SUPPLEMENTAL MATERIAL

Table of Contents

Expanded Methods and Materials

Reagents and antibodies

The following reagents were purchased from the indicated suppliers: ADP-Glo Kinase Assay (Promega, Madison, WI, USA), Phos-tag Acrylamide (Wako, Tokyo, Japan), Silver Stain MS Kit (Wako), and Cal-520 (ab171868, Abcam, Cambridge, UK). Full-length human cMLCK protein (BTN-caMLCK, #02-460-20N) was purchased from Carna Bioscience. 2'-(or-3')-O-(N-Methylanthraniloyl) Adenosine 5'-Triphosphate, Trisodium Salt (Mant-ATP) (M12417) was purchased from ThermoFisher Scientific. The antibodies that were used in this study are listed in Supplementary Table 1. We generated anti-human cMLCK.

Generation of the Mylk3 mutant knock-in (KI) mice

We applied the CRISPR/Cas9 technology for the KI of the *Mylk3* mutant (c.1951-1G>T) into its original locus of C57BL/6 zygotes. Founders carrying the KI variant were generated by combining guide RNA (gRNA), Cas9 mRNA, and single-stranded oligodeoxynucleotides (ssODNs) donor co-injection into mice fertilized eggs and were crossbred according to a previous study[30]. To design the gRNA, software tolls (crispr.genome-engineering.org) predicting unique target sites throughout the mouse genome were used. The target site and sequences of the ssODNs in the mouse genome are shown in Supplementary Figure 1. In short, using a micromanipulator, 100 ng/μl Cas9 mRNA, 50 ng/μl gRNAs, and 50 ng/μl ssODNs of CR9 were microinjected into the pronuclei of embryos collected from superovulated C57BL/6N female mice. Injected embryos were cultured overnight, and divided two-cell embryos were transferred into pseudopregnant C57BL/6N females. F0 mice were mated with wild-type C57BL/6N mice for germline transmission, and F1 mice were genotyped by PCR using genomic DNA extracted from the tail. KI of the *Mylk3* variant was validated by Sanger sequencing at the splice acceptor site of exon9 of *Mylk3*. Primers used for the genotyping and Sanger sequencing are shown in Supplementary Figure 1 and Table 2.

Vector construct and production of AAV9-EGFP-T2A-MYLK3

The coding sequences of *Mylk3* (NM_182493.3) were amplified by polymerase chain reaction (PCR) from the human heart complementary DNA library (Takara Bio, Shiga, Japan) and subcloned into the Gateway pEFDEST51 vector (Invitrogen, Waltham, MA, USA). The AAV9-*MYLK3* vector construct was produced to carry an AAV9 inverted terminal repeat (ITR)–flanked genome encoding cardiac troponin T–driven enhanced green fluorescent protein (EGFP), Thosea asigna virus 2A, *MYLK3*, and simian virus 40

polyadenylation signals. AAV vectors were produced by the transfection of three constructs (pHelper, pAAV2/9n encoding AAV2 rep and AAV9 cap genes, and the ITR-flanked transgene plasmid) in human embryonic kidney (HEK) 293T cells. After 3 days, viral particles in the medium were harvested, filtered, and purified using the POROS CaptureSelect AAV9 Affinity Resin (Thermo Fisher Scientific, Waltham, MA, USA) and CsCl2 equilibrium density-gradient centrifugation. The viral titer was 6.0×10^{12} vg/ml.

Body weight and organ weights

Mice were euthanized by cervical dislocation under deep anesthesia with a mixture of medetomidine, midazolam, and butorphanol (0.3μg/g, 4μg/g, and 5μg/g, respectively intraperitoneal injection). Hearts were then excised and weighed.

Echocardiographic measurements

The echocardiographic measurements were performed under light anesthesia with 1.0%–1.5% isoflurane supplied through a nose cone with the Vevo770 ultrasound system (FUJIFILM VisualSonics, Inc., Toronto, Canada). A two-dimensional parasternal short-axis view was obtained at the levels of the papillary muscles. After it had been ensured that the imaging was on the axis, two-dimensional targeted M-mode Tracings were recorded. Based on the left ventricular end-diastolic dimension (LVDd) and left ventricular end-systolic dimension (LVDs) obtained from M-mode echocardiographic images, left ventricular fractional shortening (LVFS) was calculated as follows: LVFS (%) = (LVDd – LVDs)/LVDd.

Left ventricular pressure-volume (LVPV) measurement

LVPV measurements using a Millar catheter were performed using the standard method[34]. In short, mice were anesthetized with 1.5–2% isoflurane supplied through tracheal intubation for LVPV measurement. A Millar P-V catheter (SPR-839NR, Millar Instruments, Inc, Houston, TX, USA) was inserted into the left ventricular cavity via the LV apex. By PowerLab (ADInstruments Pty Ltd, Bella Vista, Australia), the left ventricle pressure was recorded using a Millar catheter. Electrocardiogram (ECG) in lead II was also recorded through the needle electrodes attached to the limbs. Left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), maximum and minimum values within a beat of the first derivative of left ventricular pressure (+dp/dt / -dp/dt), and heart rate (HR) was calculated from a continuously generated pressure signal for 20 min stabilization. The LV P-V loop was acquired using a Millar control unit (MPVS-300/400 system, Millar Instruments, Houston, TX, USA). The volume calibration of the catheter was performed using a calibration cuvette supplied by Millar Instruments filled with fresh, slightly heparinized blood. All analyses were performed using the Millar analysis software PVAN 3.4 (Millar Instruments) according to the manufacturer's instructions.

Mant-ATP fluorescence decay curves plots assays and analyses

Using a fluorescent ATP analog, Mant-ATP, chased with nonfluorescent ATP, we were able to deconvolute an abundance of myosins in SRX and DRX conformations from the double-exponential fluorescent decay

pattern. Mant-ATP assays were performed on the cardiac LV tissues of mice and human iPSC-CMs (day 56 differentiations) according to the protocol described previously[33]. LV tissues and monolayers of iPSC-CMs were incubated for 30 minutes (room temperature) in permeabilization buffer and flushed with glycerin solution before the administration of Mant-ATP. ZEISS LSM 880 with Airyscan (ZEISS Corporation, Oberkochen, Germany) was used with objective "Plan-Apochromat" 20x/0.8 for fluorescence acquisition. In the experiments with purified cMLCK protein, the solutions contained 0.15 μM of purified cMLCK. The fast decay in fluorescence intensity reflects myosins in DRX conformations, and slow decay reflects myosins in SRX conformations. The decay in fluorescence was fitted to the defined equation using a two-state exponential.

Generation of human induced pluripotent stem cell (iPSC) and cardiac differentiation

iPSCs were generated from peripheral blood mononuclear cells using episomal vectors, according to the protocol provided by CiRA (https://www.cira.kyoto-u.ac.jp/e/research/protocol.html). The episomal vectors were introduced into the CD34+ cells by Nucleofector 2b (Lonza, Basel, Switzerland) using the Amaxa Human CD34 Cell Nucleofector Kit (Lonza) and plated on cell culture plates coated with Laminin 511-E8 (iMatrix-511 silk, Nippi, Tokyo, Japan). After two weeks, colonies with iPSC-like morphologies were obtained and cultured by changing the medium with StemFit (Ajinomoto, Tokyo, Japan). iPSCs were differentiated into cardiomyocytes (iPSC-CMs) using an embryoid body formation protocol as described previously[1] with slight modification using the AscleStem cardiomyocyte differentiation medium kit (Nacalai Tesque, Kyoto, Japan). Differentiated cardiomyocytes were purified by metabolic selection using no glucose DMEM (Nacalai Tesque) supplemented with 4 mM L-lactate (Wako Pure Chemical Industries) and 0.5% bovine serum albumin. After the purification, iPS-CMs were replated and cultured in DMEM with 10% FBS, and all the experiments were performed approximately 50 to 60 days of culture after the start of the differentiation.

Administration of AAV vectors to iPSC-CMs

Two days after the replating, the medium was replaced with a DMEM with 10% FBS containing the adenovirus vector encoding human cMLCK or EGFP gene at MOI of $5.0x10^4$. After 14 days of incubation, cells were fixed and immunostained with anti-α-actinin and anti-FLAG or recorded under electrical stimulation.

CRISPR gene correction strategy

iPSC clones were generated with the same genetic background and precisely modified genotypes through homology-directed repair (HDR), using the CRISPR/Cas9 genome editing as described previously[32]. Single guide RNAs (sgRNAs) for HDR were designed to target both the WT and mutant alleles (Supplementary Table 2). Single-stranded oligonucleotides (ssODNs) used for genomic repair contained the wildtype sequence at the mutation site as well as synonymous mutations to remove the PAM site to prevent re-cutting of the corrected allele while preserving the amino acid sequence (Supplementary Table 2). ssODNs were

produced from a dsDNA template using the Guide-it Long ssDNA Strandase Kit (632667, TaKaRa Bio USA, Inc.). Plasmid constructs for genome editing were transfected into iPSCs by electroporation and purified by puromycin selection (0.5μg/ml,24h). After several rounds of sib selection, we obtained an iPSC clone containing homozygous WT alleles (gene corrected-iPSC).

Droplet digital PCR (ddPCR) and quantitative real-time PCR

Total RNA was extracted from heart tissues using the RNA-Bee reagent (Tel-Test, Friendawood, TX, USA) and converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's protocols. Quantitative real-time PCR was performed with SYBR technology and StepOnePlus Real-Time PCR systems (Applied Biosystems). The level of each transcript was quantified by the threshold cycle method using Gapdh as an endogenous control. ddPCR was performed using the QX200 AutoDG Droplet Digital PCR system (Bio-Rad, Hercules, CA, USA) as per the manufacturer's protocol. To specifically detect the transcripts from wild-type or c.1951-1G>T mutant allele in *Mylk3*, HEX or FAM-labeled probe was designed (Custom ddPCR HEX/FAM Assay, Bio-Rad) (Supplementary Figure 2A). Endogenous mouse *Mylk3* and exogenously expressed human *Mylk3* transcripts were also measured separately by HEX or FAM-labeled probe (Custom ddPCR HEX/FAM Assay, Bio-Rad) (Supplementary Figure 2B). After the PCR reaction, the generated droplets were detected and analyzed using a QX200 droplet reader (Bio-Rad). TBP (TATA-binding protein) was used for internal control.

Immunocytochemistry and fluorescence imaging

Frozen sections of the left ventricle were washed once with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature (RT). After washing with PBS, the tissuess were permeabilized with 0.1% Triton X-100 in PBS for 5 min at RT, and then samples were immunostained with primary antibodies (1:200 in 0.025% Triton X-100 in PBS, 40 min). Next, tissuess were washed three times with PBS for 5 min at RT. For secondary reactions, species-matched Alexa Fluor 488– or Alexa Fluor 555–labeled secondary antibody was used (1:400 in 0.025% Triton X-100 in PBS, 40 min). Fluorescence images of Alexa Fluor 647 and Alexa Fluor 555 were recorded using a ZEISS LSM 880 with Airyscan (ZEISS Corporation, Oberkochen, Germany).

Cell motion imaging of cultured cardiomyocytes

In this study, we used the cell motion imaging system SI8000[35] for the measurement of the contractility of cultured cardiomyocytes because it can provide not only the contraction speed but also the average deformation distance (ADD) that is a surrogate marker for the force development of cultured cardiomyocytes[2]. Video images of cultured monolayer cardiomyocytes were recorded under electrical stimulation (1 Hz, 15 V) as sequential phase-contrast images with a 4x objective at a frame rate of 150 frames/sec and a resolution of 2048 x 2048 pixels using the SI8000 cell motion imaging system (Sony Corporation, Tokyo, Japan). The average of the magnitude of motion vectors was plotted against time to obtain information on the contraction and relaxation motion; (1) the maximum contraction velocity, (2) the

maximum relaxation velocity, (3) the average deformation distance (ADD) that is the total area under the two peaks[35].

Generation of self-organized tissue rings (SOTRs)

SOTRs were fabricated according to the method described in a previous report[37,38]. Before cell seeding, single iPSC-CMs were filtered using a 40 μm cell strainer (BD Falcon; Becton Dickenson, Franklin Lakes, NJ, USA) and were resuspended at a final density of 2×10^6 cells/ml in serum-supplemented cardiac differentiation culture medium containing 40% high glucose DMEM (Sigma-Aldrich), 40% IMDM (Sigma-Aldrich), 20% fetal bovine serum (FBS; Gibco, USA), 1% minimum essential medium non-essential amino acid solution (Sigma-Aldrich), 0.1% penicillin-streptomycin (Gibco, USA), and 0.5% L-glutamine (Sigma-Aldrich). 7×10^5 cells were plated in each PDMS (SYLGARD 184; Dow Corning, Midland, MI, USA) culture well with a 3 mm pillar. After plating, CMs settled in the wells, aggregated, and congregated around the central pillar to form densely packed tissue rings within 7 days. The medium was changed to a 5% FBS medium from day 3. Subsequently, the old medium was replaced with the fresh medium every 4 days. Administration of LEU1154 (10 μM) or DMSO began 3 days before force measurements.

Contraction force analysis using MicroTester G2

SOTR contractility was measured using a micron-scale mechanical-testing system (MicroTester G2; CellScale Biomaterials Testing, Waterloo, ON, Canada)[38]. ReWs (re-entrant waves) were prevented by changing the medium before the measurement at room temperature. SOTR was removed from the pillar and fixed on a stage and immersed in the culture medium warmed to 37 ℃ with or without 10 μM LEUO-1154. Next, the tissue ring was fixed on a stage and immersed in the culture medium warmed to 37 ℃. A cantilever beam with a diameter of 0.40 mm was pressed onto the SOTR and was held for 50 s at each length and the force was calculated by cantilever beam deflection in response to differential displacement.

Construct design and expression and purification of recombinant smMLCK and skMLCK proteins in HEK293T cells

cDNA fragment encoding smMLCK (NM_053025) and skMLCK (NM_033118) were PCR amplified using cDNA from human placenta and skeletal muscle and inserted into pENTR/D-TOPO using Gateway Technology (Invitrogen). The primers for smMLCK and skMLCK were listed in the supplementary Table 2. Importantly, the ATG-FLAG-tag sequence was introduced to all 5' primers of target genes to add the FLAG tag at the N-terminus region of the target proteins to yield FLAG-smMLCK/pENTR and FLAG-skMLCK/pENTR. Recombinant FLAG-tagged MLCKs proteins were purified as follows: HEK293T cells transfected with pEF-DEST51/nFLAG plasmid encoding smMLCK or skMLCK were lysed in lysis buffer (10 mM Tris-HCl [pH 7.2], 0.15 M NaCl, 1% NP40, 1 mM EDTA and protease inhibitor cocktail) and immunoprecipitated with anti-FLAG M2 agarose (Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 30 min. The beads were washed three times with wash buffer (10 mM Tris-HCl [pH 7.2], 0.3 M NaCl, 1% NP40) and eluted with elution buffer (10 mM Tris-HCl [pH 7.2], 0.15 M NaCl, 1% NP40, and 0.05 mg/ml FLAG

peptide) at 4°C for 30 min. After centrifugation, the supernatants were used as recombinant FLAG-tagged proteins.

Construct design and purification of recombinant human myosin regulatory light chain proteins from E. Coli

To prepare substrate proteins, cDNA fragments encoding human MYL9 (gene Accession No NM_006097), MYLPF (gene Accession No NM_013292), and MLC2v (gene Accession No NM_000432) were PCR amplified using cDNA from human placenta, skeletal muscle, and heart. The primers for MYL9, MYLPF, and MLC2v were listed in the supplementary Table 2. A set of point mutants of human MLC2v in which Ser was replaced with Ala or Asp were generated by PCR, using the MLC2v/pENTR vector as a template and the primer pairs listed in the supplementary Table 2. A set of double mutants of human MLC2v in which Ser-15 and Ser-19 were replaced with Ala was generated by PCR, using pENTR-MLC2v-S15A as a template and the primer pairs listed in the supplementary Table 2. Amplified cDNA was subcloned into the pENTR/D-TOPO vector and inserted into the pDEST17 vector by Gateway Technology System (Invitrogen) according to the manufacturer's protocol. The pDEST17 constructs possessing each regulatory light chain sequence were transformed into the BL21 chemically competent E. coli (Invitrogen). A 5 ml culture using LB containing 100 μg/ml of ampicillin was grown overnight shaking at 37°C and used to inoculate 200 mL of LB containing ampicillin (in a 1 L flask) until the culture optical density (600 nm) reaches 0.5. Arabinose was added at a final concentration of 0.2% and subsequent culturing for 3 hours at 37°C. Cells were harvested by centrifugation at 5000 x *g* at 4°C for 10 minutes and the resulting cell pellet was resuspended in 10 mL of BugBuster Master Mix (Merck Millipore, Billerica, MA, USA) containing EDTA-free protease inhibitor cocktail (Nacalai) and rotated at room temperature for 20 minutes. The inclusion body that contains N-terminus His-tagged MLC2v protein was isolated from the supernatant by centrifugation at 16,000 x *g* at 4°C for 10 minutes. The resulting inclusion body was washed with 2-fold diluted BugBuster Master Mix one time and 10-fold diluted BugBuster Master Mix twice. The inclusion body pellet was solubilized in IMAC binding buffer (10 mM HEPES [pH 7.4], 500 mM NaCl, 1 mM MgCl2, 6 M Urea) by repeatedly passing the inclusion body through an 18g syringe and incubated at 4°C for 1 h. After centrifugation at 20,000 x g at 4°C for 20 minutes, any insoluble material was centrifuged. The resulting resuspended protein solution was loaded onto a column of TALON Metal Affinity Resin (Clontech Laboratories, Inc., CA, USA) equilibrated with IMAC binding buffer at 4°C, and a column was washed with 3 column volume of refolding buffers (10 mM HEPES [pH 7.4], 500 mM NaCl, 1 mM MgCl2, 0.1% CHAPS) with the indicated concentration of urea (3, 2, 1, 0.5, 0 M Urea), sequentially. The bound His-tagged MLC2v protein was eluted with elution buffer (50 mM Sodium phosphate [pH 8.0], 300 mM NaCl, 0.1% Chaps, 150 mM Imidazole), followed by the buffer exchange with the stock buffer (20 mM Tris-HCl [pH8.0], 300 mM NaCl, 5 mM $CaCl_2$, 0.1% Chaps) and the concentration by centrifugation at 5,000 x *g* at 4 ºC using centrifugal filter (Amicon Ultra-15, Millipore). Apply the buffer-exchanged and concentrated protein solution onto an Econo-Pac 10DG Desalting column (Bio-Rad). The fractions containing His-tagged MLC2v protein were pooled and stored at -80ºC until use.

Construct design and purification of recombinant human calmodulin

cDNA fragment of Calmodulin (*CALM1*) (gene Accession No NM_NM006888) was also amplified using cDNA from the human heart and inserted into pENTR/D-TOPO. The primers listed in the supplementary Table 2 were used to amplify the cDNA of human CALM1. Amplified cDNA was subcloned into the pENTR vector and inserted into the pDEST17 vector (CALM1/pDEST17) by the Gateway cloning system (Invitrogen) according to the manufacturer's protocol. Furthermore, the TEV protease recognition site was inserted between the Hisx6 sequence and the coding sequence of calmodulin in the CALM1/pDEST17 vector to make the Hisx6-TEV-CALM1/pDEST17 vector by PCR reaction using the primers listed in the supplementary Table 2. The Hisx6-TEV-CALM1/pDEST17 construct was transformed into the BL21(DE3) strain of E. coli. A 5 ml culture using LB containing 100 μg/ml of ampicillin was grown overnight shaking at 37°C and used to inoculate 200 mL of LB containing ampicillin (in a 1 L flask) until the culture optical density (600 nm) reaches 0.5. Arabinose was added at a final concentration of 0.2% and subsequent culturing for 3 hours at 37°C. Cells were harvested by centrifugation at 5000 x *g* at 4°C for 10 minutes and the resulting cell pellet was resuspended in 10 mL of BugBuster Master Mix (Merck Millipore) containing EDTA-free protease inhibitor cocktail (Nacalai) and rotated at room temperature for 20 minutes. Insoluble material was removed by centrifugation at 16,000 x *g* at 4°C for 10 minutes. The resulting supernatant that contains the recombinant Hisx6-TEV-calmodulin protein was loaded onto a column of TALON Metal Affinity Resin (Clontech) equilibrated with the TALON equilibration buffer (50 mM sodium phosphate [pH8.0], 300 mM NaCl) at 4°C. Then, a column was washed with 3 CV (column volume) of TALON equilibration buffer, 3 CV of wash buffer 2 (50 mM sodium phosphate [pH8.0], 300 mM NaCl, 2.5 mM imidazole), and wash buffer 3 (50 mM sodium phosphate [pH8.0], 300 mM NaCl, 5 mM imidazole), sequentially. The bound Hisx6-TEV calmodulin protein was eluted with elution buffer (50 mM sodium phosphate [pH8.0], 300 mM NaCl, 150 mM imidazole), followed by the buffer exchange with the TALON equilibration buffer and the concentration by centrifugation at 5,000 x *g* at 4 ºC using centrifugal filter (Amicon Ultra-15, Millipore) to the proper concentration.

Immunoblotting

The stacking gel was composed of 12% (wt/vol) acrylamide, 0.1% (wt/vol) SDS, 125 mM Tris-HCl [pH 6.8], 0.1% (wt/vol) ammonium persulfate, and 0.5% (vol/vol) N,N,N',N'-tetramethylethylenediamine. The resolving gel was composed of 12% (wt/vol) acrylamide, 0.1% (wt/vol) SDS, 375 mM Tris·HCl [pH 8.8], 0.05% (wt/vol) ammonium persulfate, and 0.25% (wt/vol) N,N,N',N'-tetramethylethylenediamine. Electrophoresis was performed in 0.1% (wt/vol) SDS, 25 mM Tris, and 192 mM glycine at 180 V per gel for 50 min. Proteins were transferred to PVDF membranes (0.2 μm; Bio-Rad) at 15 V for 30 min in a transfer buffer. Membranes were incubated with 3% (wt/vol) nonfat dry milk in Tris-buffered saline containing Tween [TTBS: 50 mM Tris·HCl [pH 7.8], 150 mM NaCl and 0.1% (vol/vol) Tween 20] for 1–2 h and then in primary antibody in TTBS for 2 h at room temperature. After washout of the primary antibody, membranes were incubated with secondary antibody (anti-rabbit IgG-horseradish peroxidase conjugate) in TTBS at

1:8,000 dilution for 1 h at room temperature and washed with TTBS (3 times for 5 min each) and then chemiluminescence signal detection using the ECL Western Blotting Detection Reagents (GE Healthcare Japan, Tokyo, Japan). The emitted light was detected and quantified with a chemiluminescence imaging analyzer (LAS4000, Fujifilm), and images were analyzed with Adobe Photoshop Elements 13.

Kinase activity assay

Kinase activity assays were performed as described previously[36]. Kinase activities were assayed in 20 mM HEPES [pH 7.5], 1 mM $CaCl_2$, 5 mM $MgCl_2$, 2 mM DTT, 100 μM ATP, 0.01% Tween 20, 100 nM CaM, with purified MLCKs and substrates in 40 μl total volume at 25°C. Reaction mixtures were preincubated for 5 minutes, and the kinase reactions were started by the addition of ATP and incubated for the indicated time. For the measurement of MLC K_m and V_{max} values, 5 nM cMLCK was incubated with 0.125~20 μM MLC2v at 25ºC for 3 hours. Kinase activities were measured by ADP-Glo Kinase Assay or Phos-tag SDS-PAGE.

ADP-Glo Kinase Assay

ADP-Glo Kinase Assay (Promega Corporation, Madison, WI) was performed as described previously[3]. In brief, the kinase reactions were stopped by adding 40 μl of ADP-Glo reagent. After incubation at room temperature for 40 minutes, 80μl of kinase detection reagent was added per well and incubated for 30 minutes. Luminescence was measured using an EnSpire (PerkinElmer, Inc.) or a chemiluminescence imaging analyzer TriStar2 LB942 (BERTHOLD Technologies) with an integration time of 0.5 seconds per well. Kinase reaction without MLC2v, a substrate of cMLCK, was routinely prepared as a background. Kinase activity was calculated by subtracting the relative light unit (RLU) of the background from the RLU of the reaction with MLC2v. Standard curves with defined ADP/ATP ratios were routinely performed and used to convert relative light units (RLU) into reaction velocities. K_m and V_{max} values were calculated by nonlinear fit to the Michaelis-Menten equation by using Igor Pro software.

Phos-tag SDS-PAGE

Phos-tag SDS-PAGE was performed as described previously[3]. For analysis of MLC2v phosphorylation, samples of kinase reactions were subjected to phosphate affinity SDS-PAGE using an acrylamide-pendant phosphate-binding tag (Phos-tag SDS-PAGE). Electrophoresis was performed in 0.1% (wt/vol) SDS, 25 mM Tris, and 192 mM glycine at 150 V per gel for 80 min. After electrophoresis, the gel was soaked in EDTA (+) transfer buffer (10 mM EDTA, 50 mM Tris, 380 mM glycine, 0.000375% (wt/vol) SDS, and 20% (wt/vol) EtOH) for 10 min and then in transfer buffer for 10 min. Proteins were transferred to PVDF membranes (0.2 μm; Bio-Rad) at 15 V for 30 min in transfer buffer and immunoblotted by anti-MLC2v antibody (1:4,000 dilution).

Calcium transient measurements

iPS-CMs were plated at 5×10^4 cells/well (96-well plate) and incubated overnight in DMEM with 10% FBS. The medium was exchanged for a loading medium containing Cal-520 at a final concentration of

5μM and then incubated in a cell incubator at 37℃ for 1 hour. iPS-CMs were washed with HHBS buffer, and the buffer was replaced with a recording medium that contain the chemical compound we developed. 96-well plates containing iPSC-CM were transferred to a 37℃ heated chamber. Cells were electrically stimulated at increasing frequencies (0.5 Hz, 0.75 Hz, 1Hz, 2 Hz) using an external stimulator, and whole-cell fluorescence intensities were recorded and analyzed using FDSS/μCell (Hamamatsu Photonics, Hamamatsu, Japan).

High-throughput screening for cMLCK activators

8-Hydroxyquinoline structures were identified by high-throughput screening as an activator of cMLCK in ADP-Glo and Kumagai method at 10 mM which posed >20% activation potency, while it did not exhibit any activation and inhibition of smMLCK. Primary hit compounds were further optimized to have good potency as cMLCK activators and retained good permeability. Among them, LEUO-1154 showed no cytotoxicity to iPSC-CMs at 3 mM for 14 days.

Synthesis of small chemical compounds for cMLCK activators

General. Unless otherwise noted, all reagents were commercially obtained. NMR spectra were recorded at ambient temperature by using a JEOL JMN-ECZ400S/L1 spectrometer. The ^{1}H NMR data are reported as follows: a chemical shift in ppm from internal tetramethylsilane (0.0 ppm) on the δ scale, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m =multiplet), coupling constants (Hz), and integration. ^{13}C NMR spectra were referenced to the internal solvent signals (central peak is 39.5 ppm in DMSO-d_6). LCMS analyses were performed on the Waters ACQUITY UPLC H-Class/QDa system. The mobile phase was followed: [A] 0.1% formic acid-containing aqueous solution, [B] acetonitrile. The column was a 1.7 μm, 2.1 × 50 mm ACQUITY UPLC BEH C_{18} column. Samples were run on a 3 min gradient from 10 to 95% organic at 0.5 mL/min. The PDA was set to analyze a wavelength range from 190 to 400 nm.

***N*-((8-hydroxy-5-nitroquinolin-7-yl)(1*H*-pyrrolo[2,3-b]pyridin-3-yl)methyl)pentanamide (LEU-1154).** A mixture of 1*H*-pyrrolo[2,3-b]pyridine-3-carbaldehyde (85 mg, 0.578 mmol), pentanamide (106 mg, 0.631 mmol), and 5-nitroquinolin-8-ol (100 mg, 0.526 mmol) were heated to 135 °C for 3 h. After cooling to ambient temperature, the mixture was recrystallized from AcOEt to give the desired product as an orange solid (59 mg, 27%). Further purification was performed by recrystallization from acetonitrile/DMSO. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 11.52 (s, 1H), 9.21 (dd, J = 8.9, 1.1 Hz, 1H), 9.02 (d, J = 2.7 Hz, 1H), 8.88 (d, J = 8.7 Hz, 1H), 8.77 (s, 1H), 8.22 (dd, J = 4.6, 1.4 Hz, 1H), 7.89 (dd, J = 8.9, 4.3 Hz, 1H), 7.84 (d, J = 6.9 Hz, 1H), 7.06 (dd, J = 8.0, 4.8 Hz, 1H), 7.02 (d, J = 2.3 Hz, 1H), 6.89 (d, J = 8.2 Hz, 1H), 2.21 (t, J = 7.3 Hz, 2H), 1.51 (tt, J = 7.3, 7.3 Hz, 2H), 1.26 (qt, J = 7.3, 7.3 Hz, 2H), 0.85 (t, J = 7.3 Hz, 3H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ 171.68, 157.20, 149.07, 148.82, 142.94, 137.02, 134.44, 132.90, 127.53, 126.75, 125.12, 124.14, 123.96, 121.58, 118.23, 115.37, 114.09, 42.95, 34.97, 27.56, 21.78, 13.71. MS (ESI); *m*/*z*: $[M+H]^+$ calcd for $C_{22}H_{22}N_5O_4^+$: 420.2, found 420.1.

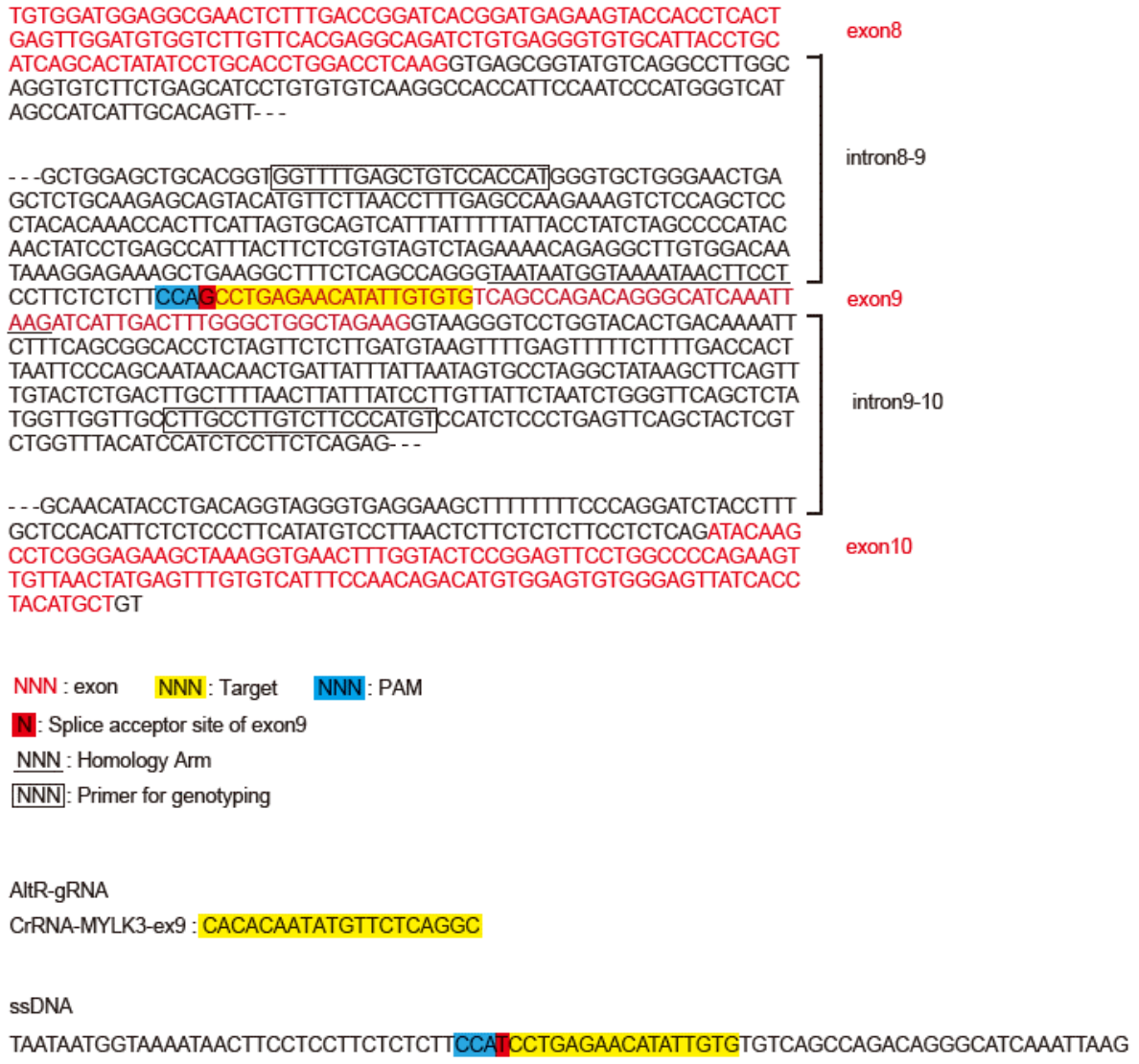
Statistics

All data are expressed as means ± SD unless otherwise indicated. For two-group comparisons, the two-tailed Student's t-test was used. For multiple-group comparisons, one-way ANOVA and Tukey correction was used. A P value less than 0.05 was considered statistically significant. Statistical analysis and graph generation were performed using GraphPad Prism (MDF, Tokyo, Japan).

Study approval

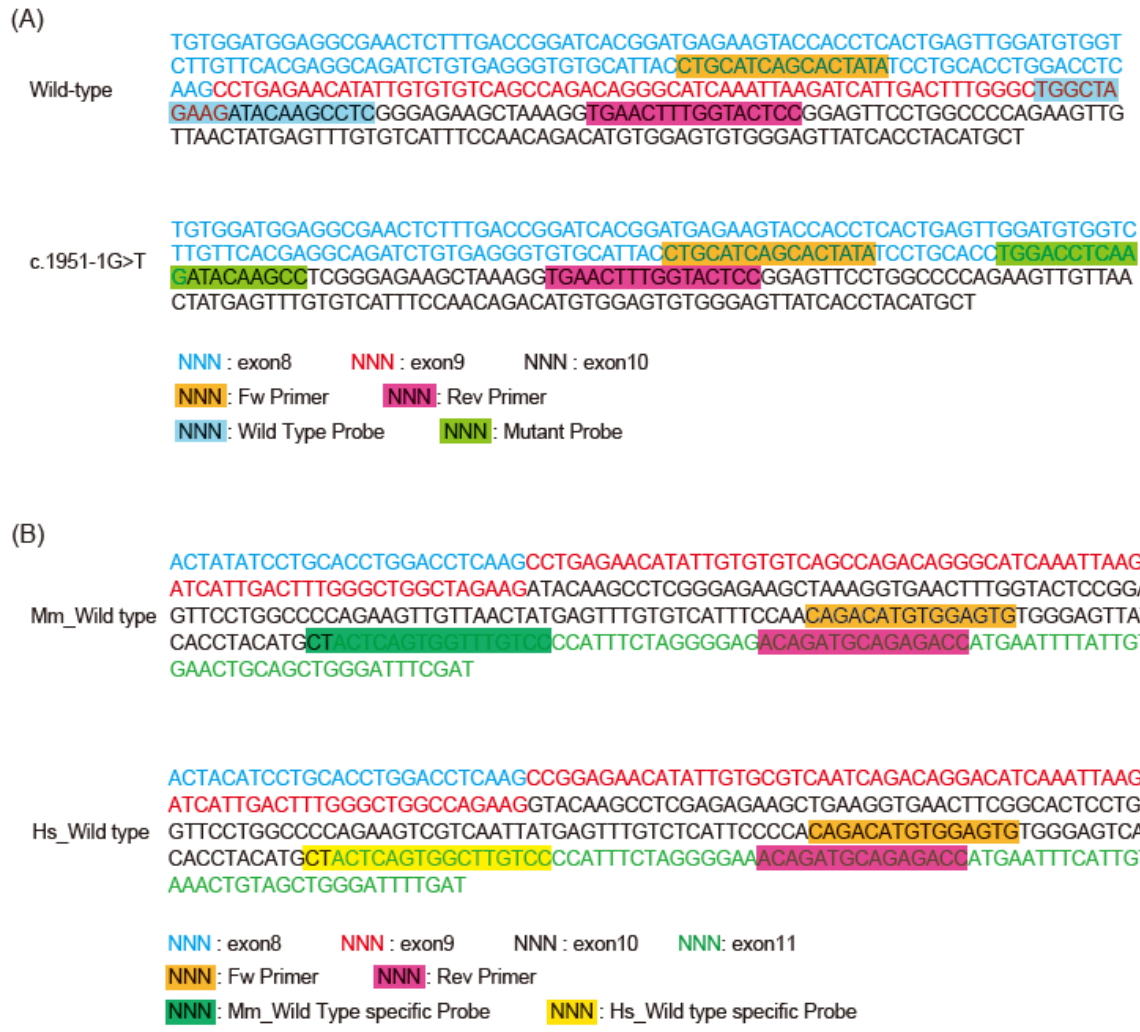
All animal experiments were performed in conformity with the Guide for the Care and Use of Laboratory Animals (NIH Publication, 8th Edition, 2011) and were approved by the Animal Care and Use Committee of the Osaka University Graduate School of Medicine. The genome research protocol was approved by the Human Genome Research Bioethical Committee at Osaka University. For the generation of iPSCs, written informed consent was obtained from the patients before inclusion in the study according to the protocol approved by the Institutional Review Board of Osaka University. Testing of human samples was approved by the Ethics Committee of Osaka University Hospital, and written informed consent was obtained from all patients before inclusion in the study.

1. Supplementary Figures



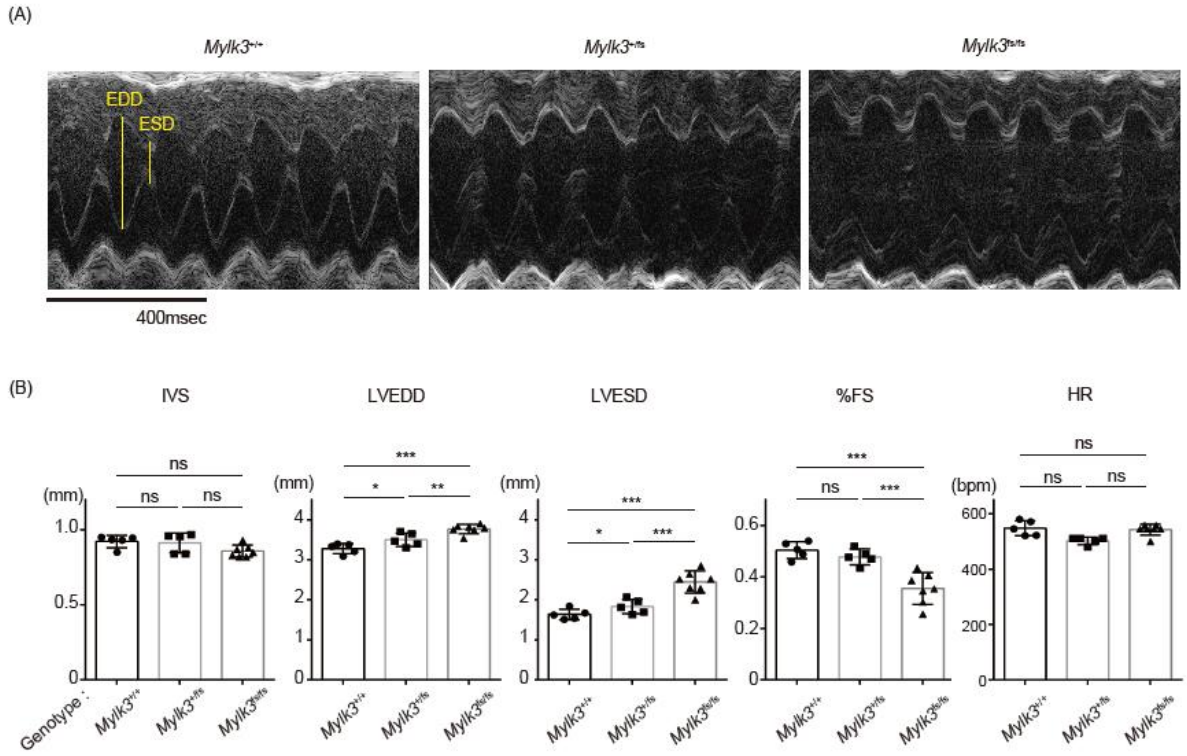
Supplementary Figure 1.

The strategy of generation of knock-in mice harboring a mutation in the *Mylk3* gene by CRISPR-Cas9-mediated homology-directed genome editing. sgRNA and donor construct of single-stranded DNA (ssDNA) targeting *Mylk3* intron 8-9 and exon 9 were designed for zygote injection. PAM motif for sgRNA is shown in blue. The targeting sequence of sgRNA is highlighted in yellow. The mutation at the splice acceptor site of exon 9 (c.1915-1G>A) is shown in red. The homology arms of the donor construct vector are underlined. Primers for genotyping are enclosed by a square.



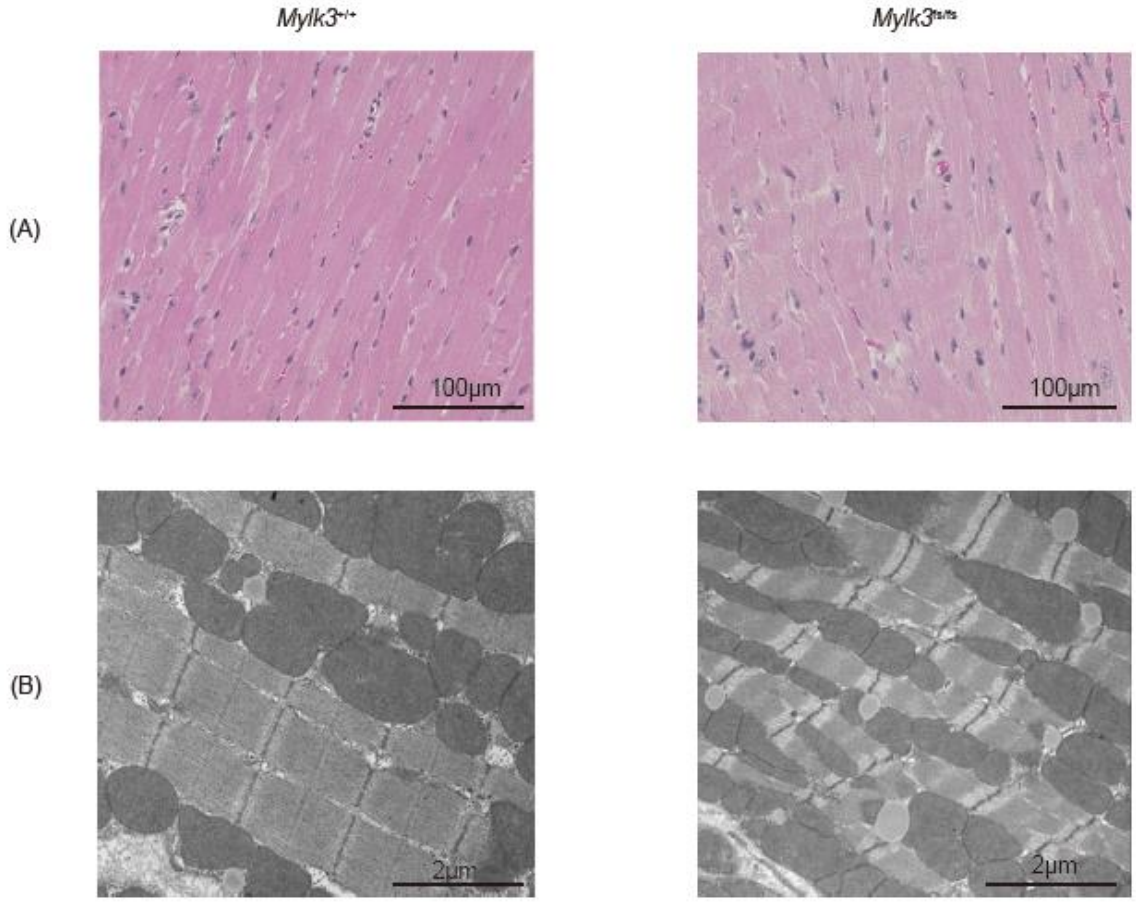
Supplementary Figure 2.

The probes and primers used in ddPCR analysis for the quantification of *Mylk3* transcripts in the hearts of mice. **(A)** The ddPCR probes and primers were designed to specifically detect wild-type or the c.1951-1G>T mutant *Mylk3* transcripts of mice. **(B)** The ddPCR probes and primers were designed to specifically detect endogenous mouse *Mylk3* or exogenous human *Mylk3* transcripts.



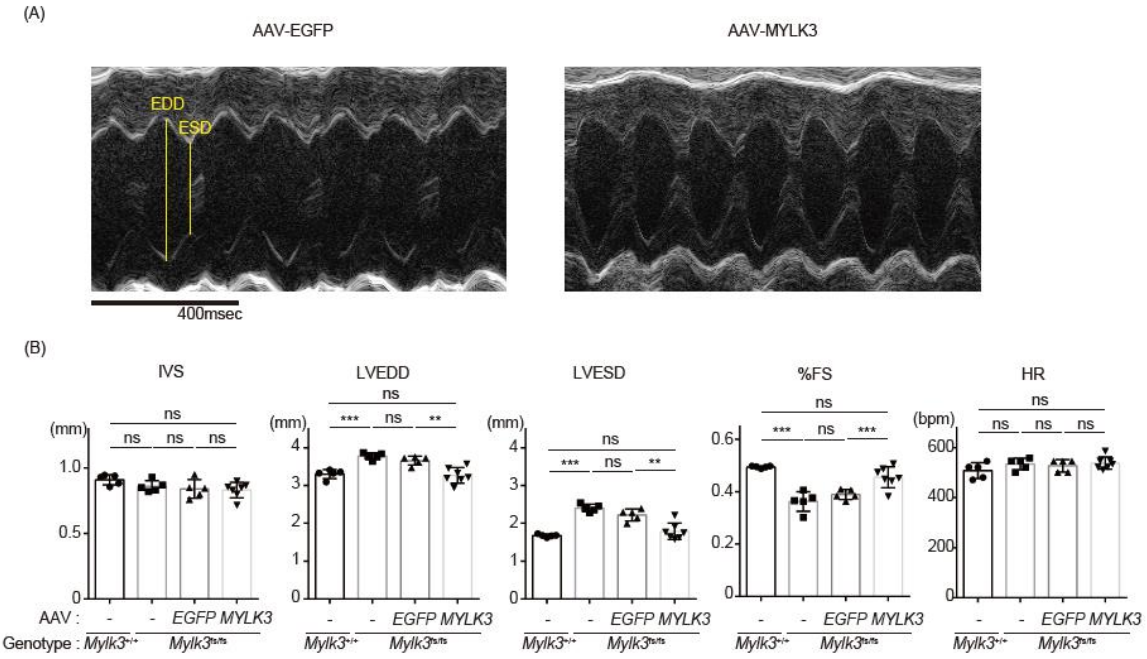
Supplementary Figure 3.

Representative M-mode echocardiograms recorded in unanesthetized mice at 12 weeks old **(A)** and summarized data **(B)**. n = 5 to 7 in each group, one-way ANOVA, values are mean ± SD, *P < 0.05 **P < 0.01 ***P < 0.001. IVS, intraventricular septal thickness; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; %FS, fractional shortening; HR, heart rate.



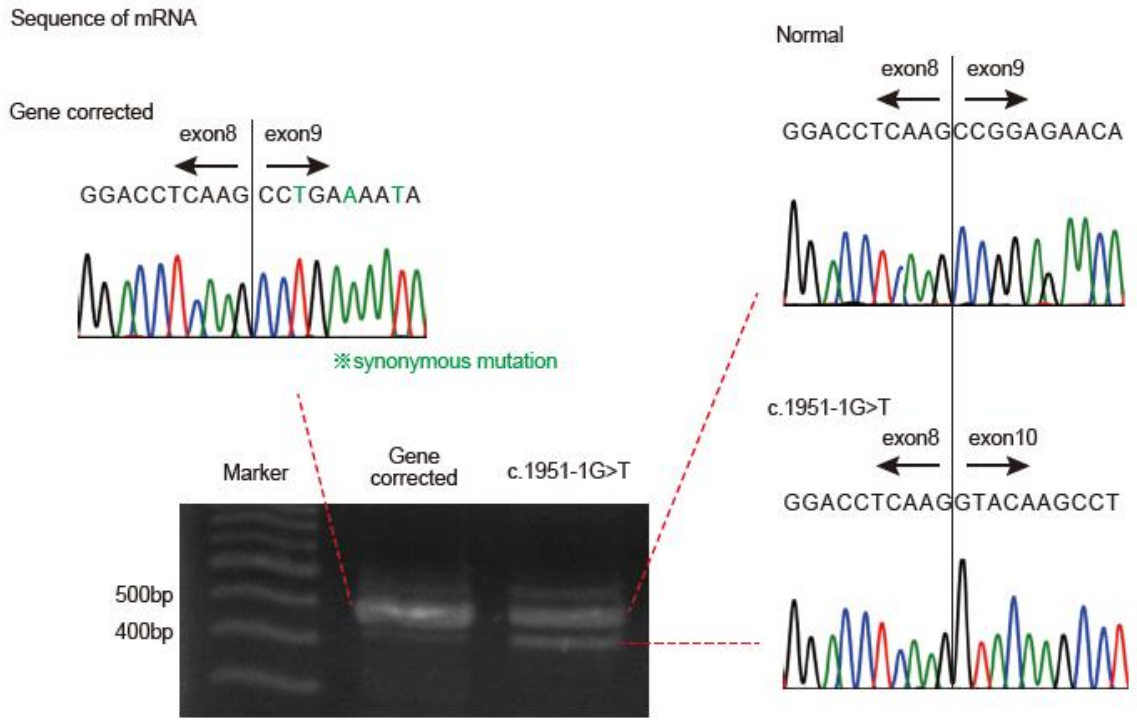
Supplementary Figure 4.

Representative HE stains **(A)** and transmission electron microscope (TEM) of the hearts **(B)** from wild-type and homozygous KI mice.



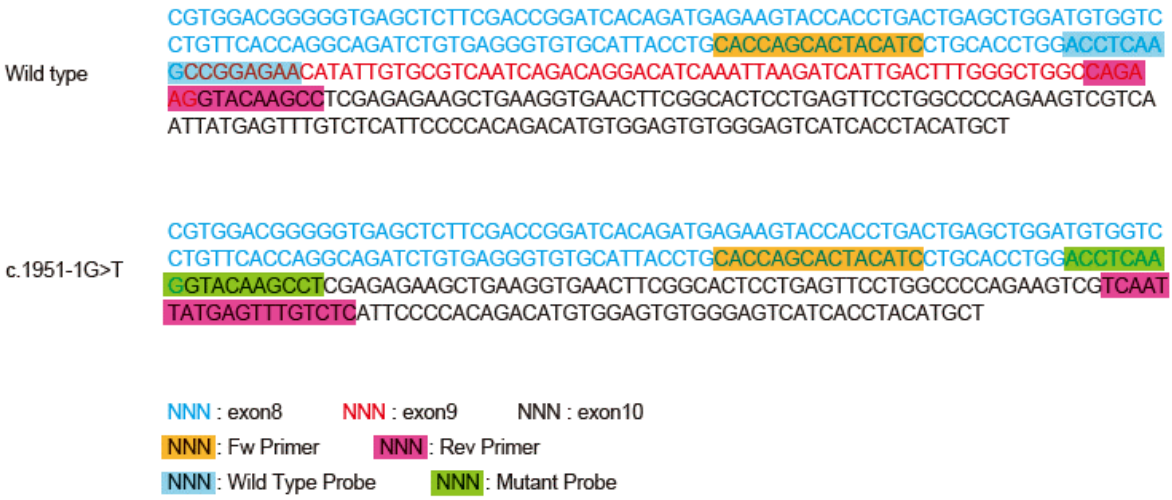
Supplementary Figure 5.

Representative M-mode echocardiograms recorded in unanesthetized mice at 12 weeks old **(A)** and summarized data **(B)**. n = 5 to 7 in each group, one-way ANOVA, values are mean ± SD, **P < 0.01 ***P < 0.001. IVS, intraventricular septal thickness; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; %FS, fractional shortening; HR, heart rate.



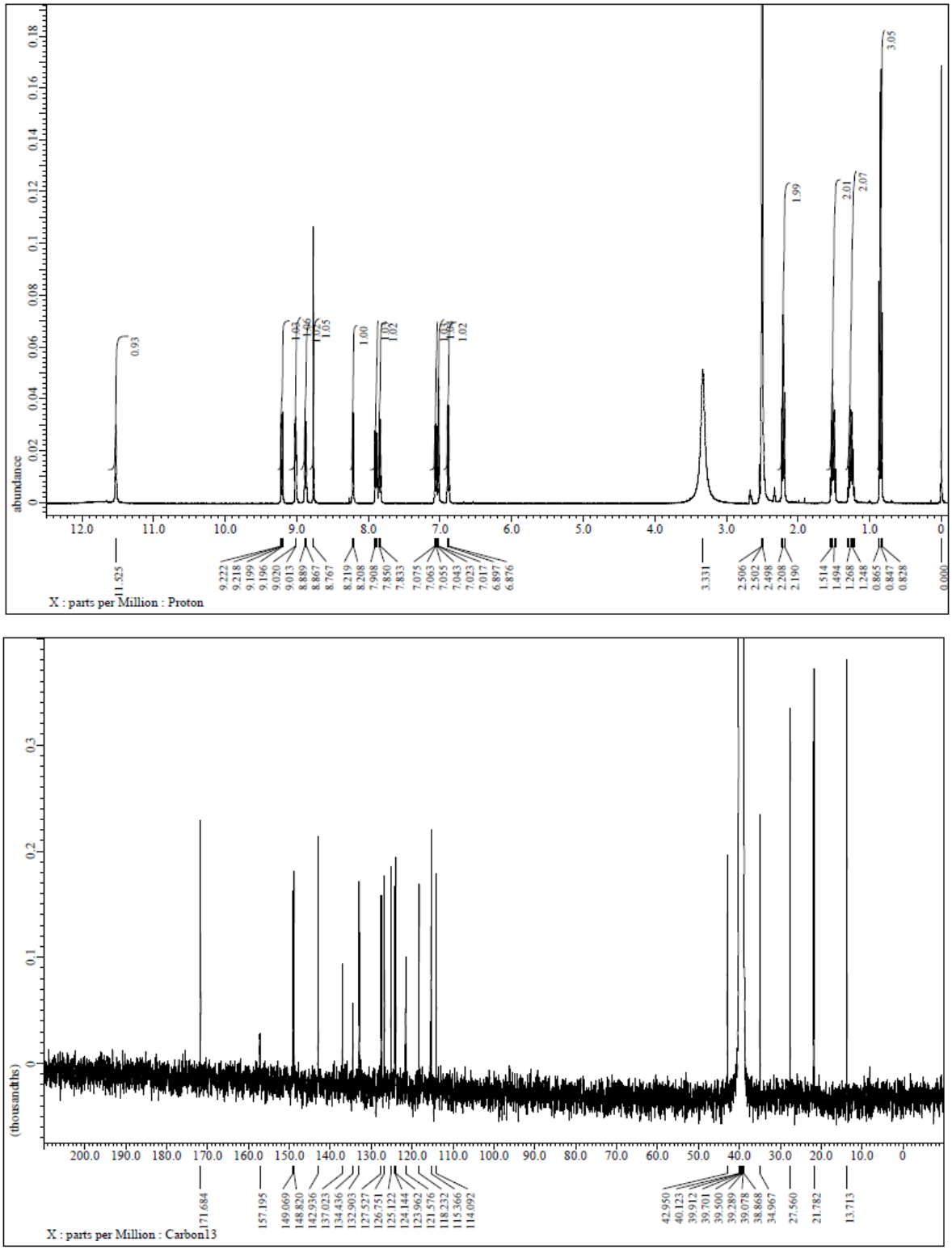
Supplementary Figure 6.

Sanger sequencing analysis of the reverse transcription PCR products verified successful CRISPR-mediated gene correction in iPSC-CMs from the carrier with heterozygous c.1951-1G>T *MYLK3* mutation. Gene-corrected iPSC-CMs expressed the corrected *MYLK3* mRNA without the out-frame skipping of exon 9 with synonymous mutations.



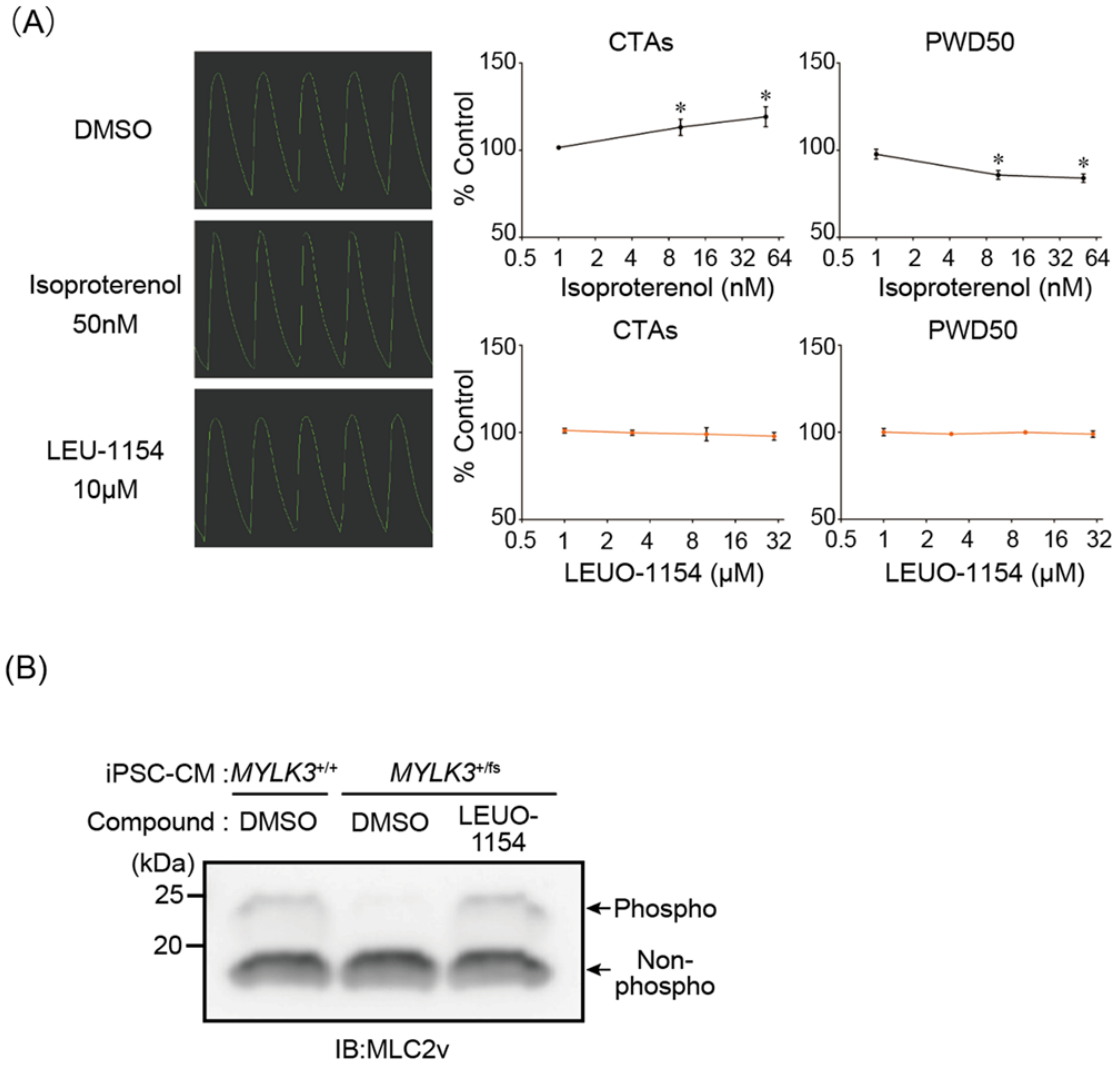
Supplementary Figure 7.

The probes and primers used in ddPCR analysis for the quantification of *MYLK3* transcripts in human iPSC-CMs. The ddPCR probes and primers were designed to specifically detect wild-type or the c.1951-1G>T mutant *MYLK3* transcripts.



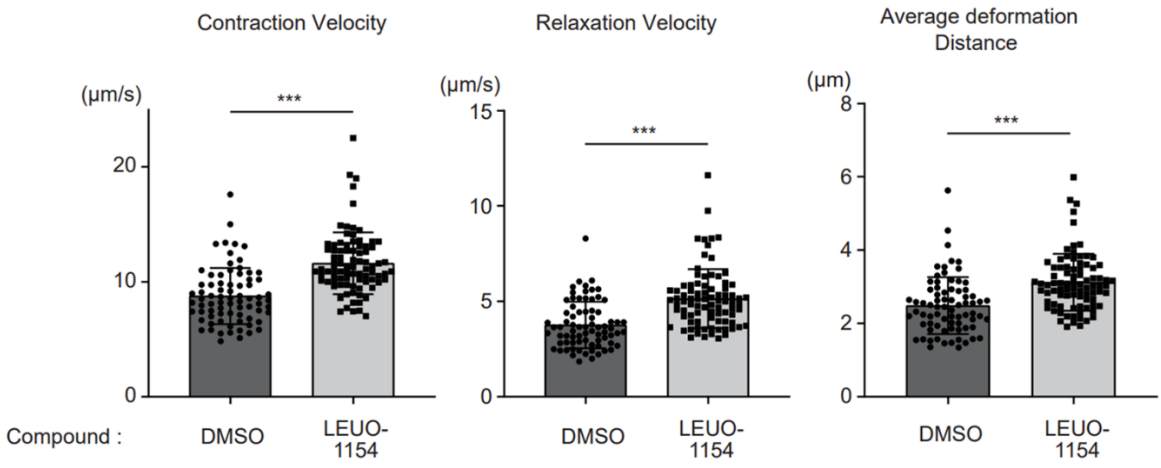
Supplementary Figure 8.

^{1}H and ^{13}C NMR spectra of LEU-1154.



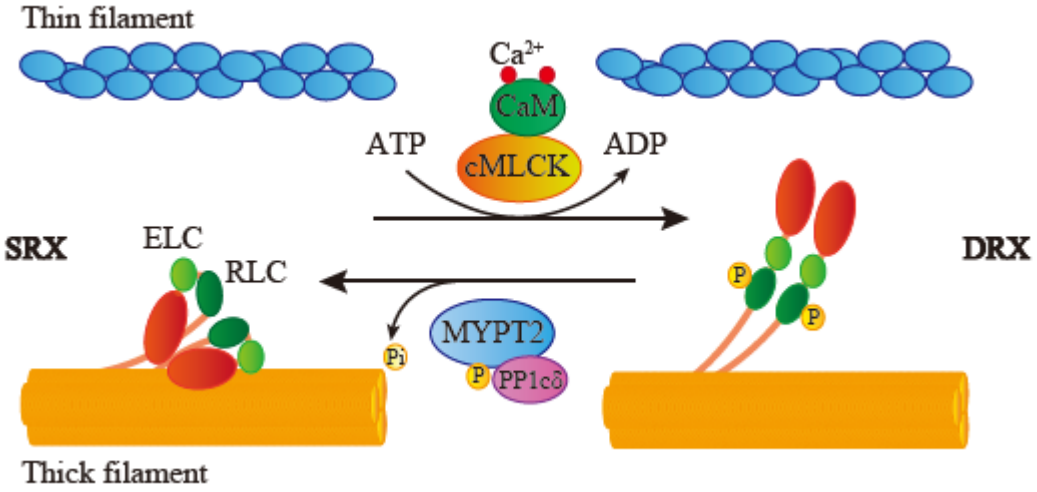
Supplementary Figure 9.

(A) Profiles of calcium transients in *MYLK3*$^{+/+}$ iPSC-CMs treated with DMSO, isoproterenol, or LEUO-1154 (10 μM). The graph shows the average changes in the amplitude of calcium transients at systole (CTAs) and pulse width duration measured at 50% CTAs (PWD50). N = 5 for each point. **(B)** Whole-cell lysates from *MYLK3*$^{+/f}$ iPSC-CMs treated with DMSO or LEUO-1154 (10 μM) were analyzed by phos-tag SDS-PAGE.



Supplementary Figure 10.

Effect of LEU-1154 (10 µM for 3 days) on cardiac contraction and relaxation in *DSG2*-R119X iPSC-CMs. N = 75 or 85 for DMSO or LEUO-1154, respectively. Values are mean ± SD. ***P < 0.001, one-way ANOVA with Tukey post hoc test.



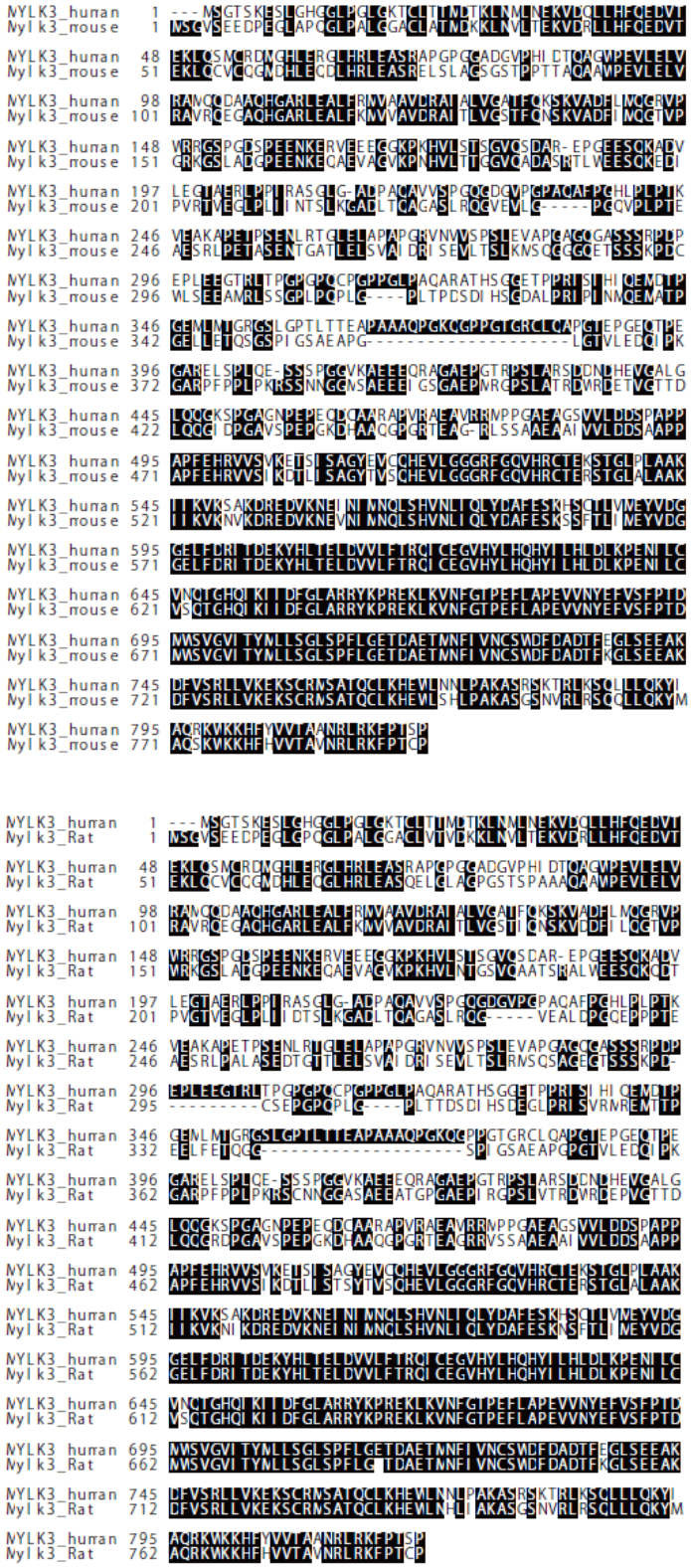
Supplementary Figure 11.

Scheme for the regulation of MLC2v phosphorylation in the heart.

Number	age	sex	LVEDD(mm)	LVESD(mm)	LVEF(%)	etiology
1	28	M	68	64	14	doxoruicin cardiomyopathy
2	23	F	63	59	19	dilated phase HCM
3	34	M	63	59	12	DCM
4	42	F	68	63	28	dilated phase HCM
5	49	F	68	62	25	DCM
6	46	M	76	64	20	ischemic cardiomyopathy
7	30	M	81	74	10	DCM
8	61	F	73	68	21	dilated phase HCM
9	38	F	60	55	27	DCM
10	58	M	79	76	28	DCM
11	59	F	62	57	24	dilated phase HCM

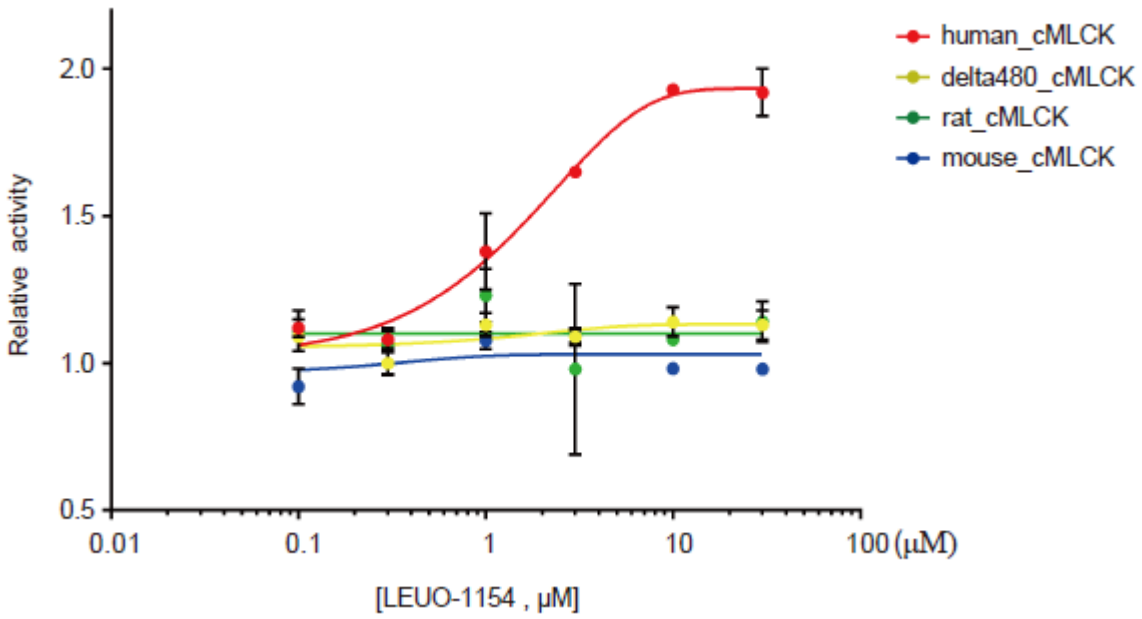
Supplementary Figure 12.

Clinical information for the patients analyzed in this study. LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; EF, ejection fraction; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy.



Supplementary Figure 13.

Amino acid sequence alignment of cMLCK with different mammalian proteins. The alignment was generated using clustalW and pyBoxshade.



Supplementary Figure 14.

The N-terminus region of cMLCK is not conserved among the species. **(A)** LEUO-1154 did not activate the N-terminus deletion mutant of human cMLCK (delta-480 cMLCK) and cMLCK from other species.

2. Supplementary Tables

Supplementary Table 1: List of antibodies used in the study

antibodies	product number	company	dilution of antibody
atni-MLC2v	ab92721	abcam	1:4,000
anti-mouse cMLCK	LS-C164492	LSBio	1:1,000
anti-GAPDH	MAB374	MEMD	1:3,000
anti-alpha Actinin	ab9465	abcam	1:200
anti-FLAG M2	D6W5B	Cell Signaling	1:800
anti–rabbit IgG	#55696	Cappel	1:8,000
anti-mouse IgG	#55550	Cappel	1:8,000

MLC2v, ventricular myosin regulatory light chain; cMLCK, cardiac-specific myosin regulatory light chain kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Supplementary Table 2: CRISPR/Cas9 sgRNA and template DNA

CRISPR-sgRNA	Site of Interest 5'→3'
sgRNA for c1951-1g>t_KI mouse	CACACAATATGTTCTCAGGC
sgRNA1 for Gene correction of iPSCs	ACCTCCTCAGCTATTGCTT
sgRNA2 for Gene correction of iPSCs	TGACGCACAATATGTTCTC
Template DNA	
ssDNA for c1951-1g>t_KI mouse	TAATAATGGTAAAATAACTTCCTCCTTCTCTCTTCCA**T**CCTGAGAACATATT GTGTGTCAGCCAGACAGGGCATCAAATTAAG
ssDNA for Gene correction of iPSCs	GAGGCCTATAGACAAGTGTGGCAGCTGCAGTCCACCACTGCATAAGCAA GCGCTAAGCAGACTTCTTTCTTTCCCAGCCTGAAAATATCTTATGTGTTAA TCAGACAGGACATCAAATTAAGATCATT

The mutation at the splice acceptor site of exon 9 (c.1915-1G>T) is shown in red.

sgRNA, single-guide RNA; KI, knock-in; ssDNA, single-stranded DNA.

Supplementary Table 3: List of primers and probes used in the study

List of qPCR primers	5'-Fw	5'-Rv	
EGFP	CCATGCCGAGAGTGATCC	GAAGCGCGATCACATGGT	
Gapdh	TCAACGGCACAGTCAAGG	CACGACATACTCAGCACC	
List of ddPCR primers	5'-Fw	5'-Rv	Probe
Mylk3 (mouse)			
Wild-type	CTGCATCAGCACTATA	GGAGTACCAAAGTTCA	TGGCTAGAAGATACAAGCCTC
c1951-1g>t	CTGCATCAGCACTATA	GGAGTACCAAAGTTCA	TGGACCTCAAGATACAAGCC
Wild-type (mouse specific)	CAGACATGTGGAGTG	GGTCTCTGCATCTGT	CTACTCAGTGGTTTGTCC
MYLK3 (human)			
Wild-type	CACCAGCACTACATC	GGCTTGTACCTTCTG	ACCTCAAGCCGGAGAA
c1951-1g>t	CACCAGCACTACATC	GAGACAAACTCATAATTGA	ACCTCAAGGTACAAGCC
Wild-type (human specific)	CAGACATGTGGAGTG	GGTCTCTGCATCTGT	CTACTCAGTGGCTTGTCC
	Assay ID		
PPP1R12B	dHsaCPE 5044710		
Tbp (mouse)	dMmuCPE 5124759		
TBP (human)	dHsaCPE 5058363		

EGFP, enhanced green fluorescent protein; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; ddPCR, droplet digital PCR; *Mylk3*, myosin light chain kinase 3(mouse); *MYLK3*, myosin light chain kinase 3(human); *PPP1R12B*, protein phosphatase 1 regulatory subunit 12B; *Tbp*, TATA-binding protein(mouse); *TBP*, TATA-binding protein(human).

4. Author contributions

T.H. primarily performed the experiments and analyzed the data. J.L, L.L, Y.K, S.H, S.O, and T.H generated iPS cells and CRISPR-Cas gene editing. N.F., S.Y., H.K., Y.A., M.T., and Y.S. acquired and analyzed clinical data. K.M. and Y.K. contributed to genome informatics analysis. O.T., K.M., and T.H. contributed to the generation of KI mice. O.T., Y.S., K.N., A.T., and R.I. performed the high-throughput screening assay for cMLCK activators. S.K., T.K., and J.H. were responsible for the design and preparation of the cMLCK activator, LEUO-1154. K.M., H.H., C.O., H.I., J.H., K.U., T.S., S.N., H.A., M.K., and S.T. analyzed the data and revised the manuscript. O.T. and S.T. were responsible for the overall direction of the project and the interpretation of the data. O.T. and T.H. wrote the paper.