heavy chains isotypes (MHC) in Gastrocnemius muscle from indicated groups (n=6, scale bar: 100 μm). **(b)** Immunostaining for MF-20 from Gastrocnemius muscle in AngII-treated mice with or without SpCRISPR-gRNA-miR-29b treatment (n=4, scale bar: 100 μm). **(c)** RT-PCR analysis the mRNA expression levels of the inflammation factors (*TNFα*, *IL-6*, and *IL-1b*) in AngII-treated mice with or without SpCRISPR-gRNA-miR-29b treatment (n=6). AngII, Angiotensin II. One-way ANOVA test was performed to compare multiple groups followed by Bonferroni or Dunnett T3's post hoc test based on homogeneity of variance test **(a-b)**. An unpaired, two-tailed Student's t-test was used for comparisons between two groups **(c)**. **p<0.01 versus respective control. Data are represented as mean±s.e.m.

Figure S5

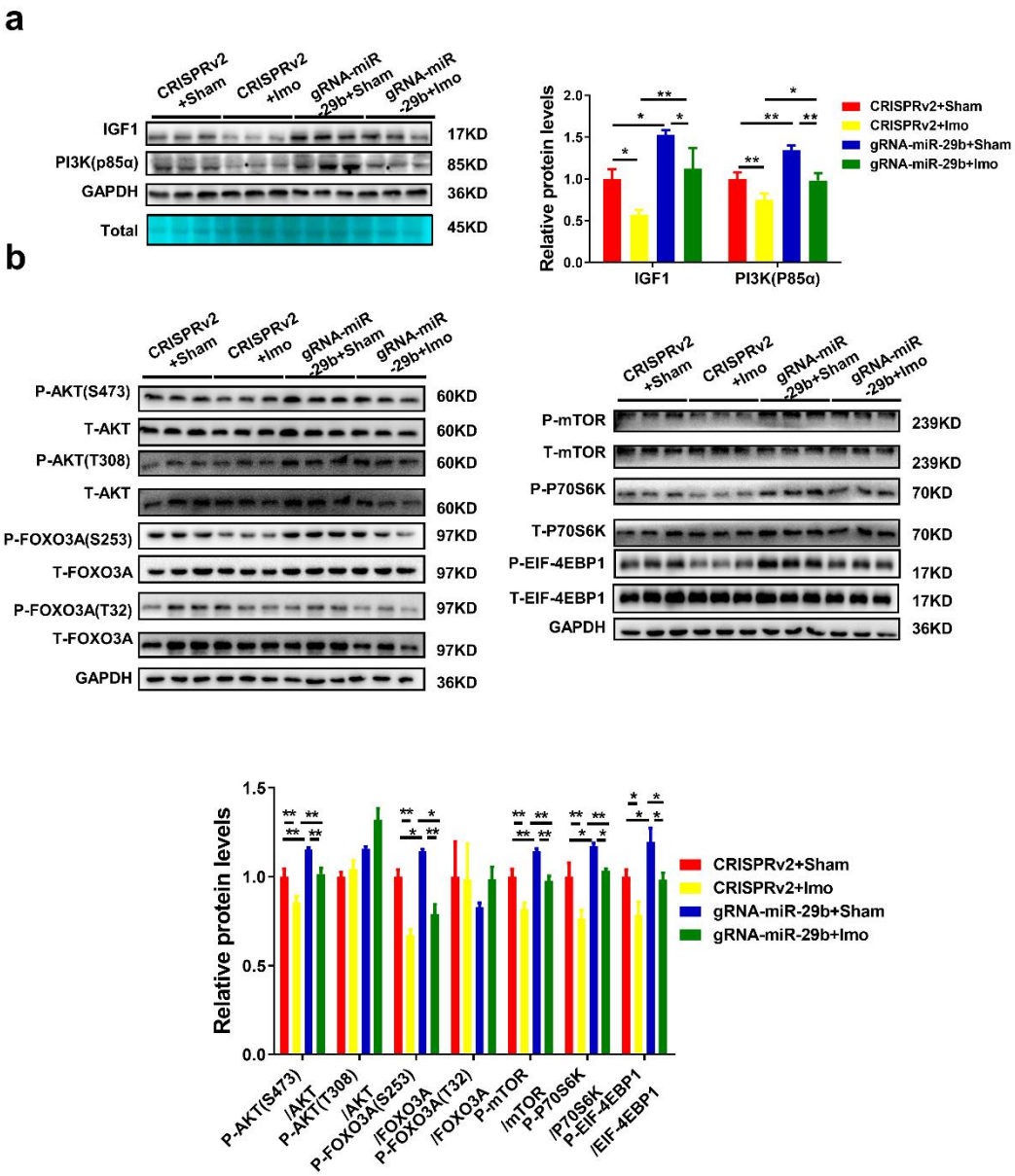


Figure S5. CRISPR/Cas9 mediated miR-29b editing prevents immobilization-induced atrophy effects through activation of the AKT-FOXO3A-mTOR signaling pathway. (a) Western blot and quantitative analysis of extracts from Gastrocnemius muscle to detect IGF1 and PI3K(p85α) protein in SpCRISPR-gRNA-miR-29b administration mice compared to control (n=3). GAPDH protein and total proteins are stained for control. **(b)** Western blot and quantitative analysis of the AKT/FOXO3A/mTOR pathway (AKT, FOXO3A, mTOR, P70S6K, EIF-4EBP1) in immobilization-treated mice with or without SpCRISPR-gRNA-miR-29b treatment (n=3). Imo, immobilization. One-way ANOVA test was performed to compare multiple groups followed by Bonferroni or Dunnett T3's post hoc test based on homogeneity of variance test. *p<0.05, **p<0.01 versus respective control. Data are represented as mean±s.e.m.

Figure S6

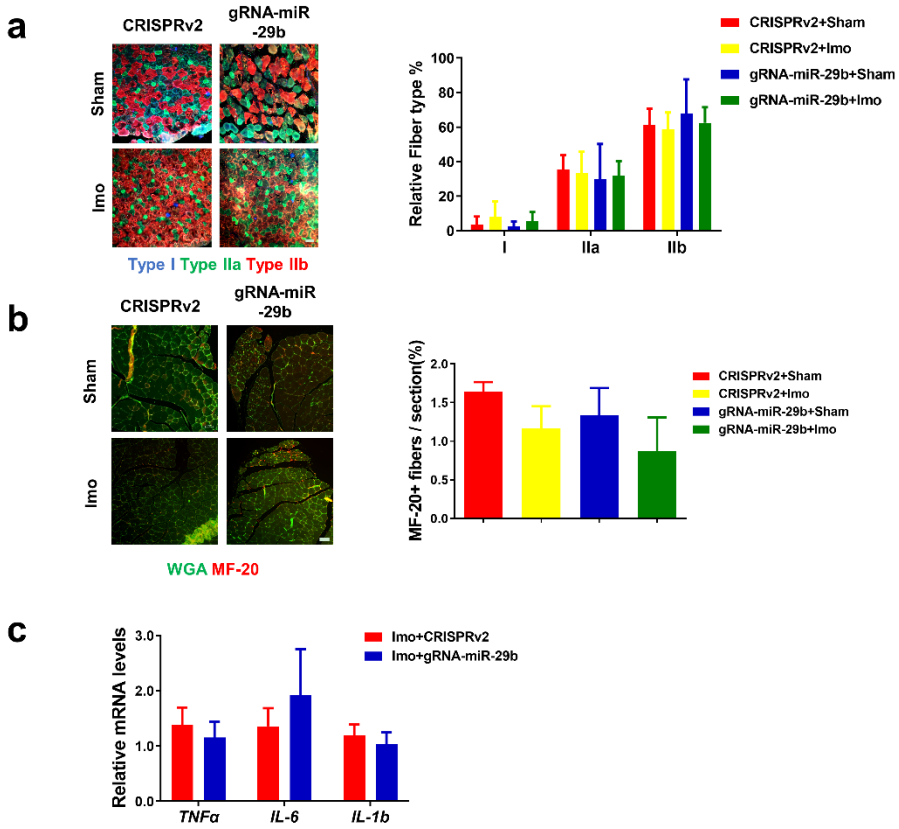


Figure S6. The effects of CRISPR/Cas9 treatment on muscle fiber composition, regeneration, and inflammation in immobilization-induced muscle atrophy. (a) The fiber type composition (n=5, scale bar: 100 μm), and **(b)** muscle regeneration (n=3,5,3,5, scale bar: 100 μm) in immobilization-induced muscle atrophy were presented by immunofluorescence staining. **(c)** RT-PCR analysis the mRNA expression levels of the inflammation factors (*TNFα*, *IL-6*, and *IL-1b*) in immobilization-treated mice with or without SpCRISPR-gRNA-miR-29b treatment (n=6). Imo, immobilization. One-way ANOVA test was performed to compare multiple groups followed by Bonferroni or Dunnett T3's post hoc test based on homogeneity of variance test **(a-b)**. An unpaired, two-tailed Student's t-test was used for comparisons between two groups **(c)**. Data are represented as mean±s.e.m.

Figure S7

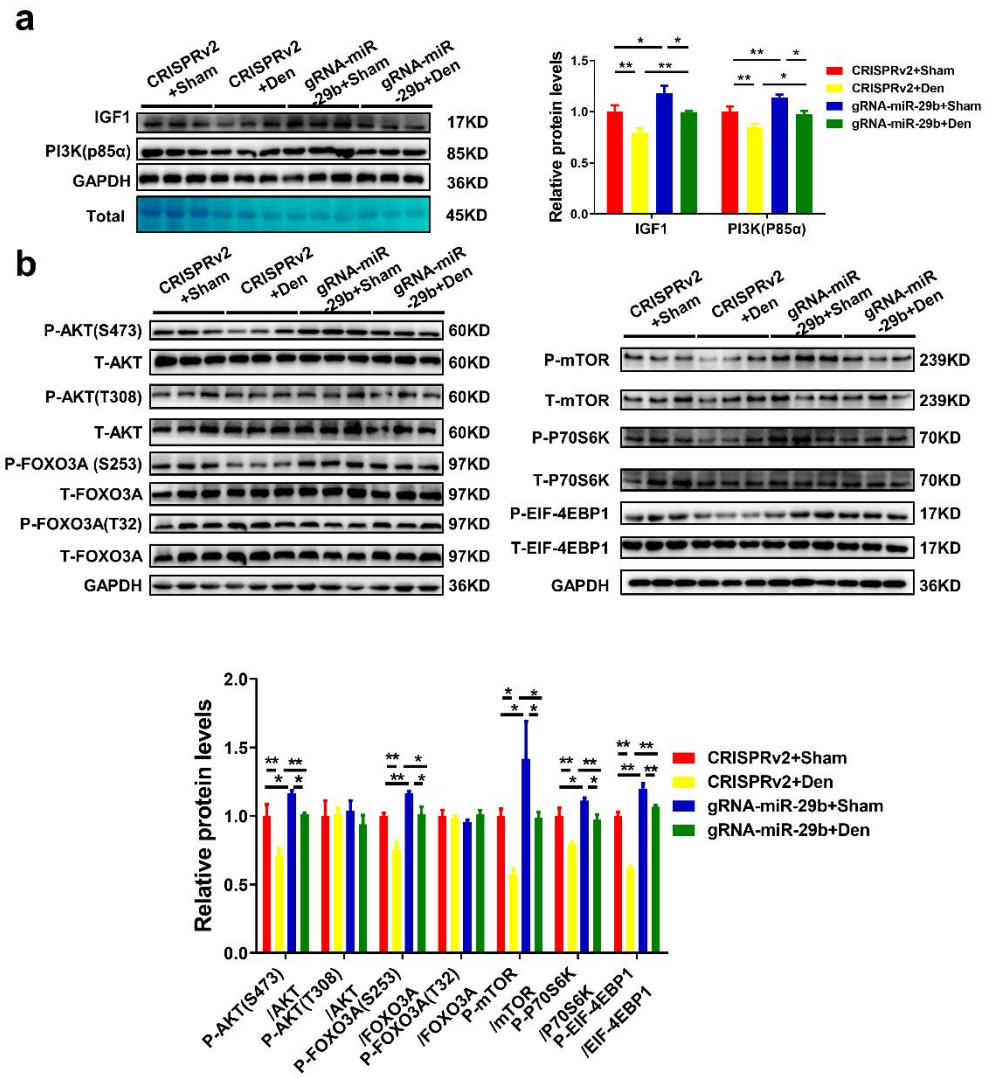


Figure S7. CRISPR/Cas9 mediated miR-29b editing prevents denervation-induced atrophy effects through activation of the AKT-FOXO3A-mTOR signaling pathway. (a) Western blot and quantitative analysis of extracts from Gastrocnemius muscle to detect IGF1 and PI3K(p85α) protein is shown in SpCRISPR-gRNA-miR-29b administration mice compared to control (n=3). GAPDH protein and total proteins are stained for control. **(b)** Western blot and quantitative analysis of the AKT/FOXO3A/mTOR pathway (AKT, FOXO3A, mTOR, P70S6K, EIF-4EBP1) in denervation-treated mice with or without SpCRISPR-gRNA-miR-29b treatment (n=3). Den, denervation. One-way ANOVA test was performed to compare multiple groups followed by Bonferroni or Dunnett T3's post hoc test based on homogeneity of variance test. *p<0.05, **p<0.01 versus respective control. Data are represented as mean±s.e.m.

Figure S8

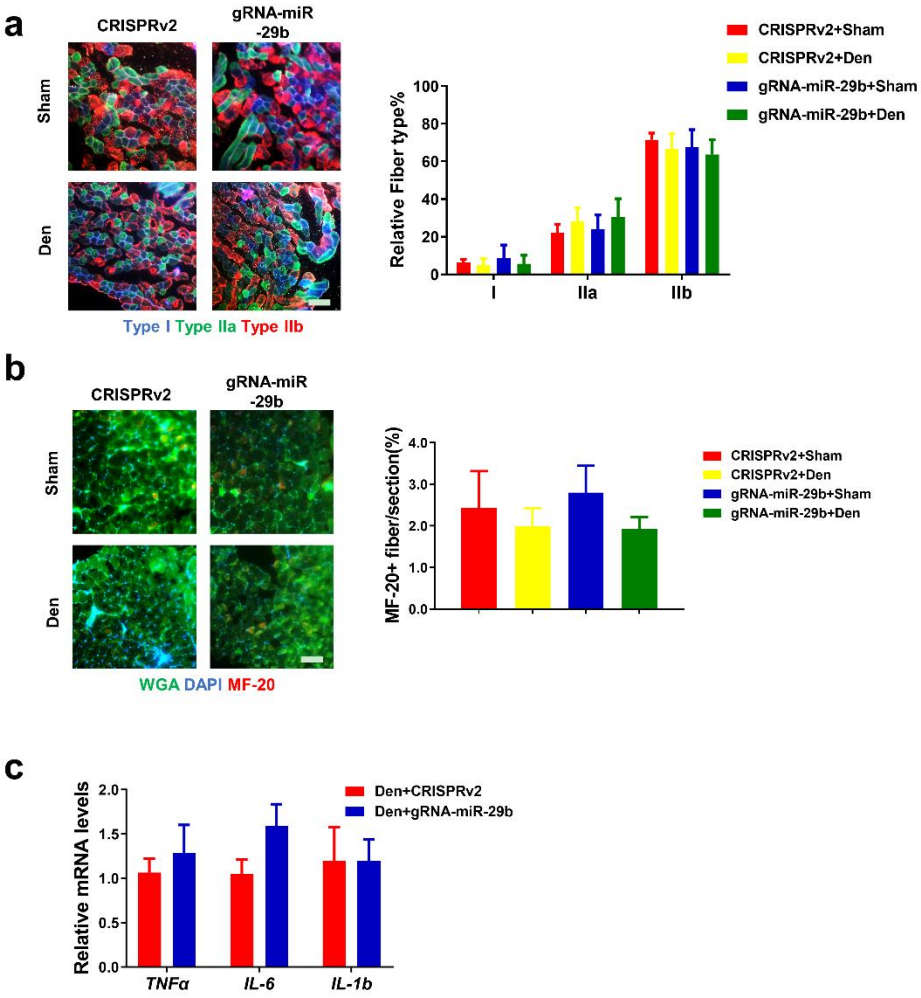


Figure S8. CRISPR/Cas9 treatment did not affect muscle fiber composition, regeneration, and inflammation in denervation-induced muscle atrophy. (a) The fiber type composition (n=6, scale bar: 100 μm), **(b)** muscle regeneration (n=4, scale bar: 100 μm), and **(c)** inflammation (n=6) in denervation-induced muscle atrophy were shown as indicated groups. Den, denervation. One-way ANOVA test was performed to compare multiple groups followed by Bonferroni or Dunnett T3's post hoc test based on homogeneity of variance test **(a-b)**. An unpaired, two-tailed Student's t-test was used for comparisons between two groups **(c)**. Data are represented as mean±s.e.m.

Figure S9

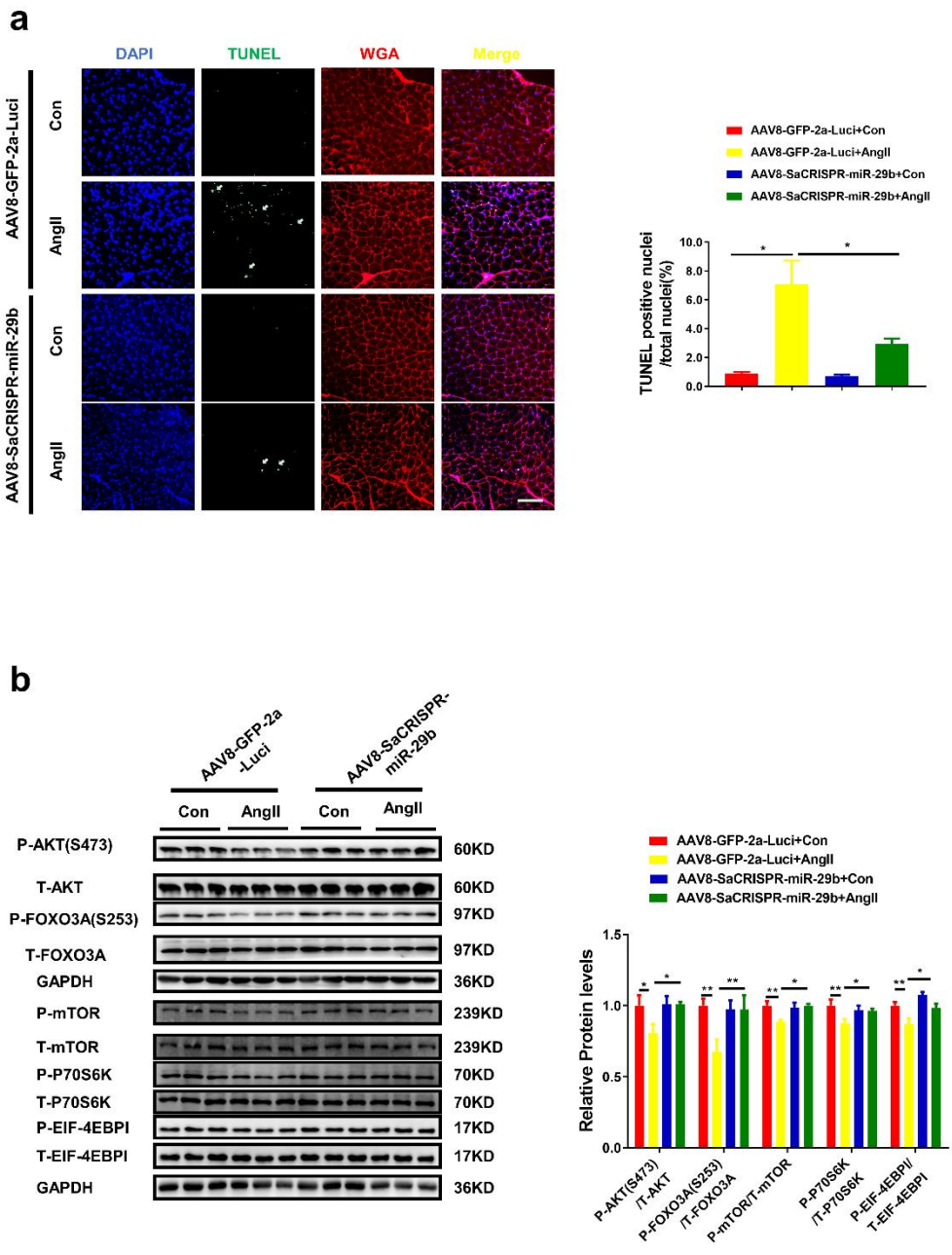


Figure S9. AAV8-CRISPR/SaCas9 mediated miR-29b editing prevents AngII-induced apoptosis and activates of AKT-FOXO3A-mTOR signaling pathway. (a) TUNEL staining of Gastrocnemius muscle in AngII-treated mice with or without AAV8-SaCRISPR-miR-29b treatment (n=4, scale bar: 100 μm). **(b)** Western blot and quantitative analysis of the relative phosphorylation levels of AKT(S473), FOXO3A(S253), mTOR, P70S6K and EIF-4EBP1 in AngII-induced muscle atrophy with or without AAV8-SaCRISPR-miR-29b-treated treatment (n=3). AngII, Angiotensin II. One-way ANOVA test was performed to compare multiple groups followed by Bonferroni or Dunnett T3's post hoc test based on homogeneity of variance test. *p<0.05, **p<0.01 versus respective control. Data are represented as mean±s.e.m.

Figure S10

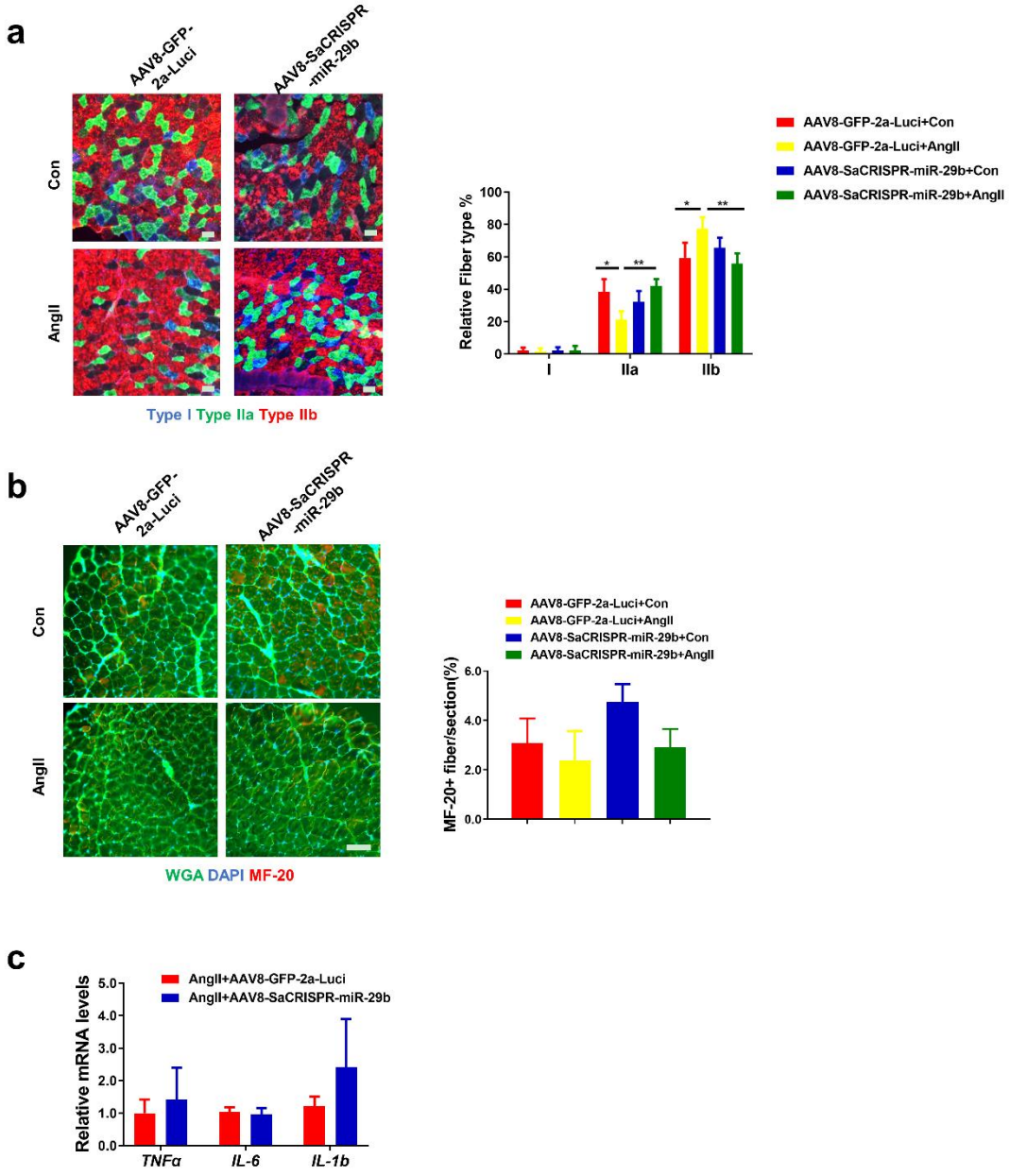


Figure S10. The effects of AAV8-CRISPR/SaCas9 treatment on muscle fiber composition, regeneration, and inflammation in AngII-induced muscle atrophy. (a) Immunofluorescence staining of myosin heavy chains isotypes (MHC) in AngII-induced atrophy are presented (n=4, scale bar: 100 μm). **(b)** Immunostaining for MF-20 is showed as indicated groups (n=4, scale bar: 100 μm). **(c)** RT-PCR analysis the expression of inflammation factors (*TNFα*, *IL-6*, and *IL-1b*) in AngII-induced muscle atrophy as indicated groups (n=8,7). AngII, Angiotensin II. One-way ANOVA test was performed to compare multiple groups followed by Bonferroni or Dunnett T3's post hoc test based on homogeneity of variance test **(a-b)**. An unpaired, two-tailed Student's t-test was used for comparisons between two groups **(c)**. *p<0.05, **p<0.01 versus respective control. Data are represented as mean±s.e.m.

Figure S11

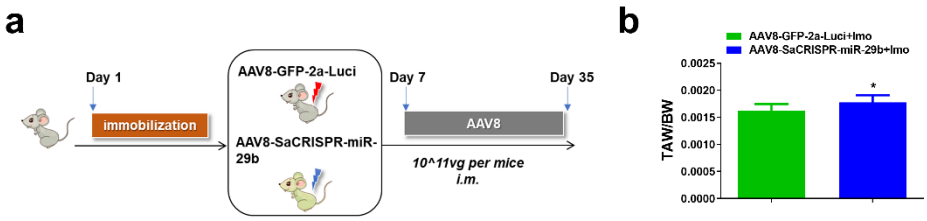


Figure S11. AAV8-CRISPR/SaCas9 mediated miR-29b editing prevents immobilization induced-muscle atrophy in vivo. (a) The schedule of immobilization (Imo)-induced atrophy mice model and virus injection establishment. **(b)**Tibialis anterior weight/body weight ratio (TAW/BW) ratio are shown in Imo-treated mice with or without AAV8-SaCRISPR-miR-29b injection treatment (n=6,7). An unpaired, two-tailed Student's t-test was used for comparisons between two groups (b). *p<0.05 versus respective control. Data are represented as mean±s.e.m.

Figure S12

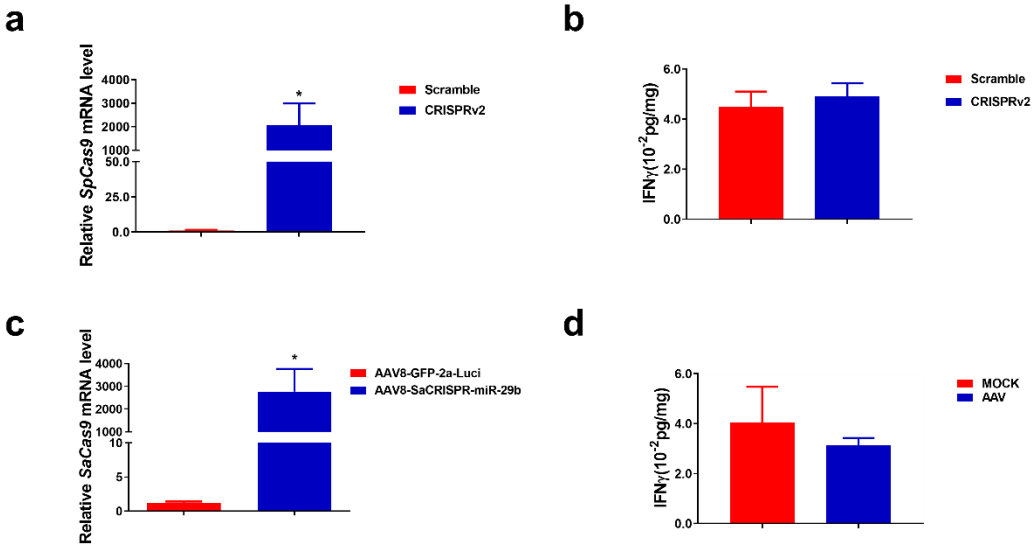


Figure S12. Expression of both the SpCas9 and SaCas9 mRNA and the production of IFNγ in the endpoints of the experiments. (a) RT–PCR analysis of *SpCas9* mRNA expression level after 2 weeks for lentivirus-delivered CRISPR/spCas9 (n=6). **(b)** The production of IFNγ was evaluated by enzyme-linked immunosorbent assay (ELISA) after 2 weeks for lentivirus-delivered CRISPR/spCas9 (n=5). **(c)** RT–PCR analysis of *SaCas9* mRNA expression level after 4 weeks for AAV8-delivered /SaCas9 (n=14). **(d)** The production of IFNγ was evaluated by ELISA after 4 weeks for AAV8-delivered /SaCas9 (n=3,6). An unpaired, two-tailed Student's t-test was used for comparisons between two groups. *p<0.05 versus respective control. Data are represented as mean±s.e.m.

Supplemental Tables:

Table S1. gRNAs sequences used in this study.

	Sequence	PAM
A	AGGAAGCTGGTTTCATATGG	TGG
B	TTCAGGAAGCTGGTTTCATA	TGG
C	CCATTTGAAATCAGTGTTTT	AGG
D	CCTAAAACACTGATTTCAAA	TGG

Table S2. qPCR primers used in this study.

Gene	Forward (5'-3')	Reverse (5'-3')
mmu-*IL-6*	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
mmu-*TNFα*	AGGCACTCCCCCAAAAGATG	CCACTTGGTGGTTTGTGAGTG
mmu-*IL-1b*	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
SaCas9	GGCGTCAGACTGTTCAAGGA	GGTCGGTCAGCAGGTTGTAA
SpCas9	CATCGAGCAGATCAGCGAGT	CGATCCGTGTCTCGTACAGG
mmu-*18s*	TCAAGAACGAAAGTCGGAGG	GGACATCTAAGGGCATCAC

Supplemental Methods:

Cultured C2C12 myotubes staining

To determine the diameter of myotubes in vitro, C2C12 myotubes were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked with 5% BSA. Then the primary antibody anti-MHC (MF-20, 1:100, DSHB) was used to specific label myotubes, FITC-AffiniPure Rabbit Anti-Mouse IgG (H+L) (1:500, Jackson) was used as secondary antibody. DAPI was subjected to nuclear staining. Images were captured by fluorescence microscope (Leica, Wetzlar, Germany) and the diameter of myotubes was measured by Image J.

Skeletal Muscle Histology and Immunofluorescence

For skeletal muscle fiber types analyses, skeletal muscle was obtained and embedded in OCT, and snap frozen in cold liquid nitrogen. Samples were cut at 10 μm per section. The section was incubated in room temperature (RT) for 20min, fixed with 4% paraformaldehyde in RT for another 20min. Then, sections were blocked with 5% BSA in PBS, incubated with anti-MyHC-I (BA-D5, 1:3, DSHB), anti-MyHC-IIA (SC-71, 1:10, DSHB), anti-MyHC-IIb (BF-F3, 1:3, DSHB) overnight at 4℃. After washed with PBS, the secondary antibodies were applied at a dilution of 1:100, the Alexa 350 anti-mouse IgG2b (Invitrogen, USA), Alexa 488 anti-mouse IgG1 (Invitrogen, USA), and Alexa 555 anti-mouse IgM (Invitrogen, USA) were used. Then the images were captured by fluorescence microscope (Zeiss, Oberkochen Germany).

For regeneration analysis, frozen sections of 10μm were used after rewarming, permeability and blocking, the sections were incubated with MF-20 (1:50, DSHB) overnight at 4℃. Then WGA conjugates (Invitrogen, USA) was incubated to show the outline of muscle fibers. Secondary antibody Cy3-AffiniPure Rabbit Anti-Mouse IgG (H+L) (1:500, Jackson, USA) was incubated at RT for 1 h to label MF-20, and 4,6-Diamidin-2-phenylindol (DAPI, Sigma, USA) was stained for nuclei. The images were captured using fluorescence microscope (Zeiss, Oberkochen Germany).