SUPPLEMENTAL METHODS

Antibodies and other reagents

Anti-MHC (clone MF20) and anti-myogenin (clone F5D) were obtained from the Developmental Studies Hybridoma bank (developed under the auspices of the NICHD, National Institutes of Health, and maintained by University of Iowa, Department of Biological Sciences). Anti-Flag-BioM2 (F9291) and gelatin were from Sigma (St. Louis, MO, USA). Anti-Myc (clone 9E10.2) and anti-HA (clone 16B12) were from Covance (Princeton, NJ, USA). Anti-tubulin (ab11304), anti-LRS (ab31534), anti-IleRS (ab31533), and anti-EPRS (ab31531), were from Abcam (Cambridge, MA, USA). Anti-IRS1 (2382), anti-phospho-IRS1(Ser307; 2381), anti-Akt (9272), anti-phospho-Akt (Thr473; 9271), anti-RagA (4357), anti-Raptor (2280), and anti-GAPDH (2118), were from Cell Signaling Technology (Danvers, MA, USA). Peroxidase-conjugated anti-rabbit (115-036-003) and anti-mouse (111-036-003) IgG antibodies and FITC-conjugated anti-mouse IgG (115-096-003) were from Jackson Immuno Research Laboratories (West Grove, PA, USA). EdU was from Carbosynth Limited (Compton, UK). FAM azide 5-isomer was from Lumiprobe (Hunt Valley, MD, USA). Akt inhibitors, Akti-1/2 and triciribine, were from Calbiochem (San Diego, CA, USA).

Plasmids

The following plasmids were previously reported: pCDNA8F-Flag-LRS-WT (1), pCDNA-Myc-LRS-WT, pCDNA-Myc-LRS-F50A/Y52A, pCDNA-Myc-LRS-K716A/K719A (2); pCMV6-myristoylated-HA-Akt (3). pRK5-HA-GST-RagB-Q99L (Plasmid #19303) and

pRK5-HA-GST-RagD-S77L (Plasmid #19308) (4) were from Addgene (Watertown, MA, USA).

Cell culture

C2C12 myoblasts were a gift from S. Kaufman at the University of Illinois and originally obtained from ATCC. Cells were maintained in DMEM containing 4.5 g/L glucose, 10% fetal bovine serum, and 1% penicillin-streptomycin, at 37°C in a humidified atmosphere of 92.5% air and 7.5% CO_2. To induce differentiation, cells were plated on tissue culture plates coated with 0.2% gelatin, grown to 100% confluence, and cultured in differentiation medium (DMEM containing 2% horse serum) thereafter. The cells were replenished with fresh differentiation medium daily for 3 days. Myoblasts were transfected by using TransIT-LT1 (Mirus, Madison, WI) or Amaxa cell line Nucleofector Kit V (Lonza, Cologne, Germany) according to the manufacturer's recommendations. Cells were selected in 1.0 mg/mL G418.

Lentivirus-delivered RNA interference

shRNAs in the pLKO.1-puro vector were purchased from Sigma-Aldrich (MISSION TRC). Clone IDs are: shLRS#1, TRCN0000253379; shLRS#2, TRCN0000253377; shIleRS#1, TRCN0000374543; shIleRS#2, TRCN0000312129; shEPRS#1, TRCN0000178427; shEPRS#2, TRCN0000178392. The following shRNAs were previously reported: RagA, TRCN0000316855; RagB, TRCN0000102657; Raptor#1, TRCN0000077472; Raptor #2 shRNA was cloned by inserting the following sequence into pLKO.1puro: 5'CCGGGGCTAGTCTGTTTCGAAATTTCTTCCTGTCAAAATTTCG

AAACAGACTAGCCTTTTTG3' (5, 6). A hairpin of scrambled sequence (shScramble) used for a negative control and lentivirus packaging were previously described (7). C2C12 cells were transduced with lentiviruses in growth medium containing 8 μg/mL Polybrene and selected in 3 μg/mL puromycin for 2 days, followed by plating into 12-well plates for differentiation.

Cell lysis and Western blotting

Cells were rinsed with ice-cold PBS and lysed in SDS sample buffer containing 5% β-mercaptoethanol or in MIPT buffer as previously described (6). Proteins were resolved on SDS-PAGE, transferred onto PVDF membrane (EMD Millipore, Darmstadt, Germany), and incubated with various antibodies according to the manufacturers' recommendations. Detection of horseradish peroxidase-conjugated secondary antibodies was performed with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Immunofluorescence microscopy and quantitative analysis of myocytes

C2C12 cells differentiated in 12-well plates were fixed and stained for MHC with DAPI as previously described (6). The stained cells were observed with a Leica DMI 4000B fluorescence microscope (Leica, Wetzlar, Germany), and the fluorescence images were captured using a RETIGA EXi camera (QImaging, Surry, BC, Canada) and Image Pro Express software (Media Cybernetics, Rockville, MD, USA). Images were analyzed by using ImageJ software (NIH, Bethesda, USA). The differentiation index was calculated

as the percentage of nuclei in MHC-positive cells. Each data point was generated by quantifying all cells in five randomly chosen microscopic fields.

Cell proliferation assay

C2C12 myoblasts or differentiating myocytes were incubated with EdU (final concentration of 1 μM) for 2 hours and then fixed with 3.7% formaldehyde. Cells were treated with 10 μM FAM azide 5-isomer, 1 mM $CuSO_4$, and 100 mM Ascorbic acid in PBS for 30 minutes. After the treatment, nuclei were stained by DAPI for 30 minutes. Fluorescence microscopy and quantification were performed in a similar way as described in “Immunofluorescence microscopy and quantitative analysis of myocytes” above.

Determination of protein synthesis by ^{35}S-Met/Cys metabolic labeling

C2C12 myoblasts ($3.5x10^5$ cells) were pre-incubated in methionine/cysteine-free DMEM medium with 10% FBS at 37°C for 30 min. ^{35}S-methionine/cysteine (Perkin-Elmer) was added into the medium at a final concentration of 200 μCi/mL and incubated at 37°C for 30 min. Labelled cells were collected in PBS and lysed, and cellular proteins were precipitated with trichloroacetic acid and subjected to scintillation counting for the determination of ^{35}S incorporation.

Injury-induced muscle regeneration and intramuscular gene knockdown

Male and female C57BL/6 wild-type mice aged 10-12 weeks (Envigo) were randomly allocated to the different experimental groups. Muscle injury was induced by injecting 50

μL of 1.2% (w/v) $BaCl_2$ dissolved in saline (35 μL when combined with 15 μL of concentrated shRNA) into mouse hindlimb TA muscles as previously described (8). To knockdown LRS, RagA, and Raptor, shLRS, shRagA, and shRaptor viruses (see "Lentivirus-delivered RNA interference") were concentrated to $1x10^7$-$1x10^8$ IU/mL via ultracentrifugation and co-injected with $BaCl_2$ into the TA muscles. The injured muscles were collected at 4, 7, or 14 days after injury.

BC-LI-0186 and Triciribine injections

BC-LI-0186 and triciribine stock solutions were dissolved in DMSO to generate 5 mg/mL and 10 mg/mL stock solutions, respectively. BC-LI-0186 diluted in 100 μL of PBS was administered to mice at 5 mg/kg body weight via an intraperitoneal injection once every 3 days. This dose did not show any detectable adverse effect on the animal physiology or behavior. Triciribine diluted in 100 μL of PBS was administered to mice at 1 mg/kg body weight (with or without 5 mg/kg of BC-LI-0186) via an intraperitoneal injection every day. All control mice received an intraperitoneal injection of an equivalent amount of DMSO in 100 μL of PBS.

Muscle tissue cryosection and analysis

Isolated TA muscles were frozen in liquid nitrogen-cooled 2-methylbutane and embedded in TBS tissue freezing medium (Thermo Fisher Scientific, Waltham, MA, USA). Sections of 10 μm thickness were made with a Microm HM550 (Thermo Fisher Scientific, Waltham, MA, USA) at –20°C, placed on uncoated slides, and stained with hematoxylin and eosin (H&E). Five to ten images of the injured areas were randomly

captured from the stained sections with a 20X dry objective (Fluotar, numerical aperture 0.4; Leica) on a Leica DMI 4000B microscope. The images were then analyzed for cross-sectional area of all centrally nucleated regenerating myofibers. All procedures were performed by investigators that were blinded to sample identification.

In situ muscle force measurement

In situ force measurements of TA muscles were performed as previously reported (9, 10) using a 1300A Whole-Animal System (Aurora Scientific, Aurora, Canada). The mouse was anesthetized and placed on an isothermal stage (38°C). TA muscle was then surgically exposed, and the distal tendon of the muscle was tied with a 3-0 suture line. After cutting the tendon, the suture line was used to connect the TA muscle to the lever arm of the force transducer. The hindlimb was stabilized by attaching the patella tendon to a fixed post using a needle insert. Electrical stimulations were elicited with 0.2-ms pulses at 0.2 mA by placing two electrodes on either side of the TA muscle. Optimal muscle length that produces a maximum twitch force was first determined, and then maximum isometric tetanic force was measured with 300-ms duration at 200 Hz. When necessary, the measurements were repeated with a 1-minute rest between stimulations. Throughout the experiments, the exposed TA muscle was kept wet with a warm PBS-soaked KimWipe. At the end of the measurements, optimal length and wet weight of the TA muscle were measured to calculate the physiological cross-sectional area (muscle mass/optimal length*muscle density 1.06 g/cm^3) which was then used to obtain specific isometric twitch and tetanic forces.

Statistical analysis

All quantitative values were expressed as mean ± SEM. The exact sample size for each experiment is described in Figure legends. Whenever necessary, statistical significance of the data was analyzed by performing two-tailed paired/unpaired t-tests for single comparison or one- or two-way ANOVA followed by planned comparisons or Student-Newman-Keuls post hoc test for multiple comparisons. Differences between groups were considered significant when $P < 0.05$. All statistical analyses were performed using Excel or SigmaPlot.

SUPPLEMENTAL REFERENCES

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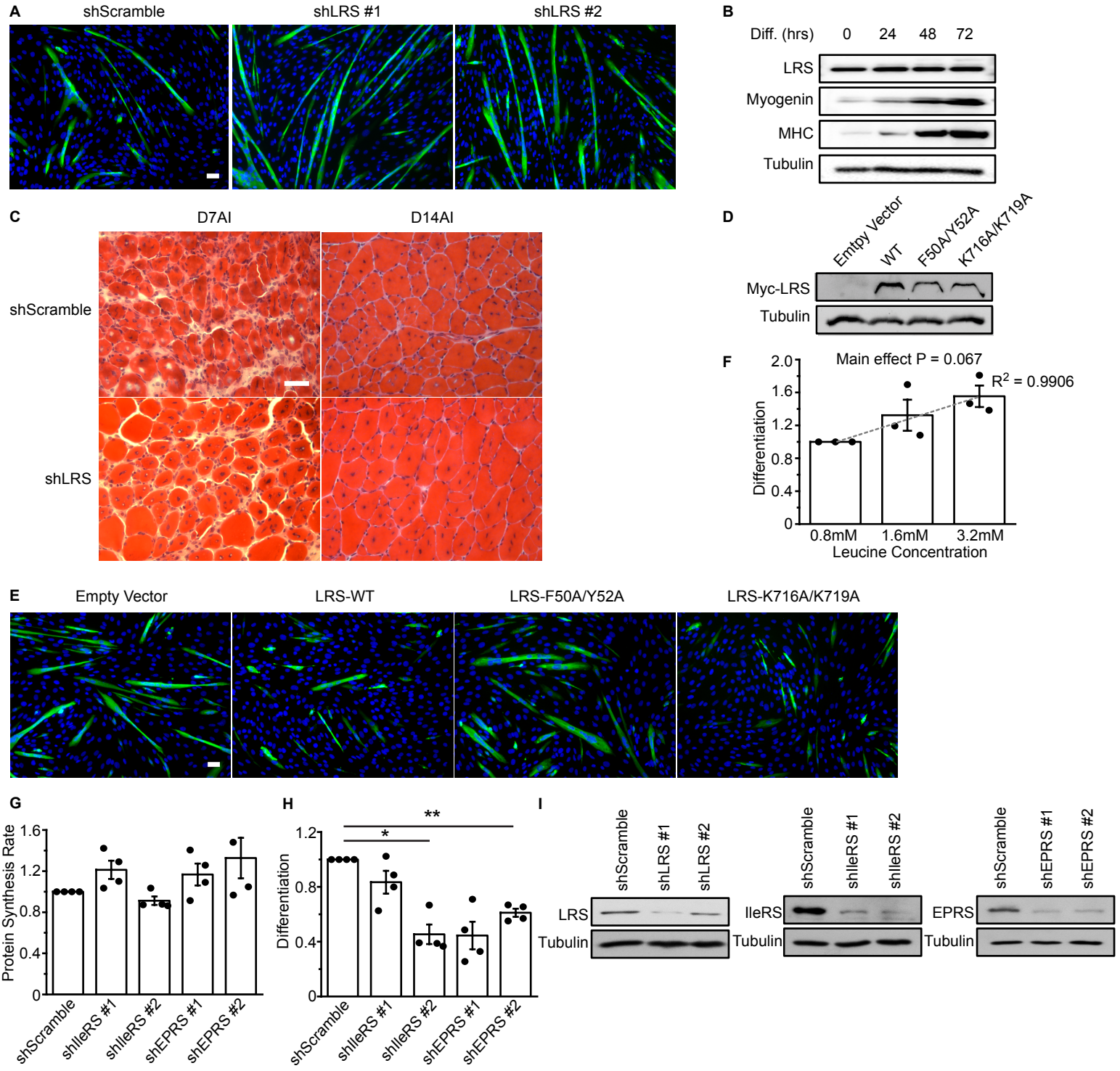
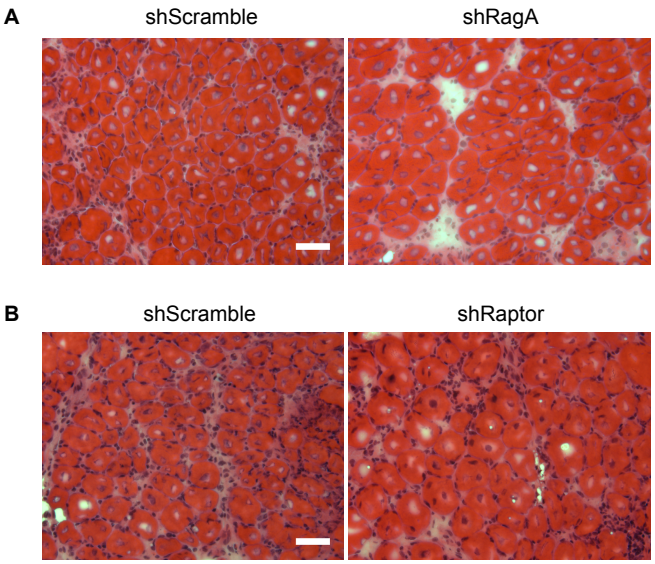
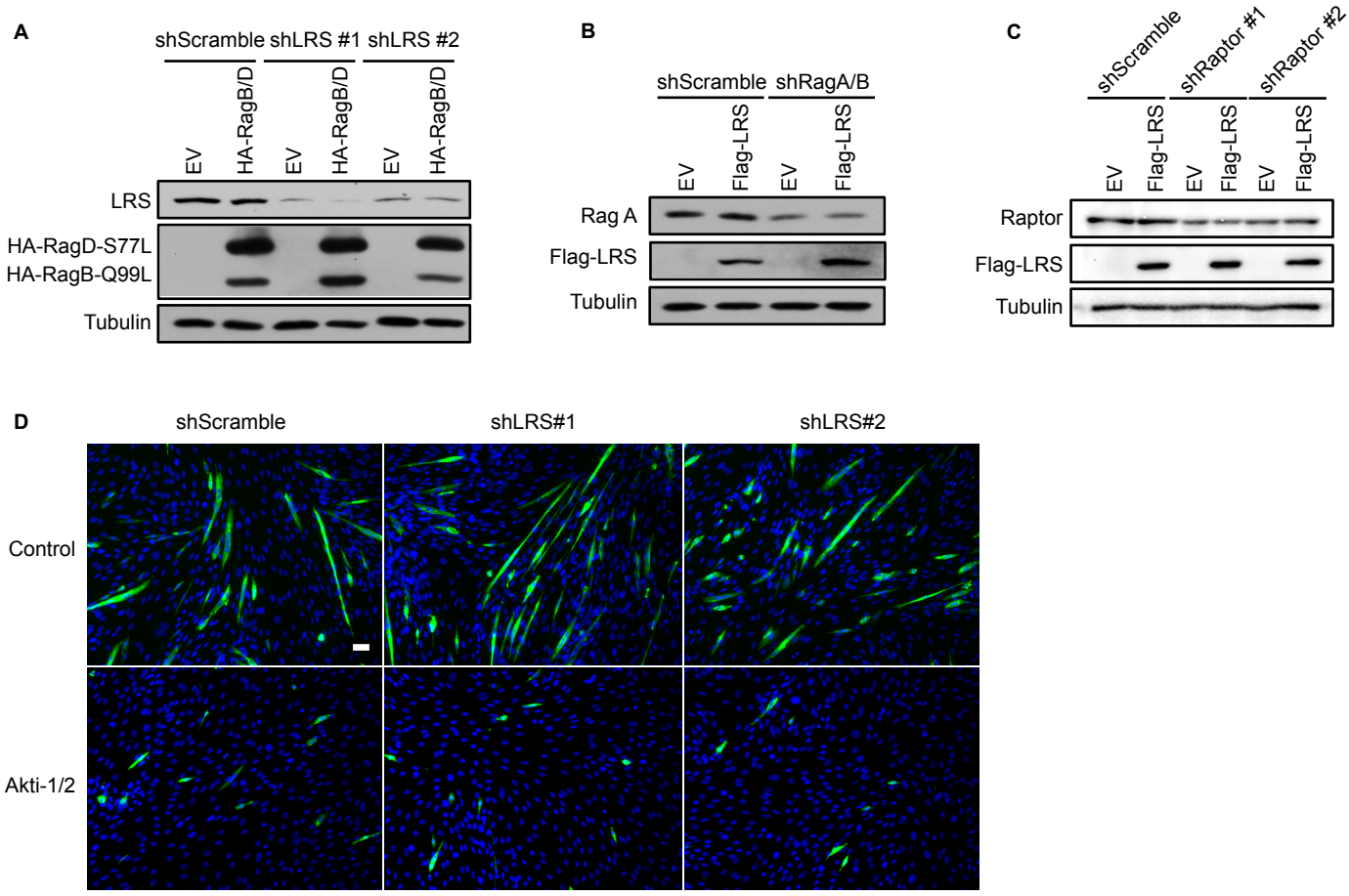


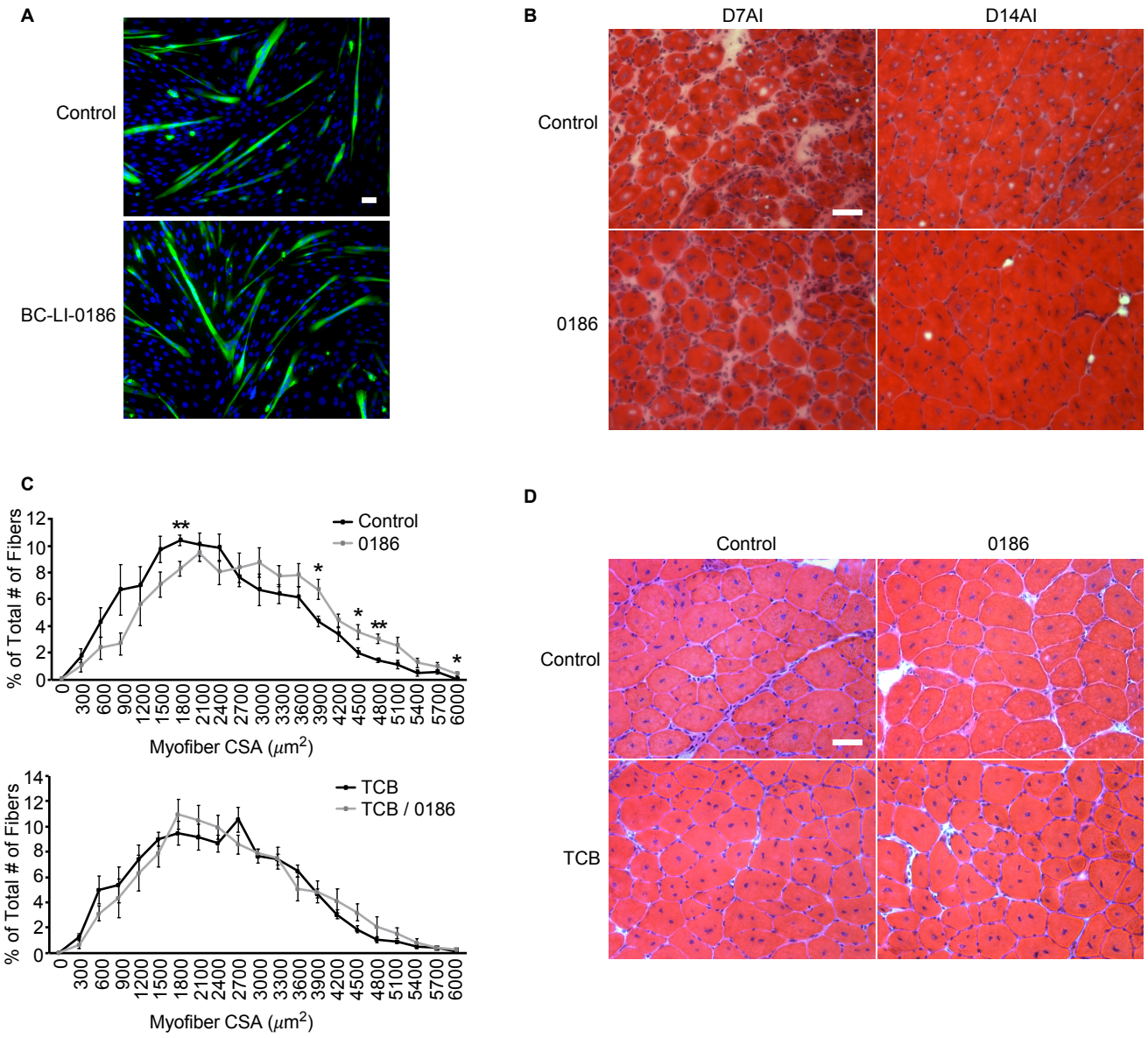
Supplemental Figure 1 (Corresponding to Figure 1). (A) Representative immuofluorescence images generated for Figure 1A (green: MHC, blue: DAPI). (B) C2C12 cells were induced to differentiate and lysed every 24 h, followed by Western blotting analysis (n=3). (C) Representative H&E images generated for data in Figure 1D. (D) Western blotting analysis of recombinant LRS and its mutants (n=3). (E) Representative immunofluorescence images generated for Figure 1E (green: MHC, blue: DAPI). (F) Cells were induced to differentiate for 72 h in differentiation media with different leucine concentrations, followed by measurement of differentiation index as described in the Supplemental Methods. Data were normalized to 0.8mM (n=3). (G) Cells were transduced with lentiviruses expressing shIleRS, shEPRS, or shScramble (control), puromycin -selected for 2 days, and subjected to measurement of protein synthesis rate as described in the Supplemental Methods (n=4). (H) Cells were transduced with lentiviruses expressing shIleRS, shEPRS, or shScramble, puromycin-selected for 2 days, and differentiated for 72 h, followed by measurement of differentiation index. Data were normalized to shScramble (n=5). (I) Cells treated as in Figure 1F and (G) were lysed and subjected to Western blotting analysis (n=4). *P < 0.05, **P < 0.01 by one-way ANOVA (F, G, H). Scale bars: 50 μm.



Supplemental Figure 2 (Corresponding to Figure 2).
(A) Representative H&E images generated for data in Figure 2A.
(B) Representative H&E images generated for data in Figure 2B.
Scale bars: 50 μm.



Supplemental Figure 3 (Corresponding to Figure 3). (A) C2C12 cells were transduced with lentiviruses expressing shLRS or shScramble, and puromycin-selected for 2 days. Cells were then transfected with either empty vector (EV) or HA-GST-RagB-Q99L and HA-GST-RagD-S77L for 24 h, and subjected to Western blotting analysis (n=3). (B) LRS was transfected in cells where RagA and RagB were knocked down as in (A), and Western blotting analysis was performed (n=3). (C) LRS was transfected in cells where Raptor was knocked down as in (A), and Western blotting analysis was performed (n=3). (D) Cells were transduced with lentiviruses expressing shLRS or shScramble (control), puromycin-selected for 2 days, and induced to differentiate for 72 h in the presence or absence of 1 μM Akti-1/2, followed by immunofluorescence staining with anti-MHC (green) and DAPI (blue) (n=3). Scale bar: 50 μm.



Supplemental Figure 4 (Corresponding to Figure 4). (A) Representative immunofluorescence images generated for Figure 4A (green: MHC, blue: DAPI). (B) Representative H&E images generated for Figure 4B. (C) Size distribution of all myofibers in Figure 4E. (D) Representative H&E images generated for Figure 4E. *P < 0.05, **P < 0.01 by two-tailed unpaired t-test. Scale bar: 50 μm.