Supporting Information

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SI Materials and Methods

Animals. All experimental procedures were conducted in accordance with the Guidelines for Animal Experimentation of Shinshu University and approved by the Committee for Animal Experimentation (approval numbers 250053 and 270060). Isolation of skeletal muscles and AAV injection were performed using 10- to 13-wk-old male C57BL/6 mice. The mice were given free access to water and standard diet throughout the study and maintained in a temperature-controlled (21–26 °C) and humidity-controlled (50–60%) room with a 12-h light/dark cycle. The experimental mice were deeply anesthetized by i.p. administration of 0.3 mg/kg medetomidine (Domitor; Nippon Zenyaku Kogyo Company), 4.0 mg/kg midazolam (Midazolam Sandoz; Novartis), and 5.0 mg/kg butorphanol (Vetorphale; Meiji Seika Pharma Company).

Molecular Cloning. The cDNAs encoding rabbit $Ca_V1.1$ and $Ca_V2.1$ were kindly provided by Dr. Manfred Grabner (Innsbruck Medical University, Innsbruck, Austria) and Dr. Bernhard Flucher (Innsbruck Medical University). The cDNA encoding rabbit $Ca_V1.2$ was kindly provided by Dr. William Catterall (University of Washington, Seattle, WA). Alanine substitution mutants were generated with the QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol. To construct bacterial expression vectors for recombinant proteins with an N-terminal GST tag, the GST sequence from pGEX4T3 was subcloned into a multicloning site of the pCold vector. The cDNA fragments encoding the indicated segments of $Ca_V1.1$, $Ca_V1.2$, and $Ca_V2.1$ were amplified by PCR and subcloned into the pCold-GST vector. The cDNAs encoding mouse JP1 and JP2, and the Pleckstrin homology (PH) domain of PLCδ were isolated by reverse transcription–PCR from a mouse skeletal muscle cDNA library. The C terminus including the transmembrane domain-deleted mutant of JP1 with C-terminal 3×FLAG-tag (JP1ΔCT-FLAG) and the N-terminal 3×FLAG-tagged-PH domain of PLCδ (FLAG-PLCδPH) were generated by PCR. The predicted cytosolic region of mouse JP1 (1–540 aa; NP_065629.1) and the PH domain of mouse PLCδ (1–175 aa; NP_062650.1) were amplified by PCR and subcloned into p3×FLAG-CMV14 and p3×FLAG-CMV10, respectively. To construct an AAV vector, the sequence of JP1ΔCT-FLAG was amplified by PCR and then subcloned into the pAAV-CMV vector. For siRNA rescue experiments, silent mutations (G1521C, T1522A, C1523G, and A1524T of JP1, NM_020604; C2389A, C2392G, C2393T, and C2395G of JP2, NM_001205076) were introduced with the QuikChange Site-Directed Mutagenesis kit (Stratagene). For the multiple alignment of Fig. 4*A*, following sequences were used: rabbit $Ca_V1.1$ (NP_001095190), mouse $Ca_V1.1$ (NP_001074492), human $Ca_V1.1$ (NP_000060), rabbit $Ca_V1.2$ (NP_001129994), human $Ca_V1.2$ (CAA84346), mouse $Ca_V2.1$ (NP_031604), and human $Ca_V2.1$ (BAA94766).

Cell Culture and Transfection. The C2C12 myoblast cell line was purchased from the American Type Culture Collection and maintained in DMEM plus GlutaMAX containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (referred to as GM-C2C12 medium). The GLT myoblast cell line was kindly provided by Dr. Bernhard E. Flucher (Innsbruck Medical University, Innsbruck, Austria) and maintained in DMEM plus 10% FBS, 10% horse serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (GM-GLT medium). To induce myotube differentiation, C2C12 myoblasts were plated on collagen-coated coverslips in GM-C2C12 and GLT myoblasts on carbon-coated coverslips coated with 0.1% gelatin in GM-GLT. One to 3 d after plating, the medium was changed to DMEM containing 2% horse serum, 100 U/mL penicillin, and 100 μg/mL streptomycin [differentiation medium (DM)]. Two days later, the cells were transfected with the indicated expression vectors using FuGENE HD (1 μg of plasmid DNA/2 mL DM in 35-mm dishes) (Roche Diagnostics) or stealth RNAi using Lipofectamine RNAiMAX (30 pmol of stealth RNAi/2.5 mL DM in 35-mm dishes) according to the manufacturer's protocol (Thermo Fisher). Stealth RNAi constructs against JP1 and JP2 were purchased from Invitrogen. The following siRNA sequences were used: JP1 #1, 5′-UACCCAUGCCUCUUGUUAUUGGCCC-3′; JP1 #2, 5′-UAGAAAUCAGGUGACAGCUCUCUGG-3′; JP1 #3, 5′-UUCAAUUGCUCUAUCCACCUUCUCU-3′; JP2 #1, 5′-AAGAUCACCAUACAGAUGAGGACGG-3′; JP2 #2, 5′-UUGACCAGCACAUUGUGGCGGUACU-3′; JP2 #3, 5′-AAAUAGGAUAGCCAGGCCGAUGUUC-3′. Seven to 10 d after plating, the myotubes were used for immunofluorescence labeling, patch-clamp analysis, or calcium imaging.

The AAVpro 293T cell line was purchased from Takara Bio and maintained in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Immunocytochemistry, Immunohistochemistry, and PLA. For immunocytochemistry, cultured myotubes or isolated FDB fibers on coverslips were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min. For muscle immunohistochemistry, isolated muscle samples were mounted on cork using gum tragacanth and frozen in liquid nitrogen-cooled isopentane. Frozen sections (10 μm in thickness) were obtained using a Leica CM1950 cryostat and immunostained without fixation. Briefly, after washing with PBS, specimens were blocked and permeabilized with PBS containing 0.2% Triton X-100 and 5% FBS for 1 h at room temperature and then incubated with the indicated primary antibodies at 4 °C overnight. After washing with PBS, cells were incubated with fluorescent dye-conjugated secondary antibodies and Hoechst 33342 (Dojindo) at room temperature for 1 h. Cells were again washed with PBS, and coverslips were mounted with Fluoromount-G (Beckman Coulter). The primary antibodies used in this study are listed in Table S1. To visualize the signals, Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 555 donkey anti-rabbit antibody, and Alexa Fluor 555 donkey anti-mouse antibody (Invitrogen) were used as secondary antibodies as indicated. Fluorescent images were acquired with a laser scanning confocal microscope LSM5 exciter (Carl Zeiss) or TCS SP8 (Leica). The fluorescent images in Fig. 5*E* and Fig. S4*D* were deconvoluted by HyVolution system (Leica).

To assess the JM targeting of JP1, JP2, LTCCs, or RyRs in myotubes, at least 20 myotubes were analyzed for each experiment, and representative photos are shown in figures.

Clusters of JP1, JP2, LTCCs, and RyRs in myotubes were quantified from confocal microscopic images by ImageJ software (NIH). Myotubes in the acquired images were selected by an ROI tool and converted to binary images using the Intermodes threshold (1). Using the Analyze Particle function of ImageJ, the numbers of particles larger than 0.198 μm^2 in the binary image were counted as clusters. The numbers of clusters per 100 μm^2 were calculated and are represented in the graphs.

PLA was performed using the Duolink system (Sigma-Aldrich) according to the manufacturer's instructions. The primary antibodies used are listed in Table S1. The signals were visualized with Duolink In Situ PLA Probe Anti-Mouse PLUS, Duolink In

Situ PLA Probe Anti-Rabbit MINUS, and Duolink In Situ Detection Reagents Orange (Sigma-Aldrich). *Z*-stack images from top to bottom of the FDB fibers (pinhole size: 1 airy unit) were acquired with a laser scanning microscope (TCS SP8), and the images were merged by the maximum projection program. For quantification of signals, merged images were converted to binary images using the MaxEntropy threshold (2), and the numbers of positive and total pixels were calculated with ImageJ.

Patch-Clamp Analysis. Ionic and gating currents of the LTCC were recorded in the whole-cell configuration at room temperature with a patch-clamp amplifier (HEKA EPC8). The currents were low-pass filtered at 5 kHz and sampled at 10 kHz. Patch pipettes (1.5–3 MΩ) were fabricated from borosilicate glass capillaries (Kimax-51; Kimble Glass) and coated with Sylgard 184 (Dow Corning Toray Company). Series resistance was always kept below 7 MΩ and routinely compensated to ∼75% using the amplifier. LTCC currents of C2C12 myotubes were measured with an intracellular solution containing the following (in mM): 145 D-glutamate, 2 $MgCl_2$, 10 Hepes, 0.1 EGTA, and 2 MgATP, balanced to pH 7.4 with CsOH. The extracellular bath solution contained the following (in mM): 10 $CaCl_2$, 145 TEACl, 5.5 glucose, and 10 Hepes (balanced to pH 7.4 with TEAOH). The membrane potential was stepped from a holding potential of −80 to −40 mV for 1,000 ms (to inactivate voltage-gated Na^+ channels and T-type Ca^{2+} channels) and then for 2,000 ms to potentials between −60 and +70 mV in 10-mV increments every 10 s. The voltage dependence of the peak Ca^{2+} current density was fitted with the following equation:

$$I(V) = G_{max}(V - V_{rev})/(1 + \exp(V_{0.5} - V)/k), \quad \textbf{[S1]}$$

where $I(V)$ is the peak current density at the command potential V, G_{max} is the maximum conductance, V_{rev} is the reversal potential, $V_{0.5}$ is the half-activation potential, and k is the slope.

Recording of gating charge movement was performed in the presence of 0.5 mM Cd^{2+} and 0.2 mM La^{3+} to block ionic currents. The membrane potential was stepped from a holding potential at −80 to −50 mV for 1,000 ms, and then for 20 ms to potentials between −50 and +70 mV in 10-mV increments every 10 s. To avoid the influence of nonspecific outward current, the voltage dependence of charge movement was calculated at "OFF" gaiting charge movement and was fitted according to a Boltzmann distribution:

$$I(V) = G_{max}/(1 + \exp(V_{0.5} - V)/k). \quad \textbf{[S2]}$$

Ca^+ Imaging. C2C12 myotubes, GLT myotubes, or FDB fibers were incubated with 5 μM Fluo-4/AM (Dojindo) plus 0.01% Cremophor EL (Sigma-Aldrich) and 0.02% BSA (Sigma-Aldrich) in serum-free DMEM for 45 min at 37 °C followed by deesterification. The dye-loaded cells were superfused with modified Tyrode solution (136.5 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.53 mM $MgCl_2$, 5.5 mM Hepes, and 5.5 mM glucose, pH 7.4) at room temperature and paced with 1-ms pulses of 50 V at 0.3 Hz (twitch) or 1-ms pulses of 50 V at 50 Hz (tetanus) across the 20-mm incubation chamber. Fluorescent images were acquired with an LSM 7 LIVE laser scanning microscope and 20×/0.8 Plan-Apochromat objective (Carl Zeiss). Fluo-4 was excited by 488-nm light, and the emission light was passed through a high-pass filter of 495 nm and captured by a CCD camera. Images of 128 × 128 pixels were acquired every 2.8 ms. The time course of Ca^{2+} transients was obtained from the fluorescence change in individual cells selected by an ROI tool. To assess the SR Ca^{2+} content of C2C12 myotubes, 30 μM CPA was applied. For estimating the SR Ca^{2+} content of isolated FDB fibers, tissues were exposed to a Ca^{2+} release mixture (ICE) containing 10 μM ionomycin, 30 μM CPA, and 100 μM EGTA in Ca^{2+}-free Ringer's solution (3).

Western Blotting. Equal amounts of protein were separated by SDS–polyacrylamide gel electrophoresis (SDS/PAGE) and electroblotted onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat skim milk in TBST (150 mM NaCl, 10 mM Tris, and 0.1% Tween 20, pH 7.4) for 1 h at room temperature and then incubated with the indicated primary antibody (listed in Table S1) overnight at 4 °C. After washing with TBST, the membranes were incubated in horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (1:10,000) or HRP-conjugated donkey anti-mouse IgG (1:10,000) (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. The bound secondary antibody was visualized with Immobilon Western (Millipore) according to the manufacturer's instructions.

Preparation of Microsomes. The gluteus and hindlimb muscles were dissected from C57BL/6 mice and ground in liquid nitrogen with a mortar and pestle. The powdered samples were homogenized in homogenization buffer (320 mM sucrose, 20 mM Hepes, pH 7.4) containing protease inhibitor mixture (Roche Diagnostics) using a grass Teflon homogenizer (Sansyo) and then centrifuged at 2,000 × *g* for 15 min at 4 °C to remove cell debris. The supernatants were ultracentrifuged at 100,000 × *g* for 60 min at 4 °C. The resultant pellets were dissolved with lysis buffer (10 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol, pH 7.4) containing protease inhibitor mixture and incubated at 4 °C on a rotator for 1 h. The lysates were centrifuged at 8,000 × *g* for 30 min at 4 °C to remove insoluble materials and stored at −80 °C until use. Protein concentrations were measured with a BCA protein assay kit (Thermo Fisher).

Immunoprecipitation. Skeletal muscle microsomes (200 μg/sample) were precleared with protein A/G plus-agarose beads (Santa Cruz Biotechnology) for 1 h at 4 °C. Two micrograms of control mouse IgG, anti-$Ca_V1.1$ antibody, or anti-RyR antibody was added to the precleared microsomes, and the mixture was incubated for 4 h at 4 °C. The beads were washed six times with PBS containing 1% Triton X-100 and then eluted with SDS sample buffer.

GST-Fusion Protein Production and Pull-Down Assay. BL21 cells were transformed with pCold-GST vectors carrying cDNA fragments of $Ca_V1.1$, $Ca_V1.2$, or $Ca_V2.1$ and inoculated into 500 mL of Luria–Bertani broth containing 100 μg/mL ampicillin. The cultures were grown to an A_{600} of 0.5 at 37 °C. Protein expression was induced by adding isopropyl-1-thio-D-galactopyranoside to a final concentration of 0.3 mM and incubating overnight at 4 °C. The cells were harvested from the cultures by centrifugation and resuspended in 20 mL of PBS and then disrupted by freezing–thawing and sonication. After centrifugation (10,000 × *g* at 4 °C for 30 min), the supernatants were saved and purified with glutathione-Sepharose 4B according to the manufacturer's instructions (GE Healthcare). The eluates were again purified with desalting column PD-10 (GE Healthcare) to eliminate glutathione and stored at −80 °C until use. The protein concentrations and molecular sizes of the sample were measured by a BCA protein assay and Coomassie blue staining, respectively (Fig. S3*A*).

Skeletal muscle microsomes were precleared with glutathione-Sepharose 4B beads for 1 h at 4 °C. Two hundred micrograms of precleared microsomes was incubated with 50 μg of GST or GST-fusion protein immobilized on glutathione-Sepharose 4B beads in lysis buffer overnight at 4 °C. The beads were washed six times with PBS containing 1% Triton X-100 and then eluted with SDS sample buffer.

AAV Production and Injection. AAV was produced by the AAVpro Helper Free system (Clontech) according to the manufacturer's protocol. Five 225-cm^2 flasks containing AAVpro 293T cells

(80% confluence) were cotransfected with pAAV-CMV-JP1ΔCT-FLAG or pAAV-CMV (mock), pRC6 (serotype 6), and pHelper vectors (120 μg plasmid/225-cm^2 flask). Transfection was performed with polyethyleneimine (3 μg of polyethyleneimine/1 μg of plasmid). The medium was changed to DMEM containing 2% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin after 6 h of transfection. The cells were harvested after 3 d, and control AAV (mock) and JP1ΔCT-FLAG-AAV were isolated using the AAVpro Purification kit (Clontech) according to the manufacturer's protocol. The isolated AAV was quantified by real-time PCR with AAVpro Titration Kit (Clontech).

The mice received intramuscular injections of control AAV or JP1ΔCT-FLAG-AAV (2×10^{10} vg in 100 μL of PBS/foot) into either the left or the right TA muscle, respectively. For FDB muscle, control AAV or JP1ΔCT-FLAG-AAV (4×10^9 vg in 20 μl PBS/foot) was injected, and the same amounts of virus were reinjected the next day to achieve high-efficiency transduction. Twenty days after AAV injection, the muscles were isolated and used for experiments.

Measurement of Contractile Forces. The contractile forces of TA muscles were measured in vivo according to the methods of a previous study with minor modifications (4). The anesthetized mice were placed into 50-mL conical tubes with their hindlimbs extending out through two holes. An incision was made in the skin above the TA muscle, and then the distal tendon was sutured to a force transducer (Nihon Kohden). The muscle was stimulated with needle electrodes placed on both sides of the TA. The TA muscles were stretched to a resting tension of 100 mN and electrically stimulated with 1-ms pulses of predetermined supramaximal voltage at 1–200 Hz. Data were collected using PowerLab 4/30 (AD Instruments). After force measurement, the TA muscles were fixed with 10% neutral buffered formalin at 4 °C overnight and used for conventional hematoxylin and eosin (H&E) staining. The cross-sectional area was calculated from the specimens using ImageJ. The specific force was calculated by normalizing the contractile force to the cross-sectional area.

Isolation of FDB Fibers. FDB fibers were dissected and digested in DMEM containing 5 mg/mL collagenase type 2 (Worthington Biomedical) with agitation at 37 °C for 1 h. After digestion, the fibers were washed four times with DMEM containing 10% FBS to eliminate the collagenase. Isolated FDB fibers were plated onto laminin-coated coverslips. After incubation at 37 °C for 30 min, the fibers were used for calcium imaging, immunocytochemistry, or PLA assay.

Transmission Electron Microscope Analysis. TA muscles were dissected and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4 °C overnight, followed by fixation in 0.1 M phosphate buffer with 1% osmium tetroxide at 4 °C for 1 h. The specimens were dehydrated in an ethanol series and propylene oxide and then embedded in Epon resin. Ultrathin sections (100 nm) were cut and stained with uranyl acetate and lead citrate. Electron micrographs were acquired with a JEM-1400 transmission electron microscope (JEOL). The distance between the T-tubule membranes and the SR membranes and the length of contact between the T-tubule membranes and the SR membranes were determined by ImageJ.

1. Prewitt JM, Mendelsohn ML (1966) The analysis of cell images. *Ann N Y Acad Sci* 128: 1035–1053.
2. Kapur JN, Sahoo PK, Wong ACK (1985) A new method for gray-level picture thresholding using the entropy of the histogram. *Comput Vision Graphics Image Process* 29: 273–285.
3. Loy RE, et al. (2011) Muscle weakness in Ryr1I4895T/WT knock-in mice as a result of reduced ryanodine receptor Ca^{2+} ion permeation and release from the sarcoplasmic reticulum. *J Gen Physiol* 137:43–57.
4. Cosgrove BD, et al. (2014) Rejuvenation of the muscle stem cell population restores strength to injured aged muscles. *Nat Med* 20:255–264.

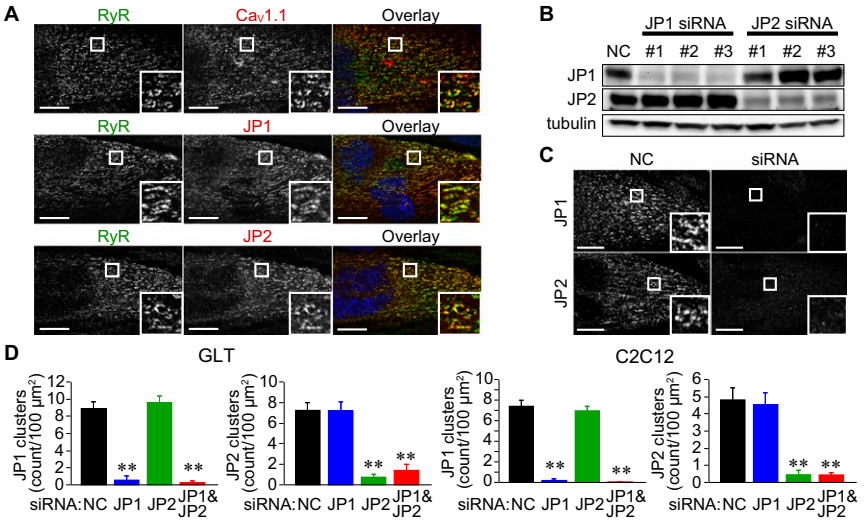

Fig. S1. Junctophilin 1 (JP1) and JP2 siRNAs suppress target protein expression in a subtype-specific manner. (*A*) Immunocytochemistry showing colocalization of the ryanodine receptor (RyR) (green), $Ca_V1.1$ (red), JP1 (red), and JP2 (red) in the junctional membrane (JM) of GLT myotubes. Nuclei were stained with Hoechst 33342 (blue). (Scale bar: 20 μm.) (*B*) Western blotting showing suppression of JP1 and JP2 expression by siRNA against JP1 or JP2, respectively. Cell lysates were isolated from GLT myotubes transfected with siRNA against JP1 or JP2, or with a negative control (NC) siRNA. (*C*) Immunocytochemistry showing loss of JP1 or JP2 clusters in GLT myotubes transfected with siRNA against JP1 or JP2, respectively. (Scale bar: 20 μm.) (*D*) Quantification of clusters of JP1 or JP2 in siRNA-transfected myotubes. Values are means ± SEM (20 myotubes from four dishes were analyzed for each group). $^{**}P < 0.01$ compared with negative control.

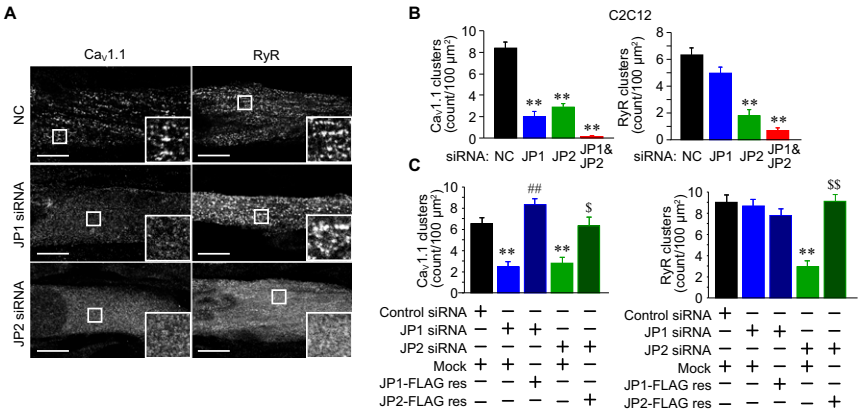

Fig. S2. Effects of JP1 or JP2 knockdown on localization and function of $Ca_V1.1$ and RyR in C2C12 myotubes. (*A*) Immunocytochemistry showing the effect of JP1 or JP2 siRNA on the JM targeting of $Ca_V1.1$ and RyR in C2C12 myotubes. (Scale bar: 20 μm.) (*B*) Quantification of clusters of $Ca_V1.1$ and RyR in siRNA-transfected C2C12 myotubes. Values are means ± SEM (20 myotubes from four dishes were counted for each group). $^{**}P < 0.01$ compared with negative control. (*C*) Quantification of clusters of $Ca_V1.1$ and RyR in myotubes. The siRNA against JPs and siRNA-resistant JP1 or JP2 were cotransfected to the GLT myotubes. Values are means ± SEM (20 myotubes from four dishes were counted for each group). $^{**}P < 0.01$ compared with negative control. $^{\#\#}P < 0.01$ compared with mock plus JP1 siRNA; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ compared with mock plus JP2 siRNA.

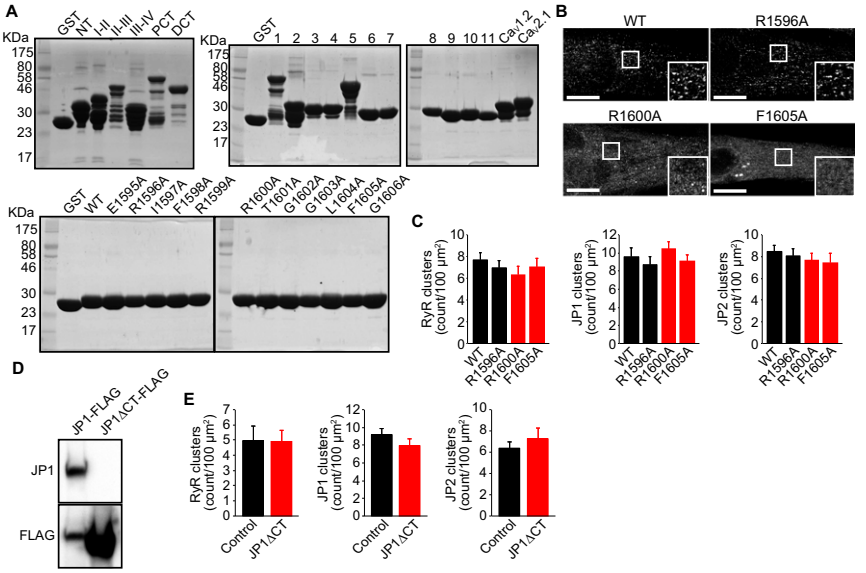

Fig. S3. Identification of JP-binding motif (JBM) of $Ca_V1.1$. (*A*) Expression of recombinant proteins. GST-fusion proteins were purified from BL21 cells. Each protein (50 μg) was separated by SDS/PAGE and stained with Coomassie brilliant blue. (*B*) Effect of alanine substitution at the JBM on channel clustering. GLT myotubes were transfected with GFP-$Ca_V1.1$ (WT), GFP-$Ca_V1.1$_R1596A, GFP-$Ca_V1.1$_R1600A, or GFP-$Ca_V1.1$_R1605A. GFP was detected with an anti-GFP antibody. (Scale bar: 20 μm.) (*C*) Quantification of clusters of RyRs and JPs in myotubes transfected with GFP-$Ca_V1.1$ (wild type), GFP-$Ca_V1.1$_R1596A, GFP-$Ca_V1.1$_R1600A, or GFP-$Ca_V1.1$_R1605A. Values are means ± SEM (20 myotubes from four dishes were analyzed for each group). (*D*) Validation of antibody reactivity of JP1ΔCT-FLAG. The expression vectors of JP1-FLAG or JP1ΔCT-FLAG were transfected to AAVpro 293T cells. Whole-cell lysates from the transfected cells were separated by SDS/PAGE and immunoblotted with anti-JP1 and anti-FLAG antibodies. (*E*) Quantification of clusters of RyRs and JPs in myotubes transfected with GFP-$Ca_V1.1$ (wild type) and PLCδPH-FLAG or JP1ΔCT-FLAG. Values are means ± SEM (20 myotubes from four dishes were analyzed for each group).

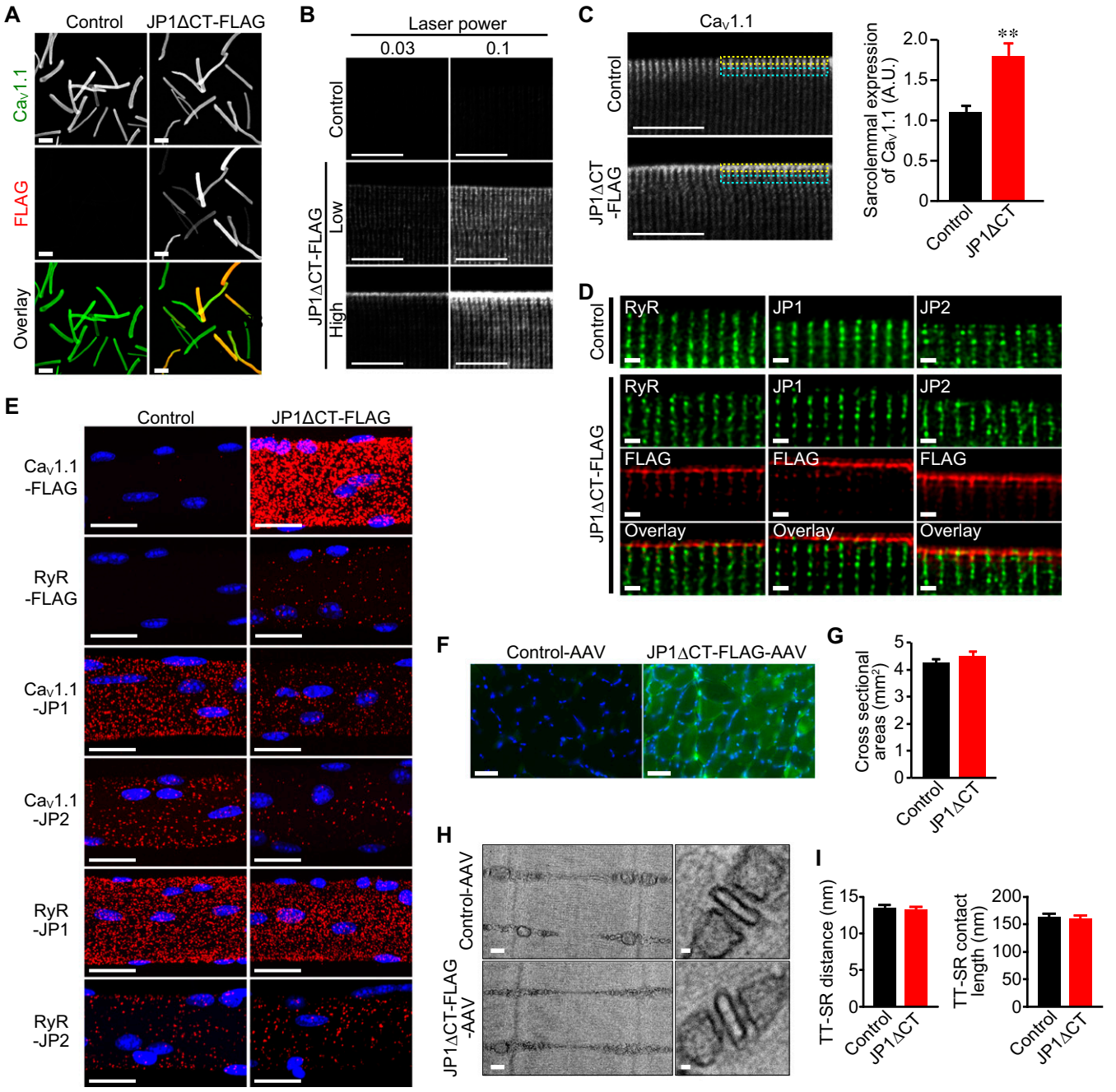

Fig. S4. AAV-mediated expression of JP1ΔCT-FLAG in mouse muscle fibers. (*A*) AAV-mediated expression of JP1ΔCT-FLAG in the FDB muscle. Mice received an intramuscular injection of control-AAV or JP1ΔCT-FLAG-AAV into the FDB muscle (4×10^9 vg/foot). Twenty days after injection, the FDB muscles were isolated and analyzed by immunocytochemistry with anti-$Ca_V1.1$ antibody (green in overlaid view) and anti-FLAG antibody (red in overlaid view). (Scale bar: 200 μm.) (*B*) Localization of JP1ΔCT-FLAG in FDB fibers. Control and JP1ΔCT-FLAG–expresed FDB fibers were analyzed by immunocytochemistry with anti-FLAG antibody. Low and high indicate expression levels of JP1ΔCT-FLAG. Fluorescent images of the same fields were obtained with different laser powers. (Scale bar: 10 μm.) (*C*) Quantification of sarcolemmal localization of $Ca_V1.1$. Control and JP1ΔCT-FLAG–expresed FDB fibers were analyzed by immunocytochemistry with ant-$Ca_V1.1$ antibody. (Scale bar: 10 μm.) The intensity of fluorescence in the sarcolemma (1×15-μm rectangular region on the cell surface, yellow boxed in the images) and T-tubules/cytosol (1×15-μm rectangular region adjacent to the sarcolemma analyzed, blue boxed in the images) was counted. The graph shows the ratio of sarcolemmal intensity to T-tubule/cytosol intensity. Values are means ± SEM (10 fibers from two animals were analyzed for each group). $^{**}P < 0.01$ compared with control. (*D*) Localization of RyRs and JPs in control and JP1ΔCT-FLAG–expressed FDB fibers. RyRs, JP1, JP2, and JP1ΔCT-FLAG in isolated FDB fibers were detected with antibodies against RyR, JP1, JP2, and FLAG. (Scale bar: 1 μm.) (*E*) Representative PLA images of control and JP1ΔCT-FLAG–expressed FDB fibers. PLA was performed with the antibody combinations indicated on the *Left* of the images. Protein–protein association is visualized as orange signals. *Z*-stack images from *Top* to *Bottom* of each FDB fiber were acquired by confocal microscopy, and collapsed *z*-stack images are shown. (Scale bar: 20 μm.) (*F*) AAV-mediated expression of JP1ΔCT-FLAG in the mouse TA muscle. The mice received an intramuscular injection of control-AAV or JP1ΔCT-FLAG-AAV into the TA (2×10^{10} vg/foot). Twenty days after injection, the TA muscles were analyzed by immunohistochemistry with anti-FLAG antibody (green). (Scale bar: 20 μm.) (*G*) Cross-sectional areas (CSAs) of TA muscle. CSAs were calculated from H&E-stained sections. (*H*) Representative electron microscopic images of TA muscles. (Scale bar: 200 nm.) The images in the right-hand panels show a representative high-magnification view of the triad. (Scale bar: 20 nm.) (*I*) The distance between the T-tubule membranes and the SR membranes (TT-SR) and the length of contact between the T-tubule membranes and the SR membranes in TA muscle fibers, calculated from electron-microscopic images using ImageJ (40 triads from four animals were counted for each group).

Table S1. List of antibodies used in this study

Antigen	Host	Supplier (catalog no./clone no.)	Application (dilution)
GFP	Rabbit-poly	Invitrogen (A11122)	ICC (1:1,000)
$Ca_V1.1$	Mouse-mono	Abcam (ab2862)	ICC/PLA (1:200), IP (2 μg), WB (1:1,000)
$Ca_V1.1$	Rabbit-poly	Sigma (HPA056416)	ICC/PLA (1:200)
RyR	Mouse-mono	Thermo Fisher (MA3-925, clone 34C)	ICC/PLA (1:500), IP (2 μg), WB (1:5,000)
RyR	Rabbit-poly	Sigma (HPA056416)	ICC/PLA (1:100)
JP1	Rabbit-poly	Invitrogen (40-5100)	ICC/PLA (1:500), WB (1:5,000)
JP2	Rabbit-poly	Invitrogen (40-5300)	ICC/PLA (1:500), WB (1:5,000)
FLAG	Mouse-mono	Sigma (F1804, clone M2)	ICC/IHC/PLA (1:500), WB (1:5,000)
FLAG	Rabbit-poly	Rockland (600-401-383)	ICC (1:500)

ICC, immunocytochemistry; IHC, immunohistochemistry; IP, immunoprecipitation; WB, Western blotting.