SUPPLEMENTAL MATERIAL

Expanded Methods:

Patient-derived induced pluripotent stem cell derivation and maintenance

Left ventricular tissues were taken from a 7-month-old patient with infantile dilated cardiomyopathy (iDCM), diced with a scalpel, and digested with collagenase IV (1 mg/ml, Invitrogen) for 2 hours at 37 °C. Primary fibroblasts were then grown in fibroblast culture medium (Dulbecco's modified Eagle's medium [DMEM] with glutamine [Invitrogen] supplemented with 10% fetal bovine serum [FBS; Invitrogen]) in tissue culture dishes (Corning). The medium was changed every other day.

The episomal vectors pEP4EO2SCK2MEN2L and pEP4EO2SET2K were used to reprogram fibroblasts from the iDCM patient. They were cotransfected into ~1.0 × 10^6 patient-derived fibroblasts via nucleofection (VPD-1001 with program U-20, Amaxa, Walkersville, MD). Transfected cells were directly plated onto 3 × 10 cm Matrigel-coated dishes (1:200) in fibroblast culture medium. After one day of transfection, the fibroblast medium was replaced with reprogramming medium supplemented with DMEM/F12, N-2 supplement (Invitrogen), B-27 supplement (Invitrogen), 100 ng/ml bFGF (Invitrogen), nonessential amino acids (Invitrogen), GlutaMAX (Invitrogen), 0.1 mM β-mercaptoethanol, PD0325901 (Stemgent), CHIR99021 (Stemgent), A-83-01 (Stemgent), hLIF (Millipore), and HA-100 for 12 days. The medium was changed every other day. On day 13, the post-transfection medium was replaced with mTeSR1 medium (Stem Cell Technologies). Individual colonies with human embryonic stem cell–like morphologies were plated on 12-well plates (1 colony per well) at day 20-30. The iPSCs were maintained in mTeSR1 medium (Stem Cell Technologies) on 1:200 growth-factor-reduced (GFR) Matrigel (BD Science) and passaged 1:10 or 1:12 every 3–4 days using 0.5 mM EDTA (Mediatech). Cell lines were used between passages 13 and 75. All cultures (primary, pluripotent, and differentiation) were maintained with 2 ml medium per well in 6-well plates.

Maintenance of iPSCs and cardiac differentiation

All iPSCs used in this study were positive for NANOG, Oct4, SSEA4, and alkaline phosphatase (Figure S2a) and were maintained on plates coated with GFR Matrigel (Corning) in mTeSR1 (Stem Cell Technologies). iPSCs were maintained at 37°C with 5% carbon dioxide and 18% oxygen. Karyotype analysis of control iPSCs confirmed that there were no chromosomal abnormalities (Genetic Associate, Figure S2b). Cardiac differentiation of control and iDCM iPSCs was performed using the small molecule–based method[45]. Control and iDCM iPSCs were grown for 4 days until they achieved 80~90% confluence. Day 0 was designated at this point; the medium was changed to basal medium RPMI 1640 (Life Technologies) plus B27 without insulin supplement (A1895601, Life Technologies), supplemented with 6-8 μmol/L CHIR99021 (Selleck Chemicals) for 2 days. On day 2, the medium was changed to RPMI 1640 plus B27 without insulin without CHIR99021. On day 3, the medium was changed to basal medium RPMI 1640 plus B27 minus insulin, supplemented with 5 μmol/L IWR-1 (Sigma). Medium was changed on day 5 and every other day thereafter until day 11 to RPMI 1640 plus B27 without insulin. At day 11~13, the medium was changed to metabolic selection medium consisting of basal RPMI 1640 without glucose (Life

Technologies) plus B27 without insulin. On day 14, the medium was changed to RPMI 1640 medium with B-27 supplement (Invitrogen), and 1% Pen-Strep (Life Technologies) until experiments were performed. More than 90% of the resulting cells were positive for cardiac troponin T (Figure S2c) or α-actinin (Figure S6b), and were cultured in in RPMI 1640 with 2% B27 complete (Invitrogen) with 1% penicillin/streptomycin (Gibco) for 30 days.

Cell Shortening Analysis

Matrigel mattress substrates were generated as described[10]. Briefly, 1 µL of undiluted Matrigel solution was arrayed on a Mattek dish (Fisher Scientific) and incubated for 10 minutes. iPSC-CMs dissociated by TrypLE Express (Invitrogen) were seeded on solid undiluted Matrigel. Video edge detection was used to assess the contractility of contracting CMs by measurement of relaxed and contracted length. CMs were visualized by a Hamamatsu-ORCA camera with 16 to 17 fps using a 20× objective. Individual cells in RPMI1640 containing 2% complete B27 supplement (Life Technology) in the presence of 1% penicillin/streptomycin were recorded. The recorded files were converted into AVI files and analyzed using ImageJ (Multi Kymograph). The numbers of iPSC-CMs used in this study: 28 and 39 cells for control-1 and -2; 59 and 48 cells for iDCM-1 and -2; 43 and 29 cells for KO-1 and -2; and 32, 35, and 42 cells for GC-1, -2, -3. For C19 treatment study, 9 cells were used for control; 32 cells for iDCM; and 36 cells for iDCM+C19 over two separate experiments for iDCM and iDCM+C19 CMs. Cells that did not shorten were excluded from the analysis.

Flow Cytometry Analysis

iPSC-CMs maintained for 30 days were detached with TrypLExpress (Invitrogen). They were singularized by slowly pipetting up and down and filtered using 2 µm cell strainers (Thermo Fisher). 5×10^5-1×10^6. Cells were fixed in 1% paraformaldehyde at room temperature for 10 minutes, permeabilized by FACS buffer (PBS without calcium or magnesium, 1% FBS, 0.1% sodium azide) with 0.1% saponin. After washing, the cells were blocked with FACS buffer for 30 min. and then treated with Troponin T antibodies (Santa Cruz Biotechnology) diluted at 50 µl/sample in FACS buffer plus 0.1% Triton X-100. Cells were incubated with primary antibodies for 1 hour at 4°C, washed once in 3 ml FACS buffer plus 0.1% Triton X-100, and then centrifuged. Secondary antibodies specific to the primary antibody IgG isotype were diluted in FACS buffer plus 0.1% Triton X-100 for a final sample volume of 100 µl at 1:1,000 dilution. Cells were incubated for 30 minutes in the dark at 4°C; washed in FACS buffer plus 0.1% Triton X-100; and resuspended in 300 µl FACS buffer for analysis. The resulting data were analyzed using FlowJo v8.5.2 and reported data are based on 10,000 gated events.

Mitochondrial morphology and function

To observe the mitochondrial morphology and function of iPSC-CMs, iPSC-CMs were treated with either 50 nM Mitotracker Green FM (Molecular Probes) or/and 2 nM tetramethylrhodamine methyl ester perchlorate (TMRM)[46] (Sigma) in RPMI 1640 without phenol red with 2% B27 complete (Invitrogen) with 1% penicillin/streptomycin (Gibco) for 30 minutes. After washing, the cells were incubated for one hour in

nonphenolic media without Mitotracker and TMRM to remove unbound TMRM and Mitotracker. Some cells were used for TMRM imaging and others were used for quantitative analysis for flow cytometry. TMRM signals were limited by low green and high red fluorescence due to autofluorescence[47].

Sarcomere structure analysis

Two methods were utilized to quantify sarcomere structure in iPSC derived cardiomyocytes. The first was using OrientationJ, an ImageJ plugin with a formula for alignment index [48]. This analysis was validated by testing with in vivo rabbit-CMs. A high value of alignment corresponds to a well-organized sarcomere. The second method for quantifying the degree of sarcomere structure was conducted through blinded evaluation of sarcomere as “mature-like” or “immature-like”.

CRISPR/Cas9 expression vector construction

Single-guide RNAs (sgRNAs) were designed using the Zhang lab CRISPR design tool. sgRNA sequence (Figure S5b) were cloned into pSpCas9(BB)-2A-puro (PX459) (Addgene, 62988) plasmid, as described[49,50]. Insertion of the sgRNA was confirmed by Sanger sequencing.

Genome modification in control and patient-derived iPSCs

iPSCs were singularized using 0.5 mM EDTA in DPBS without calcium chloride and magnesium chloride and passed through a 30-μm filter. We delivered 10 μg of the Px459 plasmid containing the sgRNA insert to 2×10^6 control iPSCs by electroporation (Epi5 Episomal iPSC Reprogramming Kit, ThermoFisher). For genome correction, iDCM iPSCs were transfected with the sgRNA in the Px459 plasmid and 10 μmol of the 160-bp single-stranded oligomers. To expand individual clones post-delivery, iPSCs were grown on 10-cm plates coated with growth factor–reduced Matrigel (1:200 dilution) and treated with puromycin (1 μg/ml) for screening 36 h after transfection. Puromycin-resistant clonal colonies were picked and manually transferred to Matrigel-coated 24-well plates for expansion. Each colony was passaged using 0.5 mM EDTA for line maintenance and DNA extraction. DNA was isolated using QuickExtract solution (EpiCentre) for PCR amplification of exon 29 of *RTTN*. Screening of genome-corrected colonies was done by digestion with *Scrf*I (New England Biolabs), which selectively cut the iDCM sequence but not the genome-corrected sequence due to altered nucleotides (G4018A) in the repaired sequence. Gene editing was confirmed in all positive clones by Sanger sequencing. KO, and GC iPSCs were maintained with mTeSR1 (Stem Cell Technologies) media.

Cas9 off-target analysis

Predicted off-target tool were amplified by PCR and evaluated by Sanger sequencing. The genomic location, nearest gene, and primers used for amplification are presented in Supplemental Tables 3 and 4.

Transmission electron microscopy analysis

Specimens were processed for transmission electron microscopy (TEM) and imaged in the Vanderbilt University Cell Imaging Shared Resource Electron Microscopy facility. For embedding, cells on coverslips

were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 at room temperature (RT) for 1 hour and then transferred to 4ºC overnight. Samples were washed in 0.1 M cacodylate buffer, incubated for 1 hour in 1% osmium tetroxide at room temperature, and then washed with 0.1 M cacodylate buffer. Coverslips were rinsed with water and then contrasted with 1% potassium ferrocyanide for 15 minutes at room temperature. Coverslips were then washed with water and subsequently dehydrated through a graded ethanol series and followed by three exchanges of 100% ethanol. Next, samples were incubated for 5 minutes in 100% ethanol and then in propylene oxide (PO), followed by 2 exchanges of pure PO. The samples were then infiltrated with 25% Epon 812 resin and 75% PO for 30 minutes at RT. Samples were then infiltrated with Epon 812 resin and PO (1:1) for 1 hour at room temperature and then overnight at room temperature. The following day, the samples went through a 3:1 (resin: PO) exchange for 3 to 4 hours and were then incubated with pure epoxy resin overnight. Samples were incubated in 2 more changes of pure epoxy resin and then allowed to polymerize at 60°C for 48 hours. For sectioning and imaging, 70- to 80-nm ultrathin sections were cut and collected on 250-mesh copper grids and post-stained with 2% uranyl acetate and then with Reynold's lead citrate. Samples were imaged using a Philips/FEI Tecnai T12 electron microscope at various magnifications.

Immunostaining

For immunofluorescence imaging, iPSC-CMs were fixed, permeabilized, and blocked as described[11]. Samples were fixed in 4% paraformaldehyde for 10 minutes, permeabilized with 0.2% Triton X-100 in PBS without calcium and magnesium for 10 minutes, and then blocked with Protein Block (Biogenex, KH112-9k) for 15 minutes at room temperature. The iPSC-CMs were incubated overnight at 4°C in primary antibodies in Protein Block solution containing 0.2% Triton X-100. After multiple washes with 0.2% Triton X-100 in PBS, secondary antibodies specific to the primary antibody IgG isotype were diluted (1:1,000) in Protein Block solution containing 0.2% Triton X-100 and incubated at room temperature for 1 hour. SASY staining was performed using the methanol method as described.[34] . Briefly, human embryonic kidney (HEK) cells , iPSCs, and iPSC-CMs were incubated in cold 100% methanol at -20 °C for 10 minutes. After washing, the cells were blocked with Protein Block (Biogenex, KH112-9k) for 90 minutes at 4°C. the cells were treated overnight at 4°C in primary antibodies in Protein Block plus 0.5% Triton X-100. After thorough washing, the cells were treated with the secondary antibody corresponding to the species of the primary antibody for one hour at room temperature. After thorough washing, all immunostained cells were mounted in Vectashield (Vector Laboratories) including DAPI, visualized by a Zeiss laser-scanning LSM 710 META Inverted confocal microscope or a Nikon A1 point laser confocal microscope and analyzed with NIS-Elements BR3.0 and Fiji software[51].

For Drosophila staining, female adults (day 1) were collected and their hearts were processed as follows, in brief: the heart was dissected and fixed in 4% paraformaldehyde (PFA) (Polysciences) in 0.1 M phosphate buffer (pH 7.4) for 20 min. The fixed specimen was permeabilized in 0.1% Triton X-100 in PBS (PBST) for 10 min; then blocked in PBST with 2% BSA for 1 hr and incubated overnight with primary antibodies at 4°C. were used to detect heart fiber. For staining of the adult heart, phalloidin conjugated

Alexa Fluor 568 (Thermo Fisher Scientific) was diluted at 1:1,000 and incubated with dissected heart overnight at 4°C. Cy3-conjugated secondary antibodies (1:1,000; Thermo Fisher Scientific) were used to recognize the actinin primary antibodies. All the fly hearts were imaged by a 63x Plan-Apochromat 1.4 N.A. oil objective (LSM900; ZEISS). For quantification and comparison of cardiac fiber intensity, same parameters were used across all conditions, set to avoid oversaturation. Each image was adjusted to get the heart tube at the middle horizon position, transferred to 8-bit image, and mean gray value of each heart tube was measured.

Antibodies

Antibodies used in this study were: cardiac Troponin T (Invitrogen, MA5-12960 and Abcam, ab45932); sarcomeric α-actinin (Sigma, A7732); α-tubulin (Invitrogen, 62204); PCNT (Abcam, mAbcam28144 and ab4448); PCM1 (Santa Cruz, sc-398365); γ-tubulin (Sigma, T-6557); rotatin (Santa Cruz I-20, sc-85129 and N-14, sc-85130; ThermoFisher, PA5-98136; R&D system, MAB9966; and Abcam, ab122710 and ab113541); ac-tubulin conjugated with Alex Fluor 488 (Santa Cruz) and anti-Actinin (Developmental Studies Hybridoma Bank)

Source of blood from proband and their parents and DNA isolation

Blood samples were obtained from the Core Lab for Translational and Clinical Research at Vanderbilt University Medical Center. The study was approved by the Vanderbilt Institutional Review Board, and written informed consent was obtained from the patient's parents. Blood samples were collected in BD Vacutainer EDTA tubes (Becton, Dickinson and Company, NJ) prior to heart transplantation. Samples were stored at -80°C until used for DNA extraction. DNA was extracted from these specimens using Qiagen DNeasy Blood & Tissue Kit (Qiagen, Cat No. 69504) according to the manufacturer's instructions.

Whole-exome sequencing of trios (patient and both parents)

We performed Trio Whole-Exome Sequencing (WES) of the proband and his parents at the Vanderbilt Advanced Genomics Core Facility using an Illumina Hi Seq 2500 with 100× coverage. Data were processed through Illumina's CASAVA v1.8.2 pipeline. We conducted thorough quality control based on the multistage quality control protocol developed at Vanderbilt[52,53]. No sequencing quality concerns were observed. Alignments were performed using Burrows-Wheeler Alignment[54] against human genome reference UCSC HG19. We marked duplicates using Picard,[55] and then performed local realignment and recalibration using the Genome Analysis Toolkit (GATK).[56] Somatic mutations were inferred using the GATK analysis pipeline. The results were further filtered based on GATK's best practices. Annotations of single-nucleotide variants were performed using ANNOVAR[57].

Confirmatory Sanger sequencing of variants identified from WES.

ANNOVAR variant calling identified 351 insertion/deletions and 29,163 single-nucleotide polymorphisms (SNPs) in the Trio analysis of the proband and his parents (see Supplementary information). Given the lack of family history of heart disease and nonconsanguinity, multiple filtering schemes were applied for hypotheses including de novo mutations, sex linkage, and compound heterozygosity. Based on filters of functional predictions (Polyphen-2), allele frequency (MAF <0.1%), and zygosity of SNPs in the parents, and the mode of inheritance, we arrived at four de novo, two X-linked, and two compound heterozygous mutations (Supplemental Table 2). We performed Sanger sequencing of these 13 variants with corresponding primer sets (Supplemental Table 5). Finally, we validated variants by Sanger sequencing and assessed expression of the transcripts in heart tissue and iPSC-CMs. Based on these criteria, we arrived at a single putative causal gene, *RTTN*.

Zebrafish injections

Single mating pairs were bred overnight, and embryos were kept at 28.5 ºC, consistent with standard practices. Injections were performed using an Eppendorf FemtoJet 4i microinjector. Needles were pulled using a Sutter Instrument Co. Flaming/Brown Micropipette Puller Model P-97 with filaments from World Precision Instruments (Item# TW100F-4) (heat = 90, pull = 30, and velocity = 7). The translational blocking morpholino oligonucleotide 5'-TCTTTATGAGTGACGATAACTCCAT-3' was purchased from GeneTools, LLC. mRNA was generated from pcDNA-dCas9 (Addgene, 47106) using the mMESSAGE mMACHINE T7 ULTRA Transcription Kit from Invitrogen. Alt-R CRISPR-Cas9 sgRNA, sgRNA #1: 5'-ATATTTCATTATCCTACAAG-3 and sgRNA #2: 5'-TTTTCCGGAGGTTCCAATGC-3', were purchased from Integrated DNA Technologies. Wild-type NHGRI embryos were injected at the 1-2 cell stage with MO (6 ng) or dCas9 mRNA (1 ng) with or without the CRISPRi sgRNA (0.5 ng). At 8 hours post fertilization (hpf), E3 water was replaced with 0.003% 1-phenyl-2-thiourea (PTU) in E3, and the embryos remained in PTU until they were phenotyped for cardiac edema, and heart morphology at 48 hpf.

Zebrafish qRT-PCR

Total RNA was collected from 48 hpf zebrafish embryos using RNA STAT-60 (Amsbio). cDNA was made using the high-capacity cDNA reverse transcription kit with RNase Inhibitor (Applied Biosystems). The qRT-PCR was run with TaqMan Universal PCR Master Mix (Thermo Fisher Scientific) on a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems). Taqman primers (Thermo Fisher Scientific) used in Zebrafish knockdown experiments are Gapdh (Dr03436842_m1) and RTTN (Custom assay ARYMM97; Chromosome 24:14068189-14067544 in GRCz10).

Rhodamine-phalloidin staining of Zebrafish heart

Embryos were screened for heart phenotypes at 2 dpf. At 4 dpf embryos were incubated in muscle relaxation buffer (20 mM imadazole, 5 mM EGTA, 7 mM MgCl2, 5 mM creatine phosphate, 10 mM ATP,

100 mM KCl in E3 media with 0.0001% methylene blue) for 3 hours at room temperature[58]. Embryos were subsequently fixed at room temperature for 2 hours in 4% paraformaldehyde with 0.1% Triton x-100, and 0.25% glutaraldehyde in phosphate-buffered saline (PBS)[59]. Samples were stored at 4^0C in 0.1% Triton x-100 in PBS. Embryos were stained with 1x rhodamine-phalloidin in 0.1% Triton x-100 in PBS for 4 hours at room temperature. Samples were washed 5 times with 0.1% Triton x-100 in PBS at room temperature for 5 mins. Hearts were collected as previously described[60].Images were captured with a Confocal Microscope A1 (Nikon).

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For mitochondrial studies mRNA was generated from pSCAC-69 (Addgene #31241) using mMessage mMachine T7 ultra transcription kit (Thermo fisher AM1345). Zygotes were injected in the single cell with 1 ng in 1 nL of mRNA and embryos were co-injected with the RTTN MO or sham. At 4 dpf embryos were treated with relaxation buffer, fixed, hearts dissected, and stained with rhodamine phalloidin, and imaged as described.

Drosophila stocks and breeding

The two mutant fly lines for ana3 were a gift from Dr. Jordan W. Raff (University of Oxford, UK)[26]. Each mutant line was crossed with w1118, Hand-GFP; Sp/CyO-actGFP to introduce the cardiac-specific marker (Hand) and GFP balancer as described previously[61,62]. Flies were raised and maintained in a controlled environment at 25°C, unless otherwise noted, and fed with standard fly food.

Determination of heart morphology and function by optical coherence tomography (OCT)

Three-day-old female flies were collected and anesthetized by CO_2. Flies were immobilized on coverslips using GelWax (Yaley Enterprises) under a regular dissection microscope. Flies were allowed to recover for 10 min and were then examined by OCT (Model: TEL220C1, Thorlabs) at room temperature. Two-dimensional B-mode images were obtained in the longitudinal direction to identify the cardiac chamber in the A4-5 abdominal segments and then the OCT image orientation was turned 90° to obtain the transverse images. M-mode OCT images were obtained in the transverse plane by stopping the scanning mirror at the midline and collecting continuous image data. After obtaining M-mode images, repeat B-

mode transverse images were obtained to ensure proper anatomic localization throughout image acquisition. All images were calibrated with a 150-μm standard.

Survival curve assay for Drosophila

Flies were maintained in a humidified, temperature-controlled environmental chamber at 25°C and live flies were counted every day for the survival assay. Each survivorship curve represents data from over 50 flies, with three independent experiments performed, for 150 flies in total.

Imaging of the larval fly brain by Stereo Microscope

At the late 3rd instar stage, the larvae were collected and dissected at room temperature in 1XPBS. The brain lobe and nerve cord were rinsed with 1XPBS, then put on a slide for visualization and image capture using a Stereo microscope (ZEISS SteREO Discovery v20; ZEISS). To examine brain morphology, differential interference contrast (DIC) images were collected at 175x magnification. Brain volume was calculated using the radius obtained from the DIC images.

scRNA-sequencing

Control and iDCM-CMs were dissociated at differentiation day 35. Following culture and dissociation, we prepared samples for cell capture using the 10x chromium 3' v3 workflow, and sequenced on a NovaseqS4 flow cell. We integrated between conditions using the SCTransform workflow implemented in Seurat (3.1.4)[63–65] . As active cell cycling has an impact on contractile gene expression[66], we filtered out actively cycling cells in addition to non-CMs for further analysis. After filtering, we began analysis on 4,671 control and 1,080 iDCM-CMs. We performed differential gene expression testing using the Wilcoxon Rank Sum test with Bonferroni-correction-adjusted p-values < 0.05. Gene Ontology analysis was done using the Gene Ontology Resource online tool [67,68].

C19 treatment

D25 control and iDCM-CMs were dissociated and plated on Matrigel mattress coated Mattek plates. At D30, we treated with 10 μM C19[28,35] for 48 hours. Sarcomere and cell size analysis were conducted on Matrigel mattress 10 days after start of treatment with confocal imaging and ImageJ analysis. Centrosome state analysis was conducted on D32. Contractility analysis was conducted using edge detection as previously described.

Statistical analysis

Statistical differences among more than 2 groups were assessed using one-way analysis of variance followed by Tukey correction. For zebrafish data, multiple comparisons were made between the vehicle and control or treatments using Fisher's exact test with Bonferroni's correction. For Drosophila, statistical analysis was performed by Prism 7 (GraphPad Software). Statistical data are expressed as mean ± SD and Student t test was applied to determine the significance of any differences between the two groups. A

value of $P<0.05$ was considered statistically significant. For Figure 3, statistical differences among two groups were tested with two-tailed Fisher's exact test.

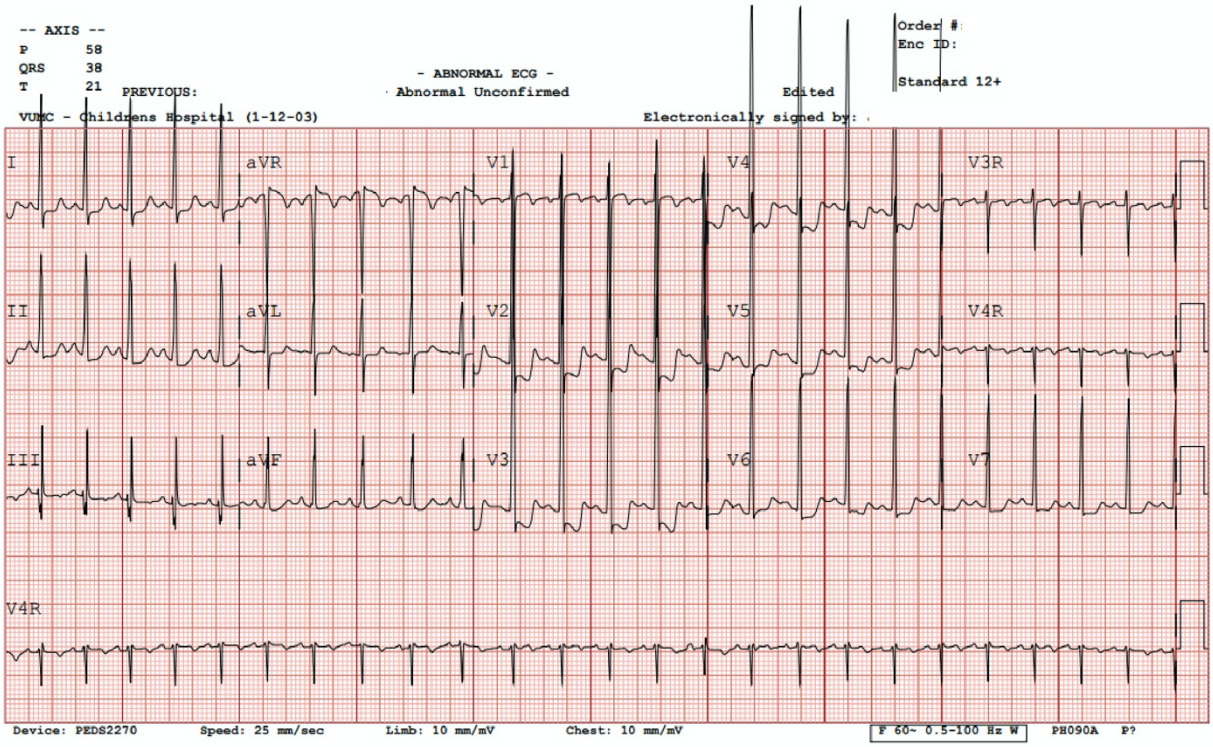

Figure S1 ECG of the infant demonstrates sinus rhythms with biventricular hypertrophy.

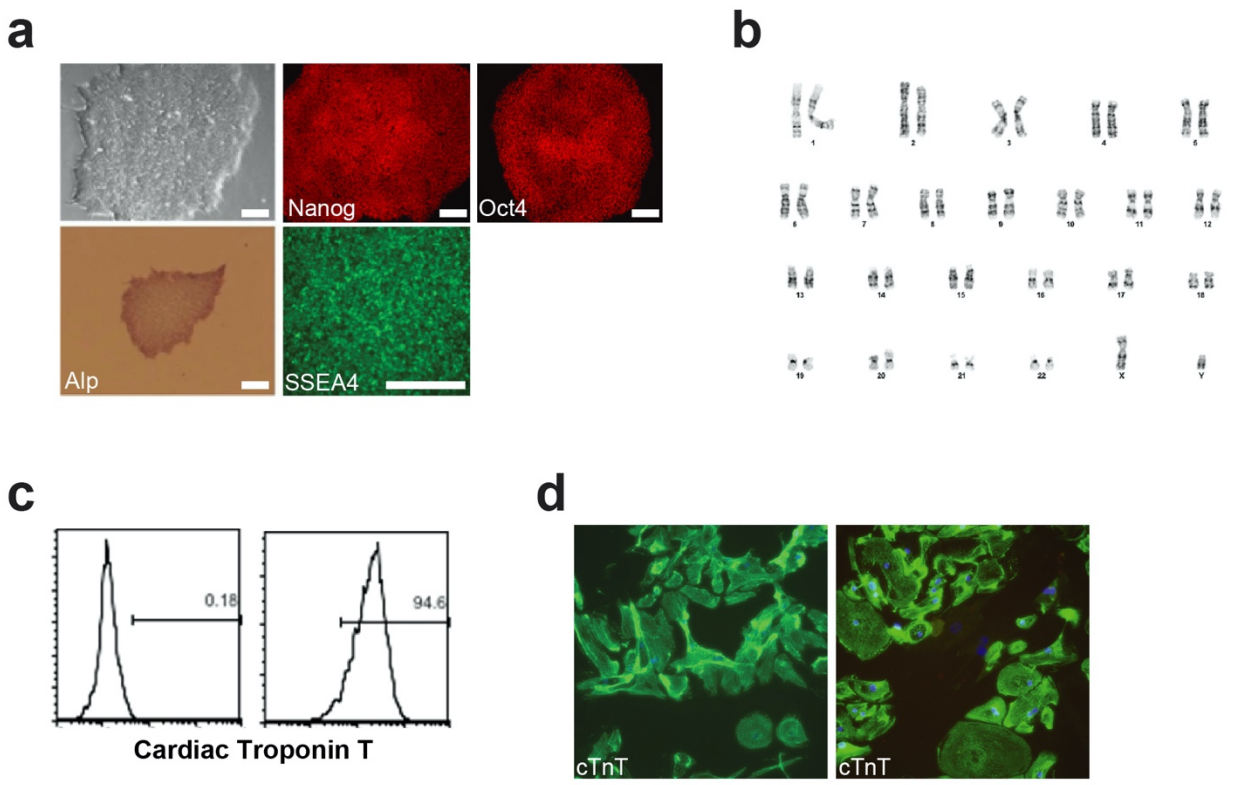

Figure S2 Characterization of iDCM iPSCs.

a, Two independent iPSC cell lines were derived from the iDCM patient. Each line had embryonic stem cell-like morphology and expressed the pluripotent stemness markers alkaline phosphatase (ALP), *NANOG, Oct4*, and *SSEA4*. Scale bars are 10μm. **b**, Karyotypes of the iDCM iPSC line utilized. **c**, iPSC-CMs were generated through directed differentiation, and confirmed by flow cytometry. **d,** Efficient differentiation to CMs was also verified by immunofluorescence for cTnt.

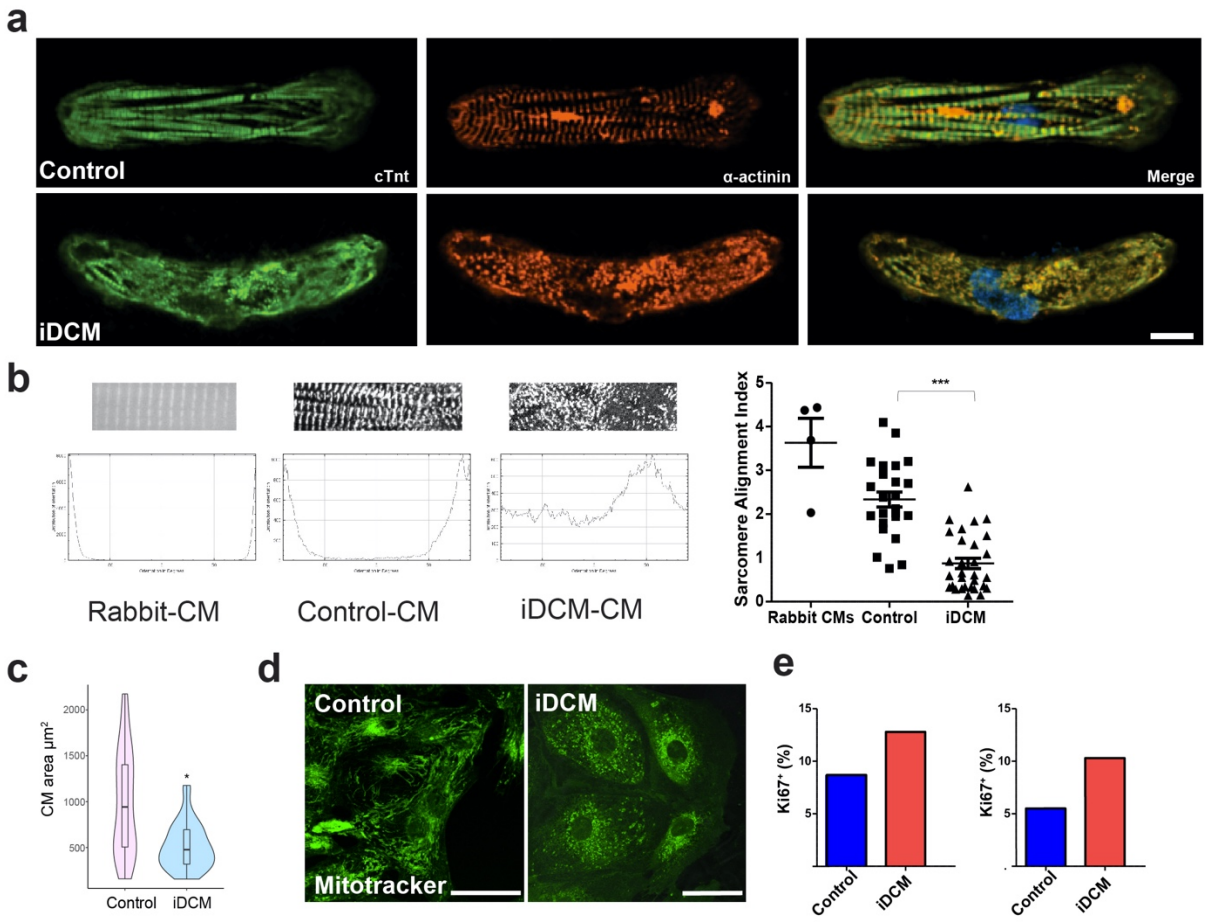

Figure S3 Sarcomere structure and mitochondrial function of control and iDCM iPSC-CMs. **a**, Control iPSC-CMs (above) exhibited organized sarcomere structures, whereas iDCM iPSC-CMs exhibited sarcomere disarray. Cardiac troponin T (green), a-actinin (orange). Scale bars are 10µm. **b,** Quantified sarcomere alignment index of Control and iDCM-CMs with in vivo Rabbit-CMs as validation of sarcomere alignment index model shows decreased levels of sarcomere alignment in iDCM-CMs. n=4 Rabbit CMs, n=25 Control-CMs, n=30 iDCM-CMs, ***p<0.0001 by One-way ANOVA with Tukey multiple comparison test. **c,** Quantified CM cell area shows a significant reduction in size of iDCM-CMs compared to control CMs. n = 71 control-CMs, 54 iDCM-CMs. **d**, Control iPSC-CMs exhibited meshwork-like elongated mitochondria (MitoTracker, green), whereas iDCM iPSC-CMs displayed globular, punctate mitochondria. Scale bars are 50µm. **e,** Quantification of proliferation rates of control and iDCM-CMs by Ki67 staining shows increased levels of proliferation in iDCM-CMs. n = 909 d21, 1872 d36, Control-CMs. n = 922 d21, 767 d36 iDCM-CMs.

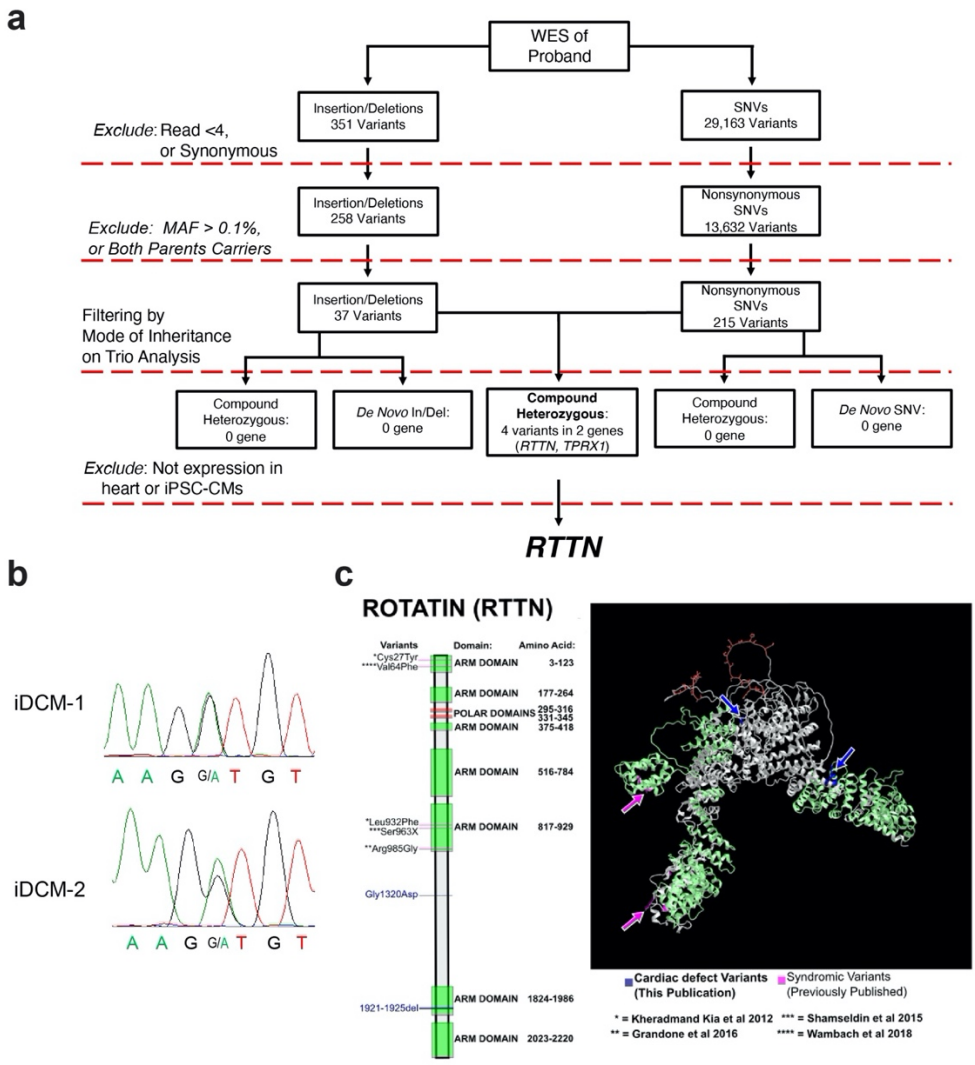

Figure S4. Workflow for WES analysis to identify the causal gene.

a, Whole exome sequencing (WES) of the trio (proband and both parents) were performed to identify a *de novo* mutation or compound heterozygosity. After filtering for a read depth of > 4 for quality and excluding synonymous variants, the proband's variant calling identified 258 Indels and 13,632 nonsynonymous SNPs. Next, variants that were not rare (minor allele frequencies, MAF > 0.1%), and variants where both parents were heterozygous carriers or either parent was homozygous were excluded, resulting in 37 indels and 215 SNPs as candidates. Finally, a 3-fold filter of functional prediction (exclusion of benign prediction using polyphen2), expression in cardiomyocytes, and validation by Sanger sequencing yielded a single gene candidate, *RTTN* (encoding rotatin). **b**, Sanger sequencing data verifying mutations in both iDCM derived iPSC lines. **c**, A schematic and a model (Alphafold) of Rotatin with annotations of predicted functional domains, armadillo domains (green) and polar domains (red). Previously described variants are indicated in magenta which cause microcephaly, short stature, and polymicrogyria with or without seizures (MSSP, OMIM:614833), while our iDCM related variants are in blue.

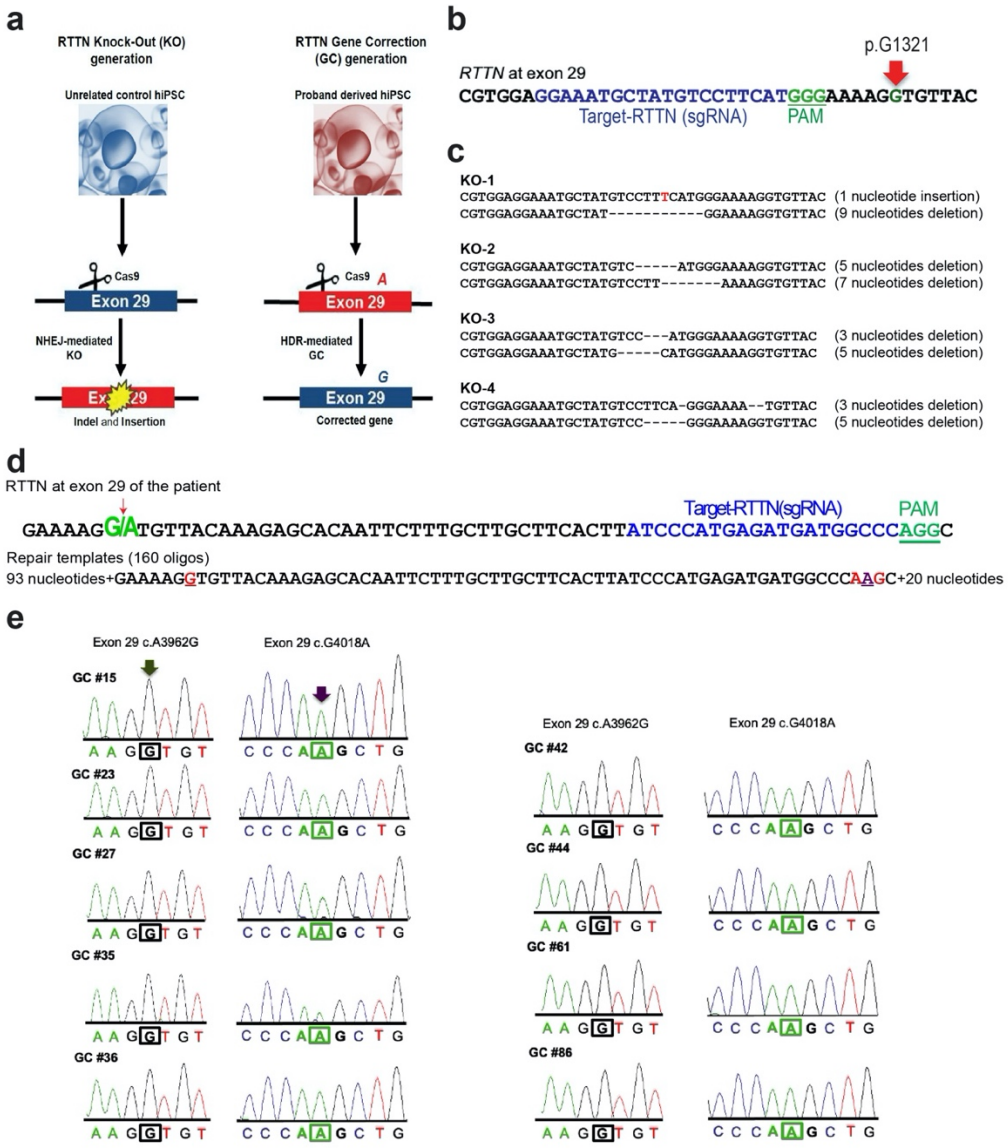

Figure S5. CRISPR-Cas9 mediated generation of *RTTN* KO and gene corrected (GC) lines to confirmation RTTN as the causal gene.

a, General strategy for generation of *RTTN* KO and GC iPSC-CMs. The region corresponding to p.G1321 on exon 29 was targeted for KO in control iPSCs by NHEJ, and the region containing p.G1321D mutation in iDCM iPSC edited by HDR. **b**, Single guide RNA (sgRNA) targeting the PAM site (green) on the *RTTN* exon 29. **c**, We generated 4 independent KO lines that were confirmed by Sanger sequencing. Dotted lines indicate "*deletion*" and red color letter presents "*insertion*". **d**, sgRNA for genome correction in iDCM iPSCs targets the PAM site (green) near the region on exon 29 which corresponds to the p.G1321D mutation (violet). The repair template contains the reference sequence at guanine-4018 (violet), corresponding to p.G1321. The repair template also contained a "beacon" (red), which does not change amino acid sequence, to confirm gene repair. **e**, In total 9 gene-corrected iPSC lines generated and confirmed by Sanger sequencing.

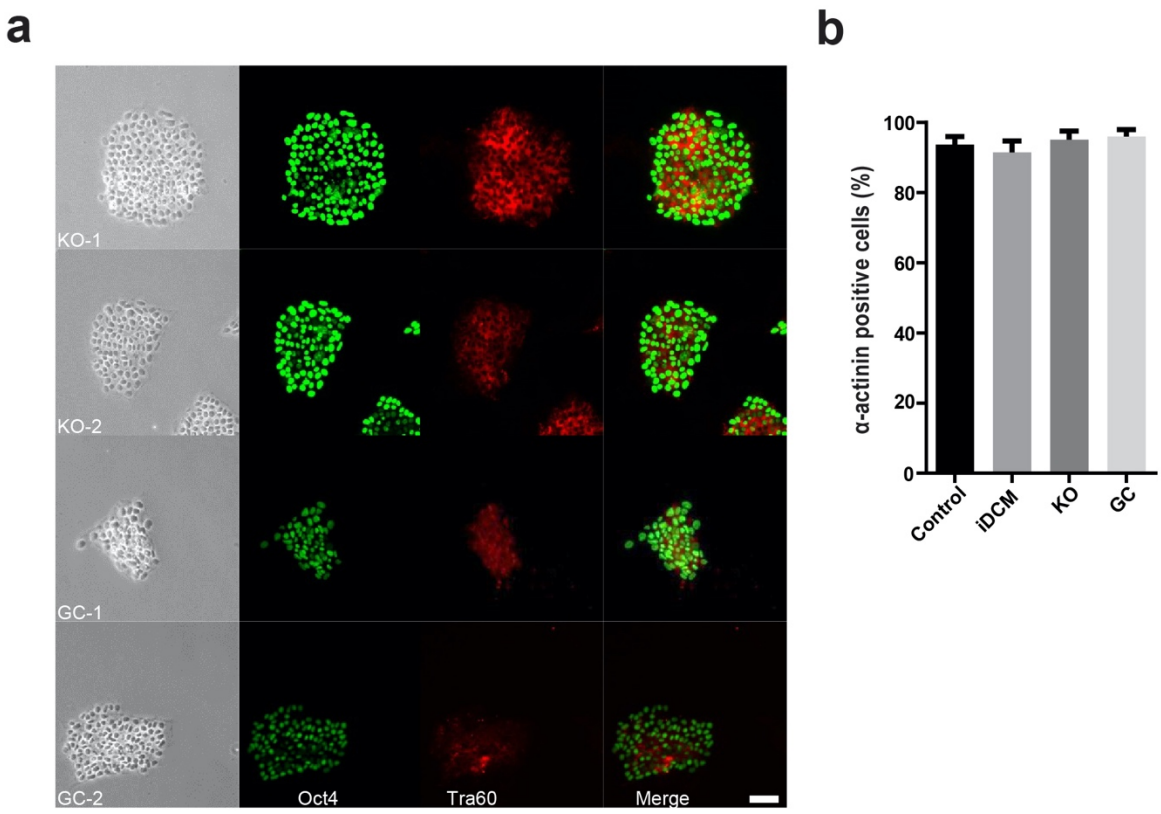

Figure S6. Confirmation of stemness markers and cardiac differentiation in *RTTN* KO and GC iPSC lines.

a, Representative immunofluorescent images show that KOs and GCs iPSC lines were Oct4- and TRA-60-positive. **b**, Control, iDCM, KO, and GC lines exhibited more than 90% α-actinin positive cells at 30 days after cardiac differentiation. Scale bar is 10μm.

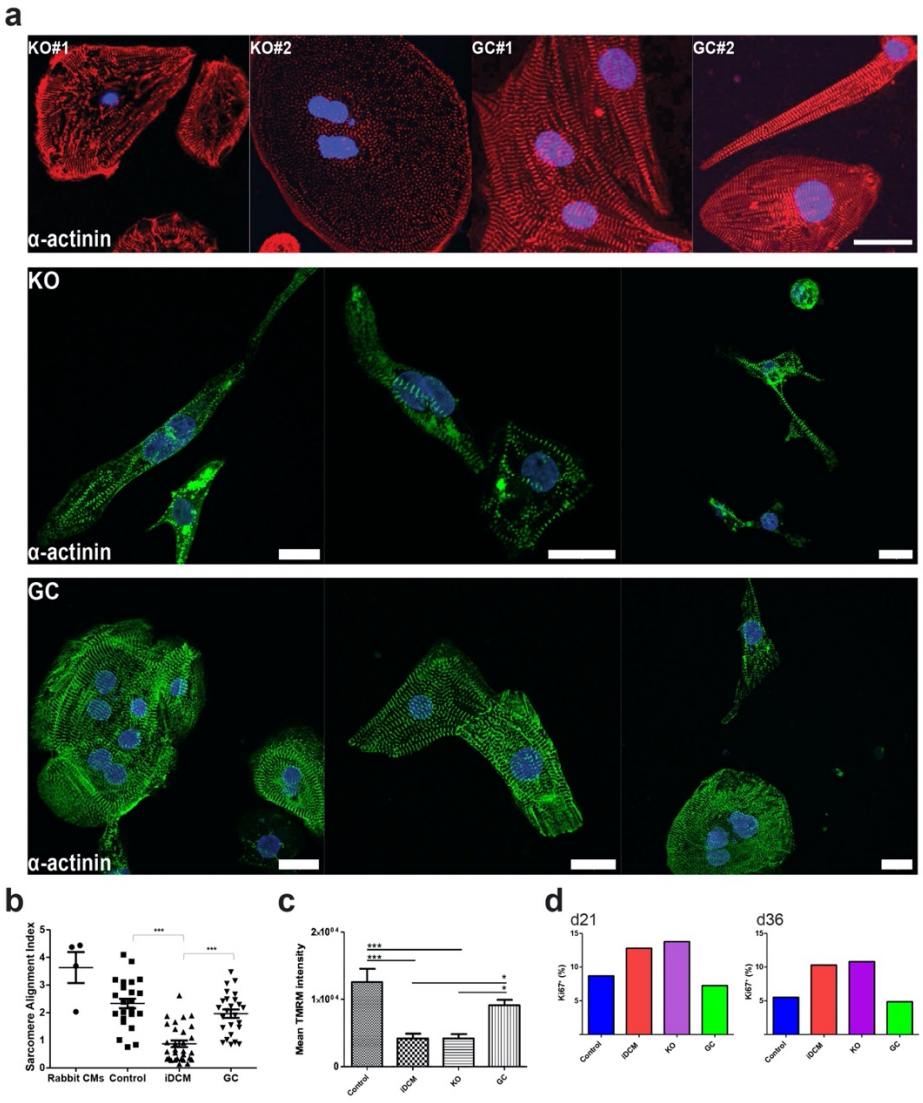

Figure S7. *RTTN* KO iPSC-CMs exhibit sarcomere disarray, and RTTN gene corrected (GC) iPSC-CMs exhibit restoration of normal sarcomere structure.

a, *RTTN* KO-CMs exhibited sarcomere disarray (red, a-actinin staining of Z-line), whereas *RTTN* GC-CMs exhibited grossly normal sarcomere structure. Scale bars = 20μm. **b,** Quantified sarcomere alignment index of Control and iDCM-CMs with in vivo Rabbit-CMs as validation of sarcomere alignment index model. n=4 Rabbit CMs, n=25 Control-CMs, n=30 iDCM-CMs, n=25 GC-CMs. ***p<0.0001 by One-way ANOVA with Tukey multiple comparison test. **c,** Quantification of mean TMRM intensity by FACS analysis. This experiment was validated by one-way analysis of variation followed by Tukey correction. Control n=12, iDCM n=12, KO n=13, GC n=17. **d,** Quantification of proliferation rates of control, iDCM, KO, GC-CMs by Ki67 staining shows increased levels of proliferation in CMs with mutant RTTN (iDCM, KO), and normalized proliferation in GC-CMs. n = 909 d21, 1872 d36 Control-CMs. n = 922 d21, 767 d36 iDCM-CMs. n = 1395 d21, 722 d35 KO-CMs. n = 1422 d21, 1378 d36 GC-CMs.

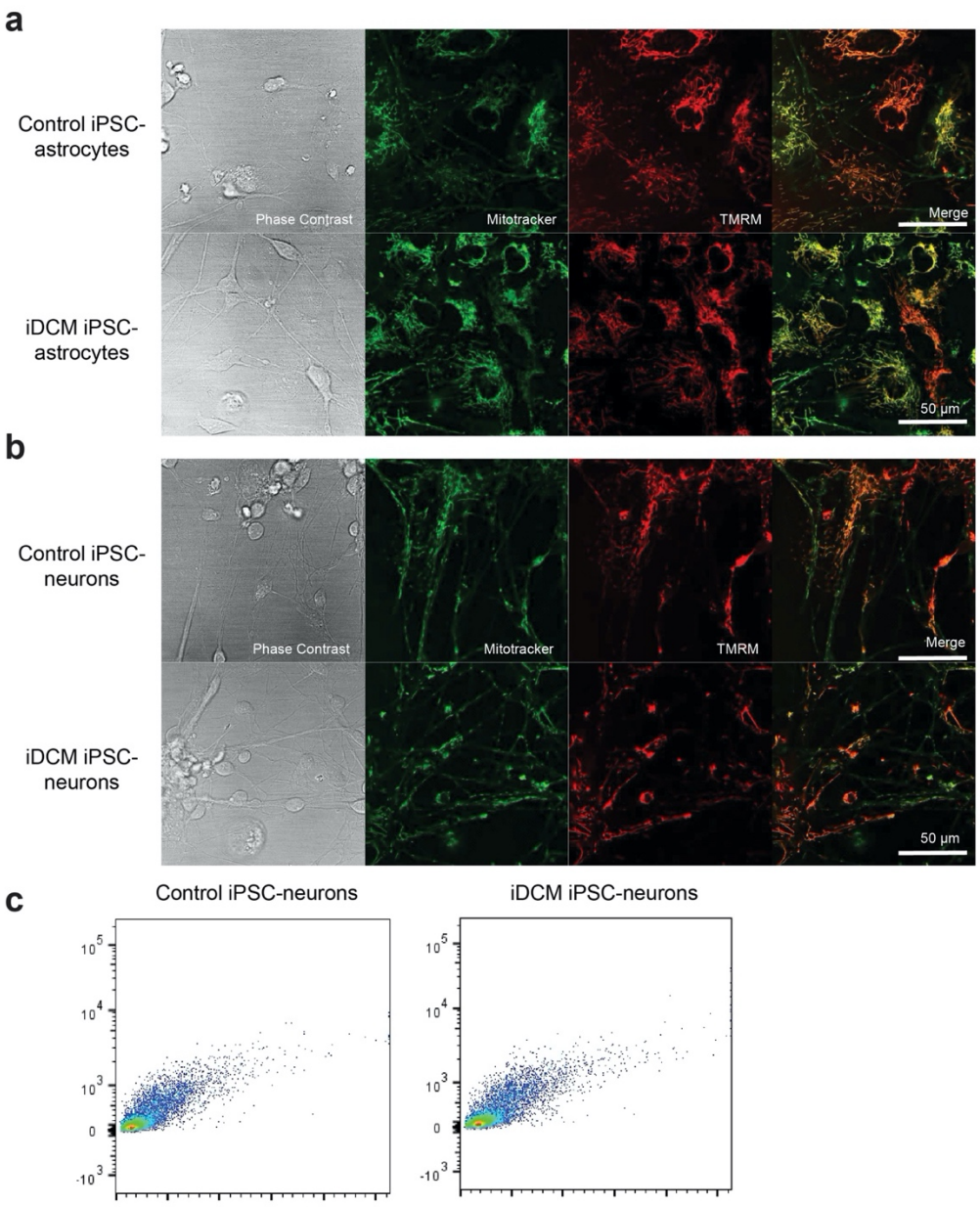

Figure S8. Mitochondrial structure and function of control and iDCM iPSC-derived astrocytes and neurons.

a, Control and iDCM iPSC-derived astrocytes exhibited no apparent difference in gross or mitochondrial morphology (MitoTracker, green), and mitochondrial function (TMRM, red). **b**, Control and iDCM iPSC-derived neurons exhibited no apparent difference in gross morphology or mitochondrial morphology (MitoTracker, green), and mitochondrial function (TMRM, red). Scale bars = 50µm. **c**, FACS analysis indicate that the mitochondrial function of control and iDCM iPSC-neurons are quantitatively similar.

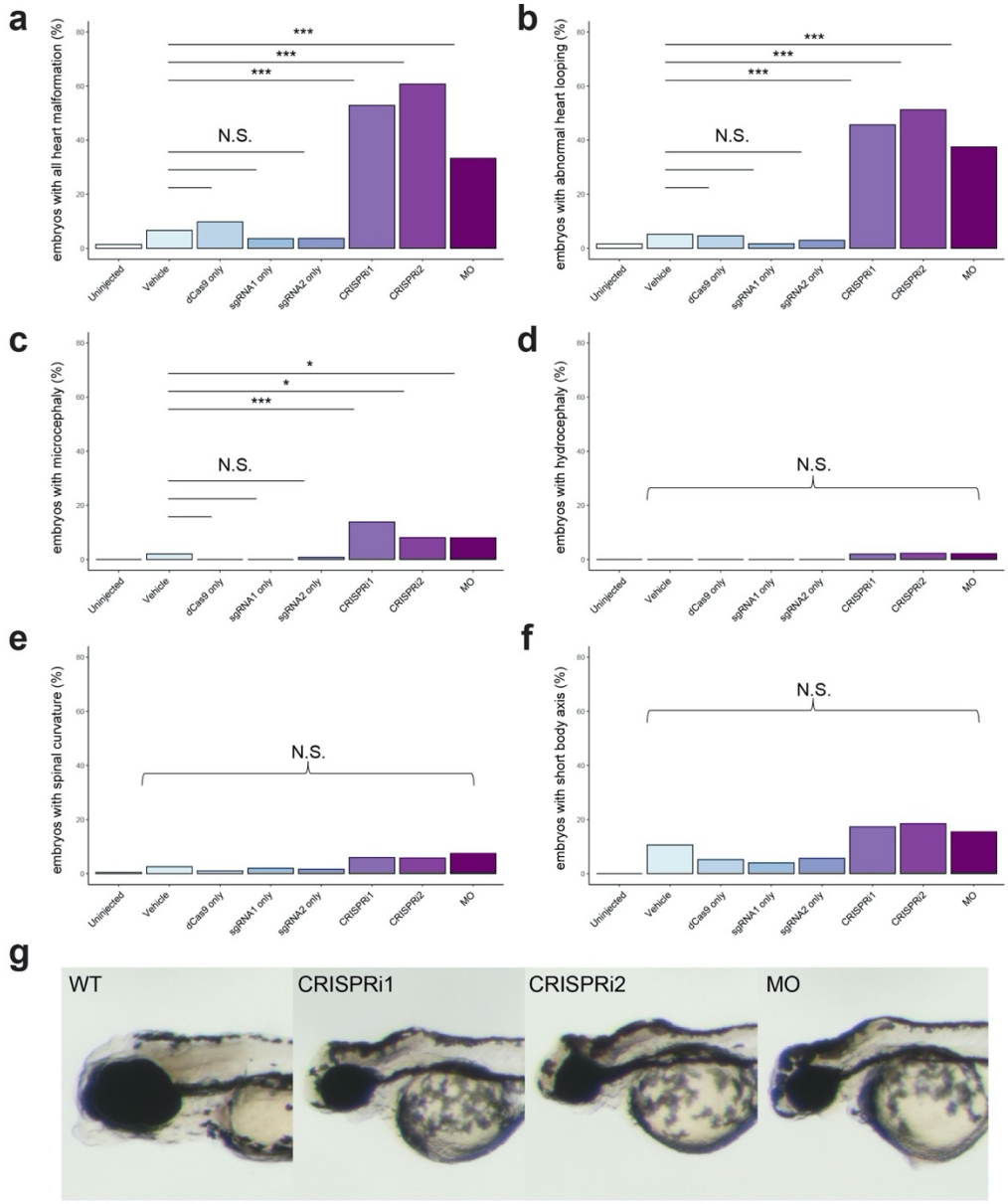

Figure S9. Knockdown of RTTN may result in phenotypes consistent with ciliopathies in Zebrafish.

a-b, Knockdown of RTTN resulted in pericardial edema, heart looping defects, and heart malformations. **c-f**, Additionally, we observed higher incidence of hydrocephaly, microcephaly, abnormal spinal curvature, and severe anterior-posterior patterning defects. Only trends of higher microcephaly reached levels of significance in this study. Embryos were assessed at 3dpf. **g**, Representative microcephaly phenotype in injected embryos. n ≥ 101 embryos from n ≥ 7 biological replicates using embryos from n ≥ 2 breeding pairs. Vehicle was 0.08% phenol red + PBS in ultrapure water. Multiple comparisons were made between the vehicle and treatments using Fisher's exact test with Bonferroni's correction. * $\rho <$ *0.0085,* *** $\rho <$ *0.00017,* N.S. is no significance.

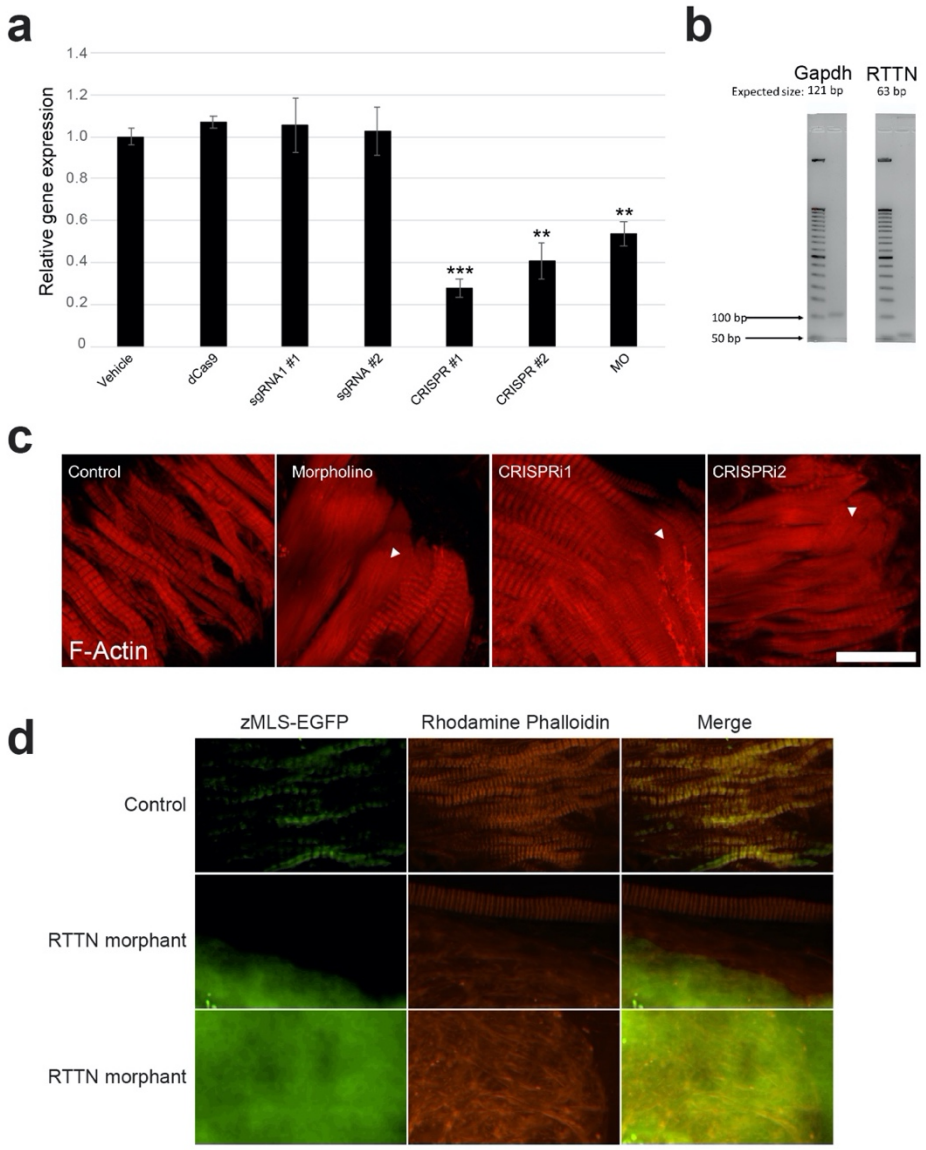

Figure S10. Validation of RTTN knockdown and sarcomere malformation in zebrafish

a, Relative expression of RTTN in knockdowns validated through RT-qPCR in Morpholino and Crispri injected embryos. Expression of RTTN was normalized to GAPDH. Representative data from n=6 biological repeats using n=3 individual embryos per treatment. b, PCR products from the vehicle demonstrate that only a single product of the predicted size was amplified. Samples were run on a 2% agarose gel with E-gel 50 bp DNA Ladder (Invitrogen). **c**, Sarcomere staining visualized through F-Actin staining in morpholino and Crispri injected embryos at 4 dpf shows disrupted sarcomere structure. White arrows indicate example regions of disrupted F-actin. **d,** Cross sections of WT and RTTN morphant hearts expressing mitochondrial localized EGFP. Cross sections of the morphant heart demonstrate non-specific localization of EGFP suggesting disruption of the mitochondria. Scale bar = 30μm.

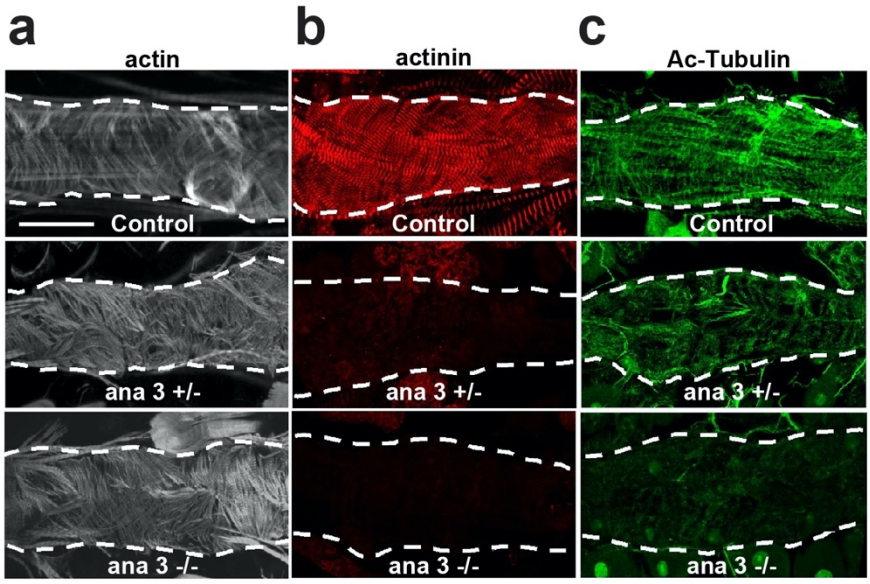

Figure S11. Knockdown of *ana3* in *Drosophila* leads to impaired sarcomere and microtubule formation.

a-c, Immunofluorescence images of actin, actinin and acetylated (Ac) alpha-tubulin in adult heart (segment A4) from control (w1118), ana3+/- and ana3-/- flies. Scale bar = 50 μm.

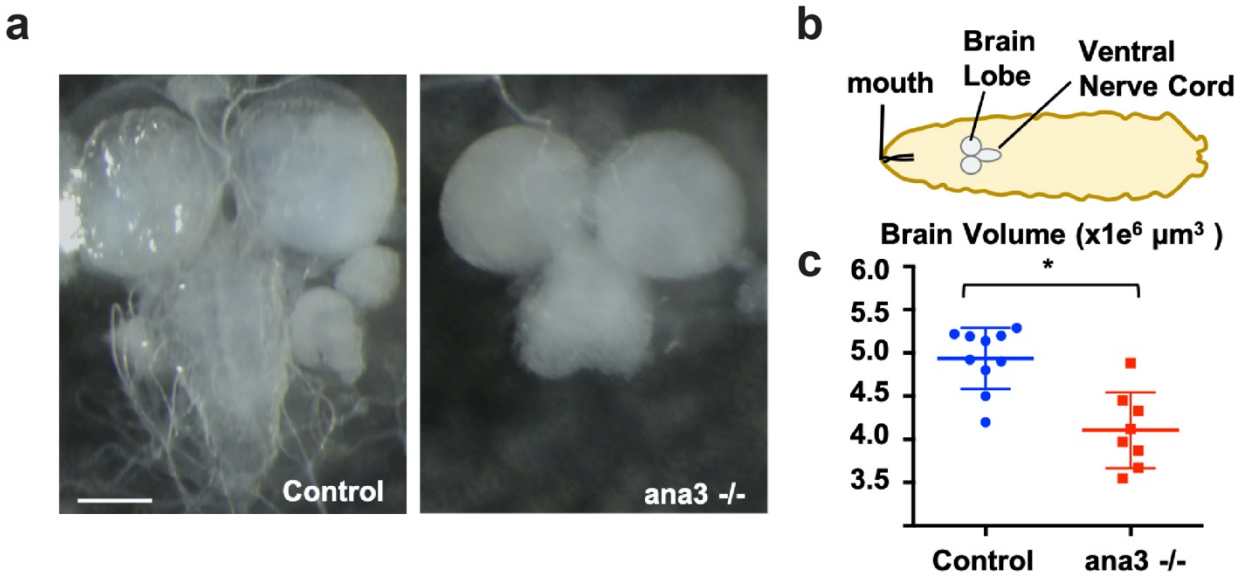

Figure S12. Knockdown of *ana3* in *Drosophila* also leads to microcephaly-like phenotype.

a, Homozygous ana3 mutant showed minor microcephaly and severe early dissociated nerve cord. Bright field images of 3rd instar larvae brains from control (w1118) and ana3-/- flies. Scale bar: 100 µm. **b**, Diagram of Drosophila melanogaster 3rd instar larvae brain. **c**, Quantification of brain lobe volumes for control (w1118) and ana3-/- flies. Individual data points with group mean (horizontal bar) ±SD are displayed for each genotype. Statistical significance (*) defined as $p<0.05$.

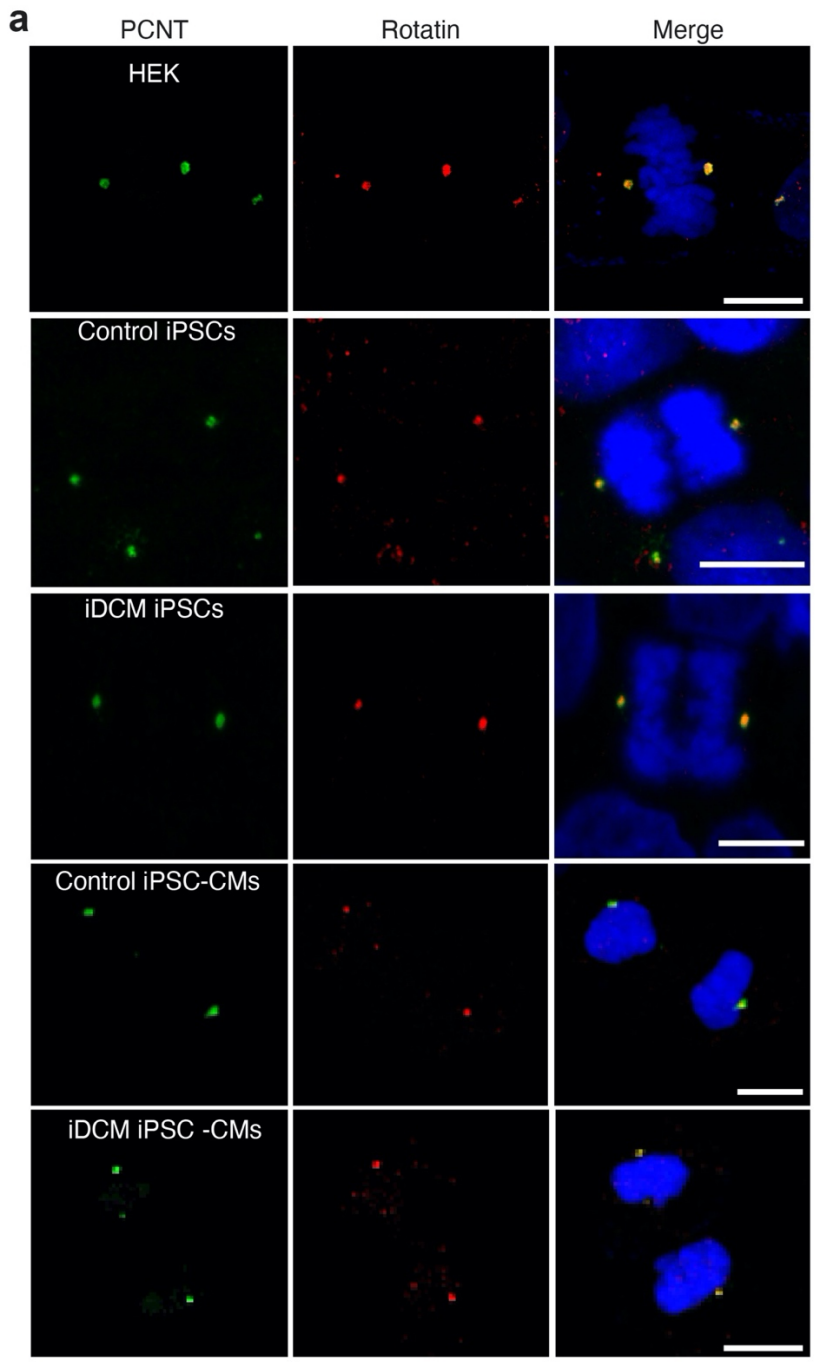

Figure S13. Rotatin localized in centrosome.

Rotatin (RTTN) is co-localized to the centrosomes in HEK293 cells, iPSCs and iPSC-CMs. RTTN was detected using the SASY antibody, which is directed against the N-terminal region of RTTN. In HEK293 cells, SASY detected RTTN in both proliferating and quiescent cells; however, in iPSCs and iPSC-CMs, SASY detected RTTN predominantly in proliferating cells. There was no gross difference between RTTN localization in control and iDCM iPSCs and iPSC-CMs, as detected by SASY antibody. Scale bars = 10μm.

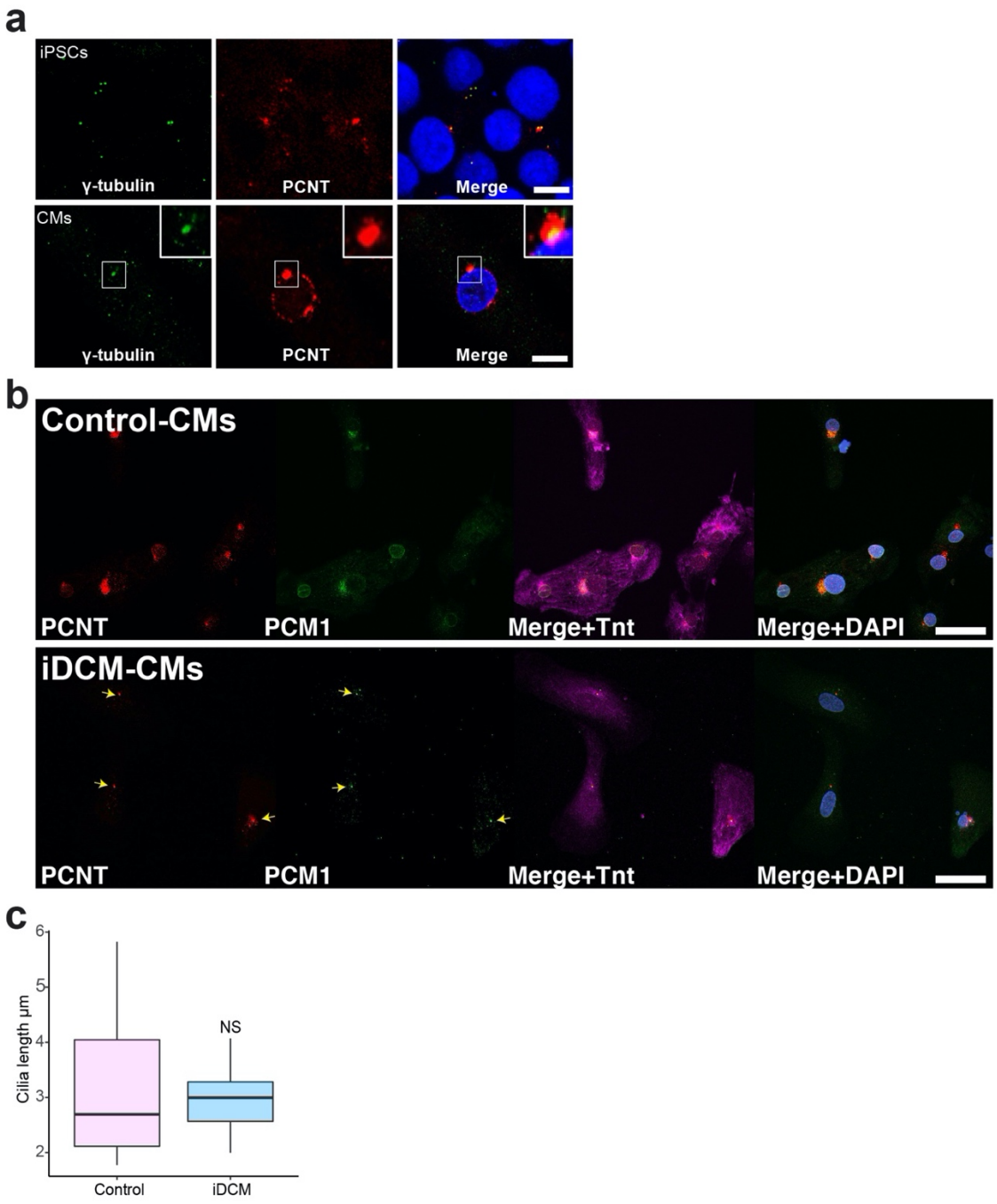

Figure S14. Localization of pericentrin (PCNT) and pericentriolar material 1 (PCM1) in iPSC-CMs. **a**, In iPSCs, PCNT is localized to the centrosome, along with γ-tubulin. Following cardiac differentiation, PCNT becomes partially redistributed to the perinuclear region of iPSC-CMs. Scale bars = 10µm.**b**, PCNT and PCM1, two of the pericentriolar material components, are redistributed to the perinuclear region in control-CMs, but not in the iDCM-CMs. Scale bars = 20µm **c,** Quantified cilia length by Arl3b staining and imageJ analysis shows no significant difference in cilia length between control and iDCM-CMs. n = 10 control-CMs, 8 iDCM-CMs. Scale bars are 10µm. Scale bars = 10µm.

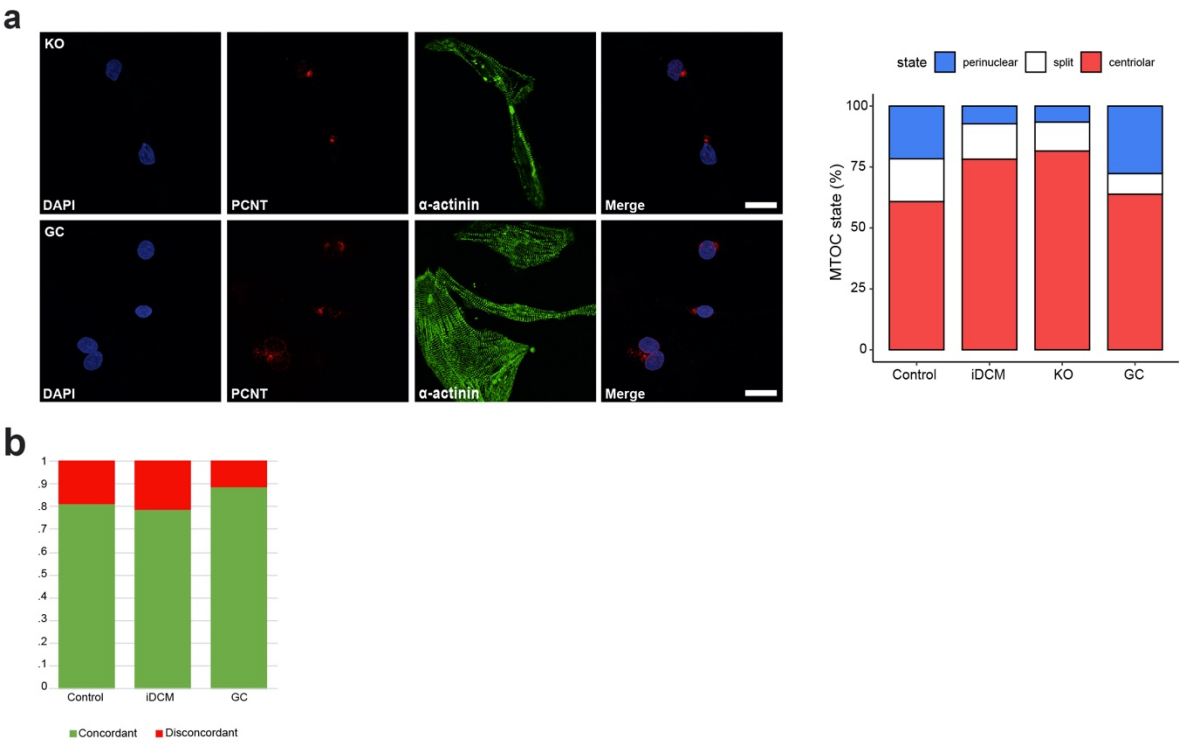

Figure S15. Localization of PCNT in KO and GC CMs

a, Immunofluorescence and subsequent quantification of initiation of perinuclear MTOC in KO and GC-CMs shows decreased perinuclear localization of PCNT in KO-CMs (similar to iDCM-CMs) and increased levels of perinuclear PCNT localization in GC-CMs (similar to control-CMs). n = 76 KO-CMs, 47 GC-CMs. Scale bar = 25μm. **b,** Concordance testing of proper centrosome reduction and mature sarcomere structure shows significant correlation in control, iDCM, and GC-CMs. n = 141 control-CMs, 62 GC-CMs, 160 iDCM-CMs. $p < 0.0001$ between observed and expected results by Chi-square test.

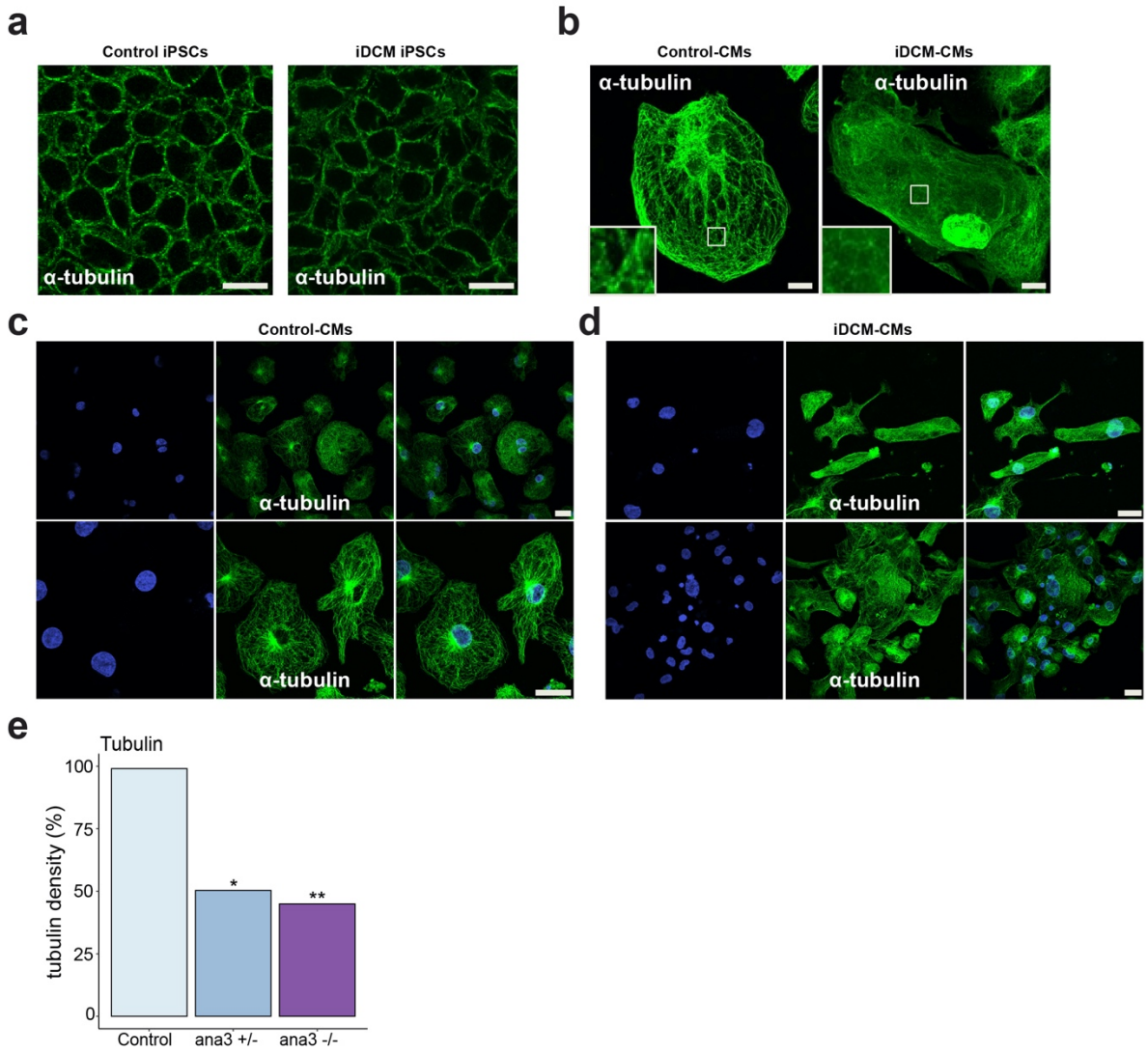

Figure S16. Defective microtubule network in *RTTN* mutant iPSC-CMs.

a, There was no significant difference in the morphology of microtubule structures in control and iDCM iPSCs. Scale bar = 20μm. **b**, Control iPSC-CM displayed prominent network of thick microtubule (MT) fibers (a-tubulin, green) emanating from the perinuclear region. By contrast, iDCM-CMs displayed thinner, shorter, and fainter MT fibers without a clear organizing center. The rectangular insets show enlarged images. Scale bar = 10μm. **c,** Additional replicate images depicting intact microtubule networks in control-CMs. Scale bar =20μm **d,** Additional replicate images depicting disrupted microtubule networks in iDCM-CMs. Scale bar =20μm. **e,** Quantification of tubulin density in control, ana3 +/- and ana3 -/- drosophila hearts.

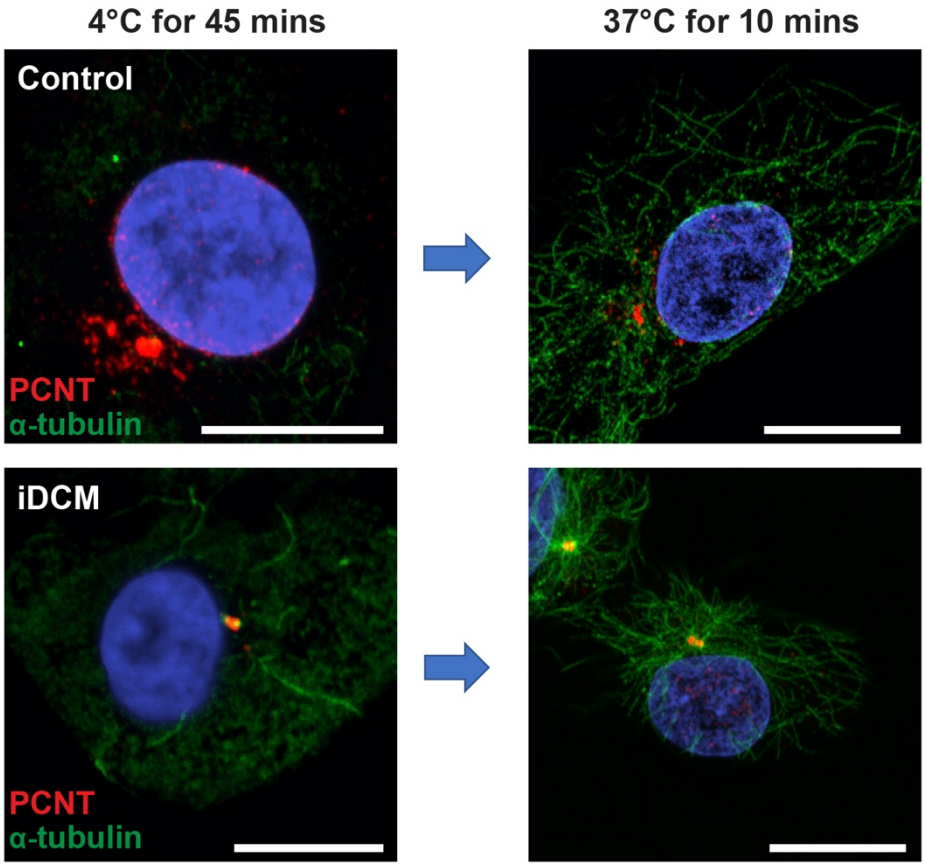

Figure S17. Microtubule regrowth assay of control and iDCM-CMs

a, iDCM-CMs retain the ability to regrow microtubules after 3 hours of cold treatment when compared to control-CMs. Scale bars = 10μm.

123 Gene list associated with dilated cardiomyopathy

ABCC6	KLHL24	MT-TT
ABCC9	LAMP2	MT-TV
ACTA1	LDB3	MT-TW
ACTC1	LEMD2	MT-TY
ACTN2	LMNA	MYBPC3
ALMS1	LMOD2	MYBPHL
ALPK3	LRRC10	MYH6
APOA1	MLYCD	MYH7
BAG3	MT-ATP6	MYL4
CASZ1	MT-ATP8	NKX2-5
CHRM2	MT-CO1	NRAP
DES	MT-CO2	PCCA
DMD	MT-CO3	PCCB
DOLK	MT-CYB	PKP2
DPM3	MT-ND1	PLEKHM2
DSC2	MT-ND2	PLN
DSG2	MT-ND3	PPCS
DSP	MT-ND4	PRDM16
DYSF	MT-ND4L	QRSL1
EEF1A2	MT-ND5	RAF1
EMD	MT-ND6	RBCK1
EPG5	MT-RNR1	RBM20
ETFA	MT-RNR2	RMND1
ETFB	MT-TA	SCN5A
ETFDH	MT-TC	SPEG
FBXO32	MT-TD	TAB2
FHOD3	MT-TE	TAZ
FKTN	MT-TF	TBX20
FLNC	MT-TG	TBX5
FOXD4	MT-TH	TCAP
GATA4	MT-TI	TMEM43
GATA6	MT-TK	TNNC1
GATC	MT-TL1	TNNI3
GBE1	MT-TL2	TNNI3K
GLB1	MT-TM	TNNT2
GSK3B	MT-TN	TOR1AIP1
HAND1	MT-TP	TPM1
HCN4	MT-TQ	TTN
ILK	MT-TR	TTR
JPH2	MT-TS1	VCL
JUP	MT-TS2	VPS13A

Supplemental Table 1. List of 123 genes known to be associated with cardiomyopathy

Incorporation of iPSC-CM expression profile (RNAseq) and PolyPhen2 pathogenicity prediction algorithm further narrowed the candidate gene list to RTTN

Type	Candidate Genes	Amino acid Change	Aliases	PolyPhen2 Score	Express. in iPSC-CMs
De novo	FRG2C	S62X	FSHD Region Gene 2 Family	n/a, Stop gain	No
De novo	ZNF718	I11V	Zinc Finger Protein 718	Benign	Yes
De novo	RPS6KA4	L769P	Ribosomal Protein S6 Kinase	Possibly deleterious	Yes
De novo	RPS6KA4	S772P	Same	Benign	Yes
De novo	BAGE3	I63T	B Melanoma Antigen Family, Member 3	Benign	No
X-linked	H2BFWT	I170fs	H2B Histone Family	n/a, Frameshift deletion	No
X-linked	EFHC2	G593E	EF-Hand Domain (C-Terminal) Containing 2	Benign to possibly damaging	Yes
X-linked	KCND1	T512P	Shal-type Potassium Voltage-Gated Channel	Benign	Yes
X-linked	MPP1	M163T	Membrane Protein	Benign	Yes
Compound Het: Del & SNP	RTTN	1921_1925del	Rotatin – conserved KELSI deleted.	n/a, Conserved 5AAs deleted	Yes
	RTTN	G1320D	Rotatin	Probably damaging	Yes
Compound Het: Del & SNP	TPRX1	215_219 del	Tetra-Peptide Repeat Homeobox Protein		No
	TPRX1	206_210 del	Tetra-Peptide Repeat Homeobox Protein		No

Supplemental Table 2. Incorporation of iPSC-CM expression profile (RNAseq) and PolyPhen2 pathogenicity prediction algorithm further narrowed down the list of candidate causal genes to *RTTN*.

Predicted CRISPR-Cas9 off-target sites against sgRNA of KO lines

Potential off-target sequence	Number of mismatches [variable nucleotide]	Locus	Gene name	Forward primer	Reverse primer
AGCAATGCTGTTTCCTTCATAAG	4MMs [1:3:10:12]	chr15:-63918371	*SNRPB2*	GAGACCCAGGCAGGATCTAA	GCAAGCTTAGCACTGGCAAC
GGCAATGTTCTGTCCTTCAGCAG	4MMs [3:8:10:20]	chr1:-44088160	*PTPRF*	TGAGGGATTTTAGCCTCTTCC	ACGACCAGGGATGAGTGTTC
CAAAAGGCAATGTCCTTCATCAG	4MMs [1:2:6:9]	chr19:-52920543	*ZAF528*	GTGCAACGGCTTGTAAATGA	TTTCATGGTCAGTTGGTGGA
GGAAGCCCTATATCCTTCATTGG	4MMs [5:6:7:12]	chr15:-67493281	*AAGAB*	GGTGGAACATGTTTTCCTTAAA	CCTTCATCCCATATGTCCCTA

Supplemental Table 3. Predicted CRISPR-Cas9 off-target sites against sgRNA of KO lines

Predicted CRISPR-Cas9 off-target sites against sgRNA of GC lines

Potential off-target sequence	Number of mismatches [variable nucleotide]	Locus	Gene name	Forward primer	Reverse primer
ATCCCAAGGGAAGATGGCCCCAG	3MMs [7:9:12]	chr11:-66082539	*CD248*	CAGGTCCCCTGTGTCTCCT	ACGTTGGCACCAGGAGTG
CTCCCAGGAAATGATGGCCAGAG	4MMs [1:7:10:20]	chrX:-153418426	*OPL1MW*	GGAAATGCCCAGTGTCTGTT	AGTCTGACCCTGCCCACTC
CTCCCAGGAAATGATGGCCAGAG	4MMs [1:7:10:20]	chrX:-153455556	*PCSK6*	GCTTGTTGGCTCCAGTCTTC	GCCCCAGTGGTCATACATTT
CTCCCAGGAAATGATGGCCAGAG	4MMs [1:7:10:20]	chrX:-153492674	*LINC00339*	TGGTCTGTTTTTGCTGATTCTT	CCGGGAGGCTTTCTAGCAT
GTCACATGTGAGGATGGCCCAGG	4MMs [1:4:9:12]	chr15:+101845019	*PPFIA3*	CCCCGACATGACTCTTTGAA	TCTCCTCCCAAGTCCTTGTC

Supplemental Table 4. Predicted CRISPR-Cas9 off-target sites against sgRNA of GC lines

Supplementary Clinical Information

The male patient was born full term to a G2P2 mother following an uncomplicated gestation. He had no syndromic features and was discharged home after uncomplicated initial hospital stay. At 3 months of age, the patent presented to the referring hospital in Tennessee with failure to thrive. At that time, the patient was found to have dilated left ventricle (LVEDI 63ml/m2), and markedly reduced ejection fraction of 18% (normal >55%) with global hypokinesis. He was ruled out for LV noncompaction and found to have normal coronary anatomy. Global metabolic panel was negative for primary metabolic disorders and genetic consult revealed no chromosomal abnormalities. There was no family history of congenital heart disease, sudden cardiac death, arrhythmias, neurologic disorders, metabolic disorders, or unexplained childhood deaths. Maternal grandmother had an "enlarged heart." His older sibling was a healthy teenager. Heart biopsy performed at 4 months of age was negative for acute inflammation, infiltrative process, and viral inclusions. The biopsy was notable for severe loss of myofilaments and pleiotropic, dysmorphic mitochondria. He was critically ill for 4 months, eventually requiring extracorporeal membrane oxygenation (ECMO) prior to heart transplantation at 7 months of age. Following the transplantation, the patient has done well, without clinically significant neuromuscular or cardiac issues.